Phytochemical metabolites and their effects on

\textit{in vitro} and \textit{in vivo} measures of oxidative stress

Edward George Walker

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Abstract

Phytochemicals, plant based non-nutrient compounds, are important components of the human diet and have been associated with many of the beneficial effects of fruit and vegetable consumption. Their potential ability to function as antioxidants and also to regulate cell signalling pathways and induce endogenous protective mechanisms is both an exciting and a complicated area of research. Currently it is known that phytochemicals can act to enhance cell survival in cells that are challenged with a cytotoxic stimulus, and it has recently been shown that the combination of different fruit extracts can have a synergistic enhancement on this cell survival.

There have been numerous studies into the mechanisms behind this protection, indicating that some phytochemicals may have a direct binding effect on cell signalling molecules, some may act as chemical antioxidants that directly scavenge free radicals and some may induce an adaptive response by upregulation of the body’s endogenous defence mechanisms. However, the majority of these studies have been conducted using dietary phytochemicals and have not examined the effects of the phytochemical’s blood metabolites.

In this thesis the effects of phytochemical blood metabolites on factors that regulate oxidative stress within the body are examined. It is shown that 3,4-dihydroxybenzoic acid (3,4-DHBA) pretreatment can induce an adaptive response by the upregulation of endogenous antioxidant enzymes and that this effects occurs in four different cell lines that originate from four distinct tissue types. Additionally, an animal experiment was conducted to determine if the results seen in vitro could be related to in vivo effects. The feeding of 3,4-DHBA to Sprague Dawley rats for two weeks at the non-physiological dose of 100 mg per kg induced endogenous antioxidant expression in both intestine and liver of healthy young adult rats. While this effect is not directly applicable to levels of 3,4-DHBA seen in normal diets, it does highlight the potential for a high 3,4-DHBA or 3,4-DHBA precursor functional food and suggest that long term consumption of dietary levels of these compounds may induce a similar response.
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1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione.

Dedicated to the memory of Gabbeh, my friend and companion of 15 years, you will be missed.
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Abbreviations:

3-AT 3-amino-1,2,4-triazole
8-epi-PGF2 8-Epi-prostaglandin F2
AAPH 2,2'-azobis-2-methyl-propanimidamide,dihydrochloride
AIF Apoptosis inducing factor
ALPS Autoimmune Lymphoproliferative Syndrome
ANT Adenine nucleotide translocator
AP-1 Activator protein 1
APAF-1 Apoptotic protease activating factor 1
ARE Antioxidant response element
ASK-1 Apoptosis signal-regulating kinase 1
ATCC American Type Culture Collection
ATP Adenosine-5'-triphosphate
Bad Bcl-2-associated death promoter
Bak Bcl-2 homologous antagonist/killer
Bax Bcl-2–associated X protein
BBB Blood brain barrier
Bcl-2 B-cell lymphoma 2
Bcl-W Bcl-2-like protein 2
Bcl-xL B-cell lymphoma-extra large
BCRP Breast cancer resistance protein
BH B-cell lymphoma 2 homology
Bid BH3 interacting domain death agonist
Bim Bcl-2-like protein 11
BITC Benzyl-isothiocyanate
BTB Blood testis barrier
CAD Caspase-activated deoxyribonuclease
CAPE Caffeic acid phenyl ester
CARD Caspase recruitment domain
CARET The beta-Carotene and Retinol Efficacy Trial
CDA Cyanidin derived anthocyanin
cFLIP Fas-associated protein with Death Domain -like IL-1beta-converting enzyme-like inhibitory protein
cMAX Maximum concentration
CVD Cardiovascular disease
CYP1A1 Cytochrome P450 A1
CYP1B1 Cytochrome P450 B1
DCFDA 2',7'-dichlorodihydrofluorescein
DED Death effector domain
DHBA Dihydroxybenzoic acid
DHEA Dehydroepiandrosterone
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
DNA-PK DNA-dependent protein kinase, catalytic subunit
DTNB 5,5'-dithiobis-(2-nitrobenzoic acid
DTT Dithiothreitol
EGCG Epigallocatechin gallate
EGTA Ethylene glycol tetraacetic acid
eNOS Endothelial nitric oxide synthase
ER Endoplasmic reticulum
ERK Extracellular signal-regulated kinases
ET Electron transfer
ETDA Ethylenediaminetetraacetic acid
FACS Fluorescence-activated cell sorting
FADD Fas-Associated protein with Death Domain
FBS Fetal bovine serum
FDA The American food and drug administration
FITC Fluorescein isothiocyanate
FRAP Ferric reducing ability of plasma
FTO Fat mass and obesity associated gene
GAS2 Growth arrest-specific protein 2
GI Gastrointestinal
GPx Glutathione peroxidise
GSH Reduced glutathione
GST\pi Glutathione s-transferase Pi
H2O2 Hydrogen peroxide
HAT Hydrogen atom transfer
HBA Hydroxybenzoic acid
HED Human equivalent dose
HEK Transformed embryonic kidney
HIV Human immunodeficiency virus
HPCL High-performance liquid chromatography
HSP Heat shock proteins
ICAD Inhibitor of aspase activated deoxyribonuclease
ICAM-1 Inter-cellular adhesion molecule 1
IFN-\gamma Interferon-gamma
iNOS Inducible nitric oxide synthase
JNK1 C-Jun N-terminal kinase 1
KEAP-1 Kelch-like ECH-associated protein 1
LPS Lipopolysaccharide
MCF7 Michigan cancer foundation-7
NAC N-acetyl-L-cysteine
NADPH Reduced nicotinamide adenine dinucleotide phosphate
NF-\kappaB Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS Neuronal nitric oxide synthase
NQO1 NAD(P)H:quinone oxidoreductase 1
NQO2 NAD(P)H quinone oxidoreductase 2
Nrf2 NF-E2-related factor-2
NTB 2-nitro-5-thiobenzoate
ORAC Oxygen radical absorbance capacity
P53 Protein 53
PARP Poly ADP ribose polymerase
PB Buffer solution
PBS Phosphate Buffered Saline
PC-3 human prostate cancer cell line
P-gp P-glycoprotein
PI Propidium iodide
PLA2 Phospholipase A2
PON1 Paraoxonase 1
RIP Receptor interacting protein
RNS Reactive nitrogen species
ROS Reactive oxygen spwecies
RPMI Roswell Park Memorial Institute
SEM Standard error of the mean
SOD Super oxide dismutase
TNB 5-thio-2-nitrobenzoic acid
TNF-R1 Tumour necrosis factor receptor 1
TNF-R2 Tumour necrosis factor receptor 2
TPTZ Tro-tripyridyltriazine
TRADD Tumor necrosis factor receptor type 1-associated death domain protein
TRAF2 Tumor necrosis factor receptor-associated factor 2
TRAIL Tumor necrosis factor-related apoptosis-inducing ligand
Trx Thioredoxin
TrxR Thioredoxin reductase
UV Ultraviolet
UVB Ultraviolet radiation B
VCAM-1 Vascular cell adhesion protein 1
VDAC voltage-dependent anion channel
Z-DEVD-FMK Z-DEVD-Fluor methyl ketone
Chapter One: Introduction

As we progress into the 21st century the increasing aged population of the western world will not only put more stress on health care systems but also on the younger care givers. It may not be possible to continue to merely treat diseases once they have occurred, rather it will be necessary for the aging population to maintain their own health for longer using preventative actions. As we look forward to a future with a larger aged population, we must also examine what helped the current aged generation to stay healthy and what environmental factors, particularly dietary ones, were the key to their good health and wellness well into old age. Lifestyle choices, such as diet, alcohol consumption, smoking and exercise are key determinants of quality of life. Diets which are high in botanical diversity, including different types of fruit and vegetables, are believed to protect from diseases, such as heart disease, better than one of low botanical diversity (Heber 2004; Thompson et al. 2006).

By exploring the antioxidant and cell signalling affects of fruits and vegetables we can further our understanding into how they, individually or together, work to protect cells of the body from damage and death, and how this may delay or even reverse the onset of diseases. If we can gain an understanding of the mechanisms by which fruit and vegetable extracts exert beneficial effects there is the potential to develop diets and foods that, when taken throughout life, will not only reduce rates of disease, but also improve the quality of life. While significant work has been carried out on phytochemical compounds from fruits and vegetables and their ability to kill cancer cells and also to potentiate the effect of anti-cancer drugs (Mertens-Talcott et al. 2006; Mai et al. 2007), less is known about how they mediate protective effects against cell death and oxidative damage. Less again is known about how digestion and absorption affects the bioavailability of these phytochemicals. The protective effects of fruits and vegetables are likely to be very important in the resistance to lifestyle related diseases such as diabetes and also diseases of old age such as Alzheimer’s disease. In many of these diseases there is an increase in oxidative load and also increased rates of cell death (Calabrese et al. 2006; Ceriello 2006; Yu & Chung 2006). To fully understand the potential effects of phytochemicals on the health and wellbeing of people, we
must have a strong understanding of how oxidative stress affects the body. It is also important to understand how and in what form fruit and vegetable compounds are absorbed into the blood, and where in the process of cell death they might act.

Through the course of this introduction many topics will be covered as the area of research spans several disciplines. It will include such diverse topics as: (i) the emerging industry of functional foods, (ii) the economic and social implication of disease, and how diseases of the 21st century are different from 100 years ago, (iii) the possible role of oxidative stress as both a cause and a modulator of disease, with a focus on the brain and the diseases that are related to it, and (iv) the mechanisms by which fruit and vegetable compounds are thought to mediate their beneficial effect, such as antioxidant function and regulation of cell signalling pathways, and how these compounds are absorbed and processed within the body. Finally, the types of cell death will be summarised together with the underlying mechanisms that govern cell death and survival and how these may be regulated by phytochemical compounds.

1.1 Functional foods

Functional foods have generated a thriving industry, with many consumers becoming interested in more than the macronutrient makeup of a food. There is a growing interest and demand for micronutrient ingredients that may have health benefits, such as polyphenolics from fruit, lycopene from tomatoes and omega-3 fatty acids from fish. Globally, there were over 26,000 new food products launched in the year ending June 2007 with over half of these making a beneficial health marketing message (Driggs 2007). The 10 fastest growing food or beverage categories in 2006 were all classified as ‘healthy’ and all grew by more than 1 billion dollars (US$), with drinkable yoghurt topping the list with 18% sales growth, and baby formula and dairy based drinks also up 10% (ACNielsen 2006). Sales of functional foods within the US topped 31 billion (US$), up over 10% year on year, and the sale of healthy beverages topped 17 billion with significant future growth predicted (NBJ 2007). In an interesting dichotomy, the younger age group, 18-24 years, were seen to be the major buyers of functional foods, in stark contrast to the sale of supplements where the
elderly, 65-74 years, are the primary consumers, suggesting a lack of marketing to a major potential market (Simmons 2006).

The reasons for the success of these healthy foods are clear. The public has a concern for their ongoing health, with surveys showing that around half of the population is either very or extremely concerned with heart disease, cancer, tiredness and stress (HealthFocus 2007). This concern and the potential health effects of foods has created a new target market, the “worried well”, a group within the population with not only disposable income, but also knowledge about the potential health benefits of products. Interestingly, there appears to be a polarisation of the population between those who consider the health benefits of foods and those that dismiss them. A 2006 survey showed 46% of people were purchasing food for its benefits to overall health and 30% to target prevention of a specific disease (FMI 2006). This is despite the fact that over half of those surveyed had not heard of polyphenols, prebiotics or probiotics, let alone knew what their beneficial effects might be. Historically, the food industry has released products with soft health claims that cannot be back up by solid scientific research. Although health claim regulations are changing, there is still the risk of unsubstantiated claims generating scepticism in a currently favourable market place.

The work conducted in this thesis is funded by the Wellness Foods Program (Foundation of Research, Science & Technology contract C06X0405), a joint government and industry research project. The Wellness Foods Program aims to develop both understanding about the compounds of fruits and vegetables that may be important in health and also to design economically viable concept products that can confer these health benefits to the public to ensure their “wellness”, with the hope of preventing some of the lifestyle-induced diseases of the 21st century.

1.2 Diseases of the 21st century

During the 20th century, the development of modern medicine has resulted in a large decline in death rates due to infectious diseases. This has led to not only an increase in life expectancy, but also to a shift in cause of death, from infectious diseases to lifestyle related diseases, many of which can be exacerbated by environmental factors,
particularly dietary, and are entirely avoidable. 100 years ago the major cause of death was infections, caused by either bacteria or viruses. While rates of heart disease were high, most damage to the heart was caused by infection, such as rheumatic fever and not a high fat diet as is the case today (Control. 2001; CDCI. 2002). Indeed, in 1900 supra-optimum nutrition was unlikely to be the cause of many deaths. Conversely, in the year 2000, dramatic increases in the rate of lifestyle related disease, such as stroke and cardiovascular disease (CVD), as well as cancer, type 2 diabetes and Alzheimer’s disease, which is linked irrefutably with increased age, has occurred (Garcia Garcia et al. 2001) (Table 1). Although the potential for lifestyle related diseases existed in 1900, recent changes in environmental factors, such as diet and inactivity, coupled with increased lifespan has resulted in the manifestation of genetic susceptibility to these diseases. Gene/environment interactions that were previously beneficial have become detrimental, contributing to the development of lifestyle related diseases. For example, a particular allele of the fat mass and obesity associated gene (FTO) is associated with an increased preference for high calorie foods (Cecil et al. 2008). This preference allows for the greater consumption of energy before appetite is suppressed and results in obesity and diabetes in populations that possess this allele when food is plentiful (Frayling et al. 2007). However, during times of famine, this same allele would allow for the storage of more energy from a given meal and hence be beneficial.
Table 1: Most common causes of death in the USA, year 1900 vs. 2000. (CDC 2001; CDC. 2002)

<table>
<thead>
<tr>
<th></th>
<th>1900</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pneumonia and influenza</td>
<td>Heart Disease</td>
</tr>
<tr>
<td>2</td>
<td>Tuberculosis</td>
<td>Cancer</td>
</tr>
<tr>
<td>3</td>
<td>Diarrhoea</td>
<td>Cerebrovascular diseases</td>
</tr>
<tr>
<td>4</td>
<td>Heart disease</td>
<td>Chronic lower respiratory diseases</td>
</tr>
<tr>
<td>5</td>
<td>Stroke</td>
<td>Unintentional injuries</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>Influenza and pneumonia</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>Nephritis, nephrotic syndrome, and nephrosis</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>Septicemia</td>
</tr>
</tbody>
</table>

In addition to the loss of life that can be attributed to lifestyle related diseases, there is a growing financial cost to the economies of the world. Diseases such as Alzheimer’s and other neurological diseases require long-term treatment and have a large social and economic cost. Looking solely at Alzheimer’s, a disease that was almost non-existent 100 years ago due to shorter lifespans, the worldwide cost on care and medication in 1993 was over 248 billion (US$) and is set to rise, as the population ages (Jonsson et al. 2006). Current figures have the rate of Alzheimer’s in certain western countries at 0.6% of the population between the ages 65-74, 7.9% aged between 75-84 and 16% of the population aged 85 years and older. As the population ages the amount of Alzheimer’s disease will further increase (Garcia Garcia et al.
2001). The cost of obesity and the related diseases of diabetes and CVD is also a significant economic burden on world economies. In 2007 the rate of obesity in American adults was 26.6%, having grown from 19.4% in 1997 (CDC 2007). The direct costs attributable to obesity have been estimated to be as high as 75 billion dollars in the US alone, with indirect costs being approximately double that (CDC 2004). Potentially, a dietary change that resulted in either decreased rates of obesity or reduced the medical complications of obesity could be highly economically beneficial.

Many of the lifestyle related diseases people are dying of today are suggested to be at least in part caused by increases in oxidative stress in the body (Halliwell & Cross 1994).

1.3 What is oxidative stress?

Oxidative stress is defined as the excessive oxidative load put on the body by disruption of the balance between oxidant and antioxidant function. This can be caused by an unmatched increase in oxidant factors or by a relative suppression of antioxidant factors. Increases in oxidative stress have been described in many different disease states including CVD, diabetes and the neurological disorders Parkinson’s disease and Alzheimer’s disease (Toyokuni 1998; Dhalla et al. 2000; Perry et al. 2002; Jenner 2003; Faraci & Lentz 2004). Many compounds from fruits and vegetables have been shown to function as antioxidants and as such, their ability to decrease oxidative stress within the body may have an important role in the prevention of said diseases. It is of course important to note that oxidants also have beneficial cellular roles, such as cell signalling, regulating vasodilatation and cytotoxic killing of either diseased or aberrant cells (Frostell et al. 1991; Poli et al. 2004; Szabo et al. 2007). However, for the purpose of this introduction, oxidative stress will be discussed when it is either inappropriate or detrimental and hence its prevention would broadly be considered beneficial.
1.3.1 Important oxidants

Essentially an oxidant is a compound that can either easily transfer oxygen to other atoms or accept electrons and, when in excess, can induce oxidative stress. There are many biologically significant oxidants that are generated during both normal homeostasis as well as during diseases states. Common oxidants generated within the body include: superoxide (\(^{\bullet}O_2^-\)) (Heikkila et al. 1976), hydrogen peroxide (H\(_2\)O\(_2\)) (Riley & Behrman 1991), singlet oxygen (Niedre et al. 2002), the hydroxyl radical (\(^{\bullet}OH\)) (Chiueh et al. 1994), organic hydroperoxide (ROOH) (Girotti 1998), alkoxy radicals (RO\(^{\bullet}\)) (Simic et al. 1989), peroxy radicals (ROO\(^{\bullet}\)) (Simic et al. 1989), hypochlorous acid (HOCl) (Weiss et al. 1982) and peroxynitrite (ONOO\(^{-}\)) (Yasmin et al. 1997). Some of these oxidants are known as free radicals, compounds with an unpaired electron, and some are formed when they react with free radicals or with reactive nitrogen species (RNS) such as nitric oxide (NO) (Beckman et al. 1990). When in excess they all have the capacity to induce cellular damage that may, over time, result in the development of chronic diseases.

1.3.2 Sources of oxidant generation in the body and antioxidant protection from them.

Oxidant generation within the cells of the body results primarily from the activity of the mitochondrial electron transport chain. The electron transport chain generates ATP for use as a cellular energy source. It also, however, generates both \(^{\bullet}O_2^-\) and H\(_2\)O\(_2\), both of which can damage the cell. To deal with this production of \(^{\bullet}O_2^-\) the mitochondria has a specific enzyme, mitochondrial superoxide dismutase (SOD), that converts \(^{\bullet}O_2^-\) into H\(_2\)O\(_2\), then the H\(_2\)O\(_2\) is dealt with by various enzymes including catalase, an enzyme that converts H\(_2\)O\(_2\) into water and oxygen (Johnson & Giulivi 2005). In addition to the electron transport chain, many exogenous molecules can be involved in the generation of oxidants. Interestingly, antioxidant vitamins such as vitamin C and various phenolics can either undergo auto-oxidation, or react with a metal ion to become oxidants (de la Lastra & Villegas 2007; Horsley et al. 2007). In addition, the generation of reactive oxygen species (ROS), such as \(^{\bullet}O_2^-\), has been...
associated with the synthesis of prostaglandins that originate from dietary omega-6 fatty acids (Wang et al. 2004). As oxidants have many roles in the correct functioning of the body they are not in themselves a problem, but there is the potential for levels of oxidants to become elevated and damage tissues. Of all the tissues in the body the brain is perhaps the most sensitive to oxidative stress, as it is under a high oxidative load, has low levels of key antioxidant enzymes in some regions and is susceptible to conditions that elevate oxidative stress.

1.3.3 Specific issues within the brain

Although all cells are exposed to oxidative damage, the brain is especially susceptible and hence would garner the greatest benefit from any dietary derived antioxidant protection. The brain’s greater susceptibility to oxidative damage is due to a variety of reasons, primarily its high rate of oxygen utilisation and subsequent high rate of oxidant generation. The brain uses about 20% of the oxygen that enters the body (Floyd & Hensley 2002). This high requirement for oxygen is due, in part, to the requirement for constant recycling of signalling molecules such as calcium ions, for the modulation of action potentials, and glutamate, which acts as a neurotransmitter (Attwell & Laughlin 2001). This high energy usage coupled with other factors, including high levels of neuronal generation of \( \cdot \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) (Pou et al. 1999), high concentration of iron (Rajan et al. 1976), neurotransmitters that can react with oxygen to generate ROS and the relatively low levels of some important antioxidant enzymes, such as catalase, in certain regions of the brain (Cardozo-Pelaez et al. 2000), results in a tissue that is highly susceptible to changes in oxidative stress. Oxidative stress is associated with multiple brain diseases, both acute (El Kossi & Zakhary 2000), such as ischemic stroke, and chronic, such as Alzheimer’s (Christen 2000) and Parkinson’s disease, and also in general age related cognitive decline (Pratico et al. 2002; Yuan et al. 2007).

1.3.3.1 Ischemic stroke

Ischemic stroke occurs when there is the loss of blood flow to a portion of the brain, caused by the blockage of a blood vessel, and results in the loss of oxygen and
glucose supply (Garcia et al. 1996). Due to the nature of the blood vessels within the brain, with many smaller vesicles often cross-supplying regions, the loss of blood flow caused by a blockage is graduated. Some areas of the brain, which receive most of their blood supply from the blocked vessel, lose almost all blood supply, while others, which are cross supplied by multiple vessels, receive a decreased supply (Garcia et al. 1996). The severity and type of damage that occurs to the cells depends on the amount of blood flow that is lost and hence their location relative to the blockage. Cells will either undergo electrical failure or, if the blood flow is very low, will metabolically shut down and die via necrosis (Astrup et al. 1981). The cells that are in a state of electrical failure go on to die from apoptosis many hours after the stroke has occurred and it is these cells that it may be possible to protect using dietary antioxidant compounds (Astrup et al. 1981). For example, it has been shown that feeding rats with either resveratrol or pomegranate polyphenols can reduce tissue loss observed 7 days after a stroke. This effect is thought to be mediated by decreases in the activation of two cell death signalling molecules, caspase 3 and calpain (West et al. 2007).

### 1.3.3.2 Alzheimer’s disease

In Alzheimer’s disease oxidative stress is thought to be one of the causes of brain damage, as higher levels of lipid, DNA and protein oxidation are seen in the brains of Alzheimer’s patients relative to those of control subjects (Butterfield et al. 2001). Alzheimer’s disease is characterised by the formation of beta-amyloid plaques; these plaques can react with transition metals to generate ROS (Rottkamp et al. 2001). It has also been suggested that the increased generation of ROS leads to the formation of soluble dityrosine cross-linked dimers (Atwood et al. 2004; Smith et al. 2007). In an interesting study, an animal model of Alzheimer’s disease was used to test the effectiveness of blueberry supplementation against both the generation of amyloid plaques and also the behavioural effects of Alzheimer’s (Joseph et al. 2003). The blueberry-supplemented group performed better than the non-supplemented group and the same as the non Alzheimer’s control group, using behavioural measures such as Y-maze performance. Intriguingly, the number of amyloid plaques formed in the blueberry and non-supplemented groups were the same even though the behavioural
affects were different (Joseph et al. 2003). This suggests that the blueberry supplementation did not stop the formation of the plaques, but may have stopped the damage they do. It is possible that this decrease in behavioural decline could be due to the antioxidant ability of blueberries, preventing the formation of soluble dityrosine cross-linked dimers and hence preventing the symptoms of the disease.

1.3.3.3 Brain aging

Oxidative stress appears to play a key role in the cognitive decline seen during aging. As we age there are increases in the oxidation of proteins, lipids and DNA within the brain, and also decreases in the levels of antioxidant defence (Smith et al. 1991). Animal studies have shown that increases in life span by caloric restriction are associated with decreased oxidative load on the body (Sohal et al. 1994). Similarly, experiments on long lived strains of animals have shown they possess increased resistance to oxidative stress (Barja 2002). Further dietary supplementation with N-acetyl-L-cysteine (NAC), a thiol based antioxidant in the body, has been shown to decrease the age related decline in memory function in prematurely aged mice, by decreasing oxidation of the mitochondrial proteins and lipids of the synaptic neurons (Martinez et al. 2000). Additionally, polyphenols have been associated with beneficial effects on brain aging (Lau et al. 2005) and supplementation with blueberries help reverse the associated age related decline in balance and co-ordination (Bickford et al. 1999).

1.3.4 Oxidative stress, CVD and cancer

Oxidative stress has been implicated in the etiology of various disease states, including lifestyle and longevity related diseases such as cancer (Toyokuni et al. 1995), obesity (Furukawa et al. 2004), diabetes and Alzheimer’s disease (Baynes 1991; Markesbery 1997). In 2003, a detailed examination into the potential association and importance of oxidative stress in obesity was conducted. A large number of subjects (n=2828) from the Framingham heart study were analysed for the correlation between obesity and oxidative stress, as determined by 8-Epi-prostaglandin F2 (8-epi-PGF2) excretion, an urinary marker for systemic oxidative
stress. It was found that after adjustment for potential confounding effects, the urinary concentration of 8-epi-PGF2 positively correlated with body mass index, cardiovascular disease, smoking and blood glucose, and suggested an important role for oxidative stress in the negative health effects of obesity (Keaney et al. 2003). In addition to the correlation between obesity and increased levels of systemic oxidative stress, levels of oxidative stress have been shown to be a predictor of negative health outcomes for several other diseases (Munch et al. 1998; Rosen et al. 2001), hence the modulation of oxidative stress potentially offers protection from the complication of these oxidative stress-associated disease states.

The involvement of oxygen in cancer was first suggested by Otto Warburg, who proposed a relationship between oxygen metabolism and carcinogenesis during the 1920’s (Warburg 1956). The subsequent identification of oxidants, such as $\cdotO_2^-$, resulted in a volume of research that connected oxidative damage with the initiation of cancer. Certainly, oxidative stress has been conclusively shown to induce cellular damage that may result in mutation and potentially cancer (Yakes & Van Houten 1997). Further, recent work has highlighted the potential for oxidative stress to play a role in the progression of cancer. Multiple factors result in the elevation of oxidative stress levels within a tumour, including glucose deprivation and hypoxia (Tew 1994; Spitz et al. 2000), in addition to macrophage infiltration (Brown & Bicknell 2001).

The consequences of oxidative stress in tumours is diverse and includes: the acceleration of tumour mutation rate that is associated with both cancer progression and poor treatment outcomes, the stimulation of tumour growth by induction of cellular growth signals (Wang et al. 2000), the increased expression of antioxidant enzymes that results in resistance to certain anticancer drugs (Tew 1994; Sander et al. 2004), the increased blood supply to tumour cells, and an increased risk of metastasis (Brown & Bicknell 2001; Gerald et al. 2004). Potentially the suppression of cellular oxidative stress may offer an avenue for both the prevention and suppression of cancer. However, intervention trials that have evaluated the effects of antioxidant therapy on cancer rates have been inconclusive at best (Bjelakovic et al. 2007). Both regulators of chemical and enzymatic antioxidant action, vitamin E and selenium, have been shown to protect from cancer in some studies (Albanes et al. 1996; Clark et al. 1998), but conversely beta-carotene has been shown to increase cancer rates in
other studies (Omenn 2007). Although the exact effect of regulating oxidative stress on the progression of cancer is unknown, it is clear that it is involved in the progression of the disease and antioxidant treatments may successfully target this.

In order to fully understand why and how compounds from fruits and vegetables may be important in combating oxidative stress, it is important to examine both the potential health benefits they confer and what their active forms are in the body after absorption and digestion.

### 1.4 Health benefits of fruit and vegetables

The benefits of consuming significant levels of fruits and vegetables in the diet have long been recognized. Epidemiological studies have shown that a high intake of plant based foods is inversely linked with rates of cancer, heart disease and many other degenerative age-related disorders. Although these benefits have long been acknowledged, as the data came primarily from epidemiologic studies (Block et al. 1992; Bazzano et al. 2002), little was known about the mechanisms beneath them. It was initially thought that this protection was conferred through vitamins present in the fruits and vegetables. However, large epidemiological studies using vitamin interventions have given unexpected results. The carotene and retinol efficacy trial (CARET) study gave in excess of 18,000 current and former smokers, as well as people exposed to asbestos, vitamin A and beta-carotene supplements based on the hypothesis it would reduce incidence of cancer. The result, however, was the exact opposite. In the supplemented group the incidence of lung cancer was 27% higher and overall mortality was 17% higher compared to the control group (Goodman et al. 1993; Goodman 2000). During the six year follow up period members from the supplemented group had a 12% greater risk of lung cancer, an 8% greater overall mortality and a 2% greater risk of death from CVD (Duthie & Bellizzi 1999).

Although the exact reasons why the beta-carotene intervention in the CARET trial failed are unclear, there are several possibilities including non-antioxidant effects of beta-carotene, variations in the baseline beta-carotene levels of participants and other
non-specified actions between beta-carotene and metabolites of chemicals present in cigarette smoke.

Of these afore mentioned possibilities, perhaps the most important is the potential non-antioxidant, hormonal effects of beta-carotene. Subsequent to the CARET trial a series of experiments were conducted, examining the link between cigarette smoke and beta-carotene using a ferret model that closely represent human beta carotene absorption and metabolism (Wang et al. 1999). These studies showed cigarette smoke and beta-carotene mediated induction of several phase 1 enzymes, altered retinoic acid concentrations and upregulation of retinoic acid receptor-b expression, as well as increases in several markers of cellular proliferation (Wang et al. 1999). Importantly, these changes were shown to be greater than those in animals given either only beta-carotene or only exposed to cigarette smoke (Wang et al. 1999). Given the increased cancer rate in the CARET study was only seen in the population that both smoked and received beta carotene supplementation (Goodman et al. 1993; Goodman 2000), and the subsequent mechanistic animal study showed increased markers of cellular proliferation in animals given both beta-carotene and exposed to cigarette smoke, it appears that this mechanism would have possibly been a significant contributor to the increased lung cancer rate observed in the CARET study. Additionally, the supplementation of beta-carotene in the CARET study resulted in serum beta-carotene levels that were greatly in excess of normal physiological levels (210 µg/dL vs 5-50 µg/dL for general population) (Goodman et al. 1993, Galen et al. 2005). Given the rational for this study was based on epidemiological data that showed populations with high beta-carotene intake had lower rates of cancer, the large discrepancy between the maximal normal serum concentration and the concentration achieved after supplementation, suggest that the study design may not correctly test the authors hypothesis.

Results from the CARET study, in addition to the results from several other large vitamin intervention studies, highlight the potential dangers of large intervention trials and also suggest that vitamins are probably not responsible for the protective qualities of fruits and vegetables (Bjelakovic et al. 2007). The CARET study did, however, in some respects validate the theory that dietary compounds can have significant effects
on human health and wellbeing, albeit a negative one. Studies using other vitamins have also given mixed results, with vitamin E protecting from cancer but increasing rates of hemorrhagic stroke in one study (Goodman et al. 1993) and vitamin C having no significant effect on rates of breast, gastric and other cancers in various studies (Zhang 2004; Jenab et al. 2006).

The lack of correlation between epidemiological studies and intervention trials using vitamins has lead to the suggestion that other phytochemicals present in fruits and vegetables are responsible for the observed protection from disease. Phytochemicals, such as carotenoids, phenolics, alkaloids, nitrogen containing compounds and organosulfurs, from fruits and vegetables are thought to, at least in part, confer their beneficial effects by acting as antioxidants and reducing the incidence of disease by protecting from oxidative damage to proteins (Labieniec & Gabryelak 2005), DNA (Jenab et al. 2006) and lipids (Lapointe et al. 2006). Indeed, most of the major constituents of fruit and vegetable extracts do have redox potentials that allow for the reduction of oxygen radicals and in vitro studies have shown oxygen radical scavenging ability (Jastrzebski et al. 2007). However, studies involving the consumption of many of these extracts have failed to show a significant corresponding increase in phytochemical levels in the blood and it has now become accepted that many of these phytochemicals are modified during the digestion and absorption process (Silberberg et al. 2006). The fate of phytochemicals throughout the digestive track is either to be absorbed without modification, or to be modified by conjugation or breakdown. Neither these phytochemical conjugates nor metabolic breakdown products necessarily have a significant or biologically important effect on blood antioxidant capacity (Day et al. 2000; Feng 2006).

1.4.1 Absorption and conjugation of phytochemicals

The digestion, absorption and subsequent modification of phytochemicals in humans are highly dependent on both the class and individual properties of the compound consumed. The digestion and absorption of polyphenols is reasonably well characterized and partially depends on the level of glycosylation (Manach et al. 2005). Aglycones or polyphenols with no sugar groups can be absorbed directly from the
stomach or small intestine, as is the case of chlorogenic acid (Lafay et al. 2006a). More complex polyphenols, whether they are polymers, esters or glycosides of the aglycone, can require hydrolysis before they can be absorbed. Absorption of polyphenols that require modification by gut bacteria, such as polyphenols with rhamnose groups (Nielsen et al. 2006), may potentially be influenced by both the genetic and dietary factors that determine an individual’s gut microflora profile.

As most polyphenols are hydrophilic they are unlikely to cross the gut wall without some form of active transport. Currently, only a few active transport methods for phenolic compounds have been identified. Cinnamic acid, a phenolic acid found in spinach, is absorbed through the rat jejunum by a sodium ion dependent active transporter (Wolffram et al. 1995) and ferulic acid appears to be transported by the monocarboxylic transporter (Konishi & Shimizu 2003), although others have suggested that phenolics merely bind this transporter and are not transported by it (Konishi et al. 2003). It is also likely that other polyphenols are also transported across the gut by active transporters, as many have been shown to interfere with various drug transporters, suggesting either a common or closely related specificity. Recent studies have indicated that transport of many phytochemicals is mediated by the actions of the ATP-binding cassette (ABC) transport proteins that can transport compounds out of the gut epithelial cells. This family includes P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) that have been shown to regulate the uptake of various phytochemicals, particularly the flavonoids (Li et al 2010.; Sesink et al. 2005; Wang et al. 2005b). Further, several polymorphisms in these transporters have been identified (Ieiri et al. 2006; Kerb 2006), suggesting a source of inter-individual variation in the absorption of phytochemicals. It has previously been suggested that the lack of competitive inhibition seen when the absorption of three phenolic compounds, ferulic acid, genistein and hesperetin, was tested using in-situ perfusion of rat intestine at physiological concentrations is indicative of there being no specific transport system (Silberberg et al. 2006). It is possible, however, that the reason for the lack of observed competitive inhibition was due to either the phenolics being absorbed via discrete mechanisms, or that the same mechanism was used but was not saturated. Regardless of the reason, it is perhaps difficult to come to such a conclusion when only three out of many thousands of dietary polyphenols were
tested. Perhaps a more correct conclusion would have been that the tested phenolic compounds do not share a common transport mechanism that can be saturated at physiological conditions.

Once transported into the epithelial cell of the gut the polyphenols are conjugated by the actions of various detoxifying enzymes (Silberberg et al. 2006). Conjugation of dietary polyphenols occurs at multiple stages of the digestion and absorption process, but most notably during the crossing of the gut wall and first-pass metabolism in the liver, where sulfation, methylation and glucuronidation occur (Silberberg et al. 2006). Conjugation changes the polyphenol’s redox potential and in many case means it cannot act as an chemical antioxidant (Day et al. 2000). After oral consumption several polyphenols have been shown to be present in plasma, un-modified and in considerable quantities. These polyphenols are not conjugated during absorption and can potentially act as antioxidants in vivo. Epigallocatechin gallate (EGCG), a catechin present in tea, already has a gallate conjugate on the third carbon of the C ring and is not necessarily modified during absorption (Henning et al. 2006). It is likely that this gallate conjugate prevents any further conjugation by metabolic enzymes in some individuals. It is also possible that other phytochemicals, which already have conjugates that are not removed during the process of digestion, also will not be further conjugated. The conjugation of polyphenols may potentially be a protective mechanism, as quercetin, a polyphenol from onions and apple skin, was shown to induce renal toxicity when the un-conjugated form was injected directly into the blood stream (Singh et al. 2004). Phytochemicals modified during absorption may not have antioxidant properties, but instead may act as direct modulators of cell signaling pathways or inducers of endogenous antioxidant enzymes.

1.5 Cancer etiology and disease prevention by phytochemicals

Extensive research has been carried out on the ability of phytochemicals to inhibit cancer progression and disease pathogenesis. However, a significant amount of this work has been conducted in vitro at the cell culture level and has examined markers
of cell death but not elucidated the mechanisms behind them. Many possible mechanisms of action for phytochemical regulation of disease have been suggested by *in vitro* experiments, including: inhibition of cell proliferation (Park et al. 2006), upregulation of oncogene expression (Lin 2004), regulation of signaling pathways (Khan et al. 2006), induction of cell differentiation, initiation of programmed cell death (Sadava et al. 2007), tumor suppression and also protection from cellular damage by antioxidant capacity, regulation of immune function and modulation of hormones and enzyme activity. In addition to this wide range of biological effects, phytochemicals are also likely to mediate multiple cellular effects, on a given cell type, concurrently. Couple this with the complex nature of the signaling system being regulated, the differences that occur in these processes between cell types and also the differences in these pathways that occur when a cell becomes cancerous, then this is likely to result in a significant variation in possible effects.

The divergent effects that phytochemicals can have on different cell types can be highlighted by examining results obtained with the polyphenol resveratrol. Resveratrol, an antifungal polyphenol found primarily in the skin of red grape but also to a lesser extent in other fruits, has been shown to have a variety of effects on the programmed cell death of different cell types. Resveratrol has been shown to have an anti-apoptotic effect *in vitro* in human neuroblastoma cells when apoptosis is induced with the anti-cancer drug Paclitaxel (Rigolio et al. 2005). Pro-apoptotic effects, mediated through the classic apoptotic pathway, have been seen in metastatic breast cancer cells treated with resveratrol (Sareen et al. 2006), and programmed cell death has also been induced via autophagy in ovarian cancer cells (Opipari et al. 2004). The reasons for these different effects are unclear. It is possible, however, that the same signaling pathway is being regulated but is having a different effect due to inherent differences between the cell types. It is also possible that different pathways are being regulated in the different cell types. While these studies highlight the variable effects a single compound can have, *in vivo*, phytochemicals are usually consumed as a mixture and more recently work has been conducted to examine how phytochemicals work synergistically to prevent disease.
1.6 Synergies between phytochemical extracts

Synergy, a process when the total effect of two variables is greater together than the sum of the two when apart, can occur when phytochemicals are combined with other phytochemicals, drugs or natural defense proteins. Initially much of the work in this area was done with cancer drug therapy and dietary polyphenols as it was thought that some of these polyphenols possessed properties, specifically estrogenic, which may have a significant effect on cancer progression and treatment. More interestingly, recent work has focused on the interactions between different phytochemicals and how these might synergistically protect from or enhance the progression of programmed cell death.

Genistein, a soy isoflavone, and beta-lapachone, a member of the naphthaquinones family, have both been shown to induce apoptosis and inhibit angiogenesis in cancer cells (Fotsis et al. 1993; Planchon et al. 1995; Li et al. 1999; Kung et al. 2007). Synergistic effects on apoptosis were shown when these two phytochemicals were administered at certain concentrations to human prostate carcinoma cells. Mechanistically, apoptosis induced by genistein was mediated primarily through the activation of the caspase 3 governed apoptotic pathways. Cell death induced by the treatment of the human prostate cancer cells by beta-lapachone was mediated primarily through a caspase-independent NAD(P)H:quinone oxidoreductase (NQO1) regulated pathway (Kumi-Diaka et al. 2004). The synergistic effect these two phytochemicals had on cell death is likely due to their regulation of two independent cell death pathways.

Antioxidant-based synergistic effects against interferon gamma (IFN-γ) induced nitric oxide generation in mouse macrophages has been show between EGCG and genistein when combined at low concentrations (Murakami et al. 2003). Interestingly, at higher concentrations EGCG and genistein were seen to act antagonistically, possibly due to one or both of the phytochemicals acting as oxidants, as seen with some vitamins at high concentrations. EGCG was also shown to act synergistically in combination with benzyl-isothiocyanate (BITC), a phytochemical from cruciferous plants, at low concentrations and antagonistically at high concentrations, suggesting a strong dose...
dependency for synergies between phytochemicals. The combination of all three of these phytochemicals was also seen to be synergistic at low concentrations (Murakami et al. 2003).

Furthermore, synergy can occur naturally and there is some evidence that components within a plant can work synergistically to suppress the proliferation of tumor cells. The individual components of a cranberry extract, when separated by HPLC and grouped into organic acids, anthocyanins, proanthocyanidins and total polyphenols, were found to be less effective than the whole extract (Seeram et al. 2004). A similar study, performed on fractions from pomegranates had similar results (Lansky et al. 2005). The pomegranate was divided into 3 fractions, fermented pomegranate juice polyphenols, pomegranate peel polyphenols and pomegranate seed oil. These fractions were tested by adding sub-therapeutic dosages of the peel polyphenols and oil to therapeutic dosages of the juice polyphenols. It was shown that the combination of the different groups resulted in synergistic inhibition of the proliferation of human prostate tumors.

The results from this initial research suggest several mechanisms of action for potential synergies between phytochemicals:

1. The protective synergistic effects of phytochemical antioxidants against oxidative damage when combined at a low dose.

2. The combined effect of polyphenol antioxidants at high concentrations to act synergistically as oxidants and sensitize cancer cell to cell death.

3. The induction of multiple programmed cell death pathways by multiple phytochemicals to synergistically induce cell death in cancer cells.

4. The synergistic inhibition of programmed cell death pathways by phytochemicals to protect healthy cells from oxidant, toxic or temperature related programmed cell death.
5. A combination of the above mechanisms

6. Regulation of the immune system, hormones or other physiological systems.

Further research into the mechanisms of phytochemical based synergies will allow for a better understanding of their potential health effects and may facilitate the development of designer diets and functional foods. However, past and current research has primarily evaluated non-bioavailable phytochemicals for synergistic effects and it is key that future work examines potential synergies between phytochemical metabolites when using *in vitro* systems.

Additionally, protective synergies against cell death have been demonstrated in the laboratory where the experiments presented in this thesis were conducted (unpublished data). Evaluating phytochemical metabolites for synergistic protection from cell death is perhaps the most likely system to yield biological synergies between phytochemical metabolites that are physiologically relevant.

### 1.7 Cell death

The process of cell death is complicated and by no means fully understood. There are different types of cell death and various mechanisms which induce them. The different types of cell death include apoptosis, necrosis, and autophagy and all are defined based on the morphological sequence of events that occur when the cell is dying.

#### 1.7.1 Apoptotic cell death

Of all the types of cell death perhaps the most biologically relevant is apoptosis.

Apoptosis or programmed cell death was first described in the 1970’s by Kerr, Wylie and Currie when they observed a specific regulated mechanism that was different to uncontrolled necrotic cell death (Kerr et al. 1972). It had long been accepted that there was a morphological pattern which dying cells followed, however, the work of Kerr
and his associates was the first to identify an identical series of events that occurred across multiple cell types during the process of cell death. Although this discovery was made over 30 years ago there has only recently been a surge of interest in apoptosis, as it has become apparent that the dysregulation of the apoptotic mechanisms is a key factor in multiple human diseases, such as cancer, autoimmune diseases and various neuro-degenerative disorders (Dickson 2004; Niculescu et al. 2004; Wang et al. 2005c).

A key part of the Wellness Foods program, the grant under which this research is being undertaken, is researching the potential anti-apoptotic benefits of phytochemical compounds and determining the molecular mechanisms that may be important factors for phytochemical related improvements in human health. In order to fully appreciate the possible effect that the regulation of apoptosis might have, it is important to understand both the morphological process of apoptosis and also the involvement of cell signaling pathways and key cell organelles.

1.7.1 The process of apoptosis

Apoptotic cells typically undergo a series of morphological changes that are caused by alterations to the cyto and nucleo-skeleton within the cell. These changes to the cyto- and nucleo-skeleton result in apoptotic cells losing contact with both neighboring cells and other adjacent structures. Protrusions from the cell membrane, known as blebs, start to occur and can continue for some time. The cell membrane becomes flipped resulting in the cytoplasmic side of the membrane becoming exposed on the outside of the cell (Schlegel, 2000). Condensation of the nucleus can also be observed if it is stained with certain DNA dyes, and it normally starts to occur around the nuclear envelope and expands from there. The cell then forms apoptotic bodies, or membrane bound vesicles that vary in size and shape, that contain the contents of the cell and these are then phagocytosed in vivo. The various cell organelles appear to last for a different amount of time before they are broken down. The endoplasmic reticulum (ER) breaks down early in the apoptotic process, however, the lysosome and the mitochondria appear to stay intact for some time. The exact morphology of apoptosis varies between different cell types and is linked to the regulatory signaling
cascades that occur within the cell during apoptosis. Apoptosis can take place through several different mechanisms, including receptor mediated and mitochondrial mediated cell death (Elmore 2007).

1.7.1.2 Receptor mediated cell death

The receptor mediated cell death pathway is one of the major apoptotic pathways in the body. It is regulated by many different external stimuli and its downstream effector molecules are the caspases. The internal signaling mechanisms within the cell differ depending on both the type of stimuli used and also the particular cell type so it is important to gather as much information as possible before performing experiments on this pathway.

The death receptor mediated cell death pathway is a major pathway for the induction of apoptosis in cells. Death receptors are present on the surface of the cell and act to propagate cell death signals triggered by the binding of receptor ligands, such as Fas (Apo1, CD 95), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor alpha (TNFα). These receptor ligands are produced by specialized cells, for example TNFα by macrophages, and act to induce apoptosis in the target cell in a rapid manner. The exact level of expression of the death receptors as well as the linked cell-signaling cascade that follows the binding of a ligand differs between cell type; however, there is a general consensus for the mechanism of action for this pathway in cells.

The binding of a specific ligand to the cell death receptor results in the grouping of cell death receptors. This grouping is caused by the generation of ceramide, a lipid molecule that is typically generated from hydrolysis of sphingomyelin but can also be produced by the ER. The release of ceramide causes the formation of cholesterol-enriched regions of the membrane, known as a lipid rafts. These lipid rafts allow for the movement of death receptors into large clusters that allows for the amplification of the cell death signaling. Lipid raft formation and the subsequent clustering of cell death receptors is not required for all cell types to undergo cell death via death receptor activation (Miyaji et al. 2005).
The binding of the ligand to the cell death receptor also induces a conformational change to the intracellular region of the receptor. This change exposes the death domain that allows for the binding of tumor necrosis factor receptor type 1-associated death domain protein (TRADD), Fas-associated protein with death domain (FADD) and then ultimately pro-caspase 8 to form a complete signaling complex. It is worth noting that different caspases can be recruited to the death domain, such as caspase 10 (Kischkel et al. 2001), and further, that non-caspase molecules can also be recruited competitively with the caspases. The recruitment of the Fas-associated protein with Death Domain-like IL-1beta-converting enzyme-like inhibitory protein (cFLIP) to the death domain in the place of caspase 8 can lead to the resistance of cells to receptor induced cell death (Baumler et al. 2003). After the recruitment of pro-caspase 8 to the death domain to form the completed signaling molecule, pro-caspase 8 is cleaved to form active caspase 8, which then goes on to trigger the caspase cascade, and ultimately apoptosis. The exact mechanism of signaling depends on which of the death receptors is activated and differs slightly between TNFα, TRAIL and Fas.

1.7.1.2.1 TNFα receptor signaling

TNFα is a cytokine that can have a variety of effects on cells including induction of cellular proliferation, differentiation, tumorigenesis as well as apoptotic cell death (Liu 2005). TNFα has two receptors, tumor necrosis factor receptor 1 (TNF-R1) and tumor necrosis factor receptor 2 (TNF-R2). TNF-R1 is expressed as both a soluble and a membrane-bound form (Manach et al. 2005). TNF-R2 is only found expressed on cells from the immune cell lineage and is not as well characterized as TNF-R1. TNF-R1 is the major TNF receptor, it is expressed in most tissues and its activation by the binding of TNFα can have several different effects. TNF-R1 signaling can lead to the activation and relocation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) into the nucleus, where it can act to regulate the expression of many genes related to injury, stress and immune response (Chen & Goeddel 2002). It is worth noting that NF-κB can have both pro-apoptotic and anti-apoptotic effects. TNF-R1 signaling can also activate activator protein 1 (AP-
1), a group of leucine zipper transcription factors consisting of the Fos and Jun families, which also affect inflammation, differentiation and apoptosis (Natoli et al. 1997). Furthermore, NF-κB and AP-1 may positively regulate each other, potentiating their possible anti-apoptotic effects (Fujioka et al. 2004). The process of receptor binding causes the three intracellular death domains to trimerise allowing for the binding of TRADD (Jones et al. 1999).

TRADD is an adaptor protein that has roles both in facilitating the binding of other proteins to the signaling complex and also in the propagation TNF signaling. TRADD mediates the binding of several other proteins including TRAF-2, a NF-κB stimulating protein, and receptor interacting protein (RIP), a serine-threonine kinase, to the signaling complex (Jackson-Bernitsas et al. 2007). TRADD may also modulate TNF signaling by regulating the generation of ceramide by sphingomyelinase upon TNFα activation of TNF-R1 receptor (Schwandner et al. 1998). TRADD can also induce apoptosis by binding and cleaving pro-caspase 8 to active caspase 8. It is worth noting that activation of the TNF-R1 receptor on its own does not normally lead to the induction of apoptosis, as the activation of its death receptor often fails to cause significant activation of downstream signaling molecule caspase 3.

1.7.1.2.2 Fas Receptor Signaling

In many ways the Fas receptor works like a simplified TNFα receptor. The Fas receptor can also induce apoptosis through the cleavage of pro-caspase 8 to active caspase 8; however, it does not require the binding of TRADD to the death domain to facilitate the binding of FADD (Kischkel et al. 2000). As TRADD is not bound to the Fas receptor other proteins such as TRAF-2, which bind to the TRADD on the TNF-R1 receptor signaling complex, do not bind to the Fas receptor signaling complex. This results in the Fas signaling complex primarily affecting apoptosis signaling pathways. The major physiological roles of Fas induced apoptosis are the killing of cancer or virus infected cells by cytotoxic T-cells, the induction of apoptosis of immune cells in immune privileged sites and also the killing of activated T-cells after an infection when the immune response is no longer needed (Roberts et al. 2003). Mutation in the genes encoding either Fas or the Fas receptor can lead to Autoimmune
Lymphoproliferative Syndrome (ALPS), an autoimmune disorder where peripheral lymphocytes fail to die. This results in the enlargement of the lymph nodes and ultimately death (Oliveira & Fleisher 2004). Knock out studies in mice show that expression of FADD is essential for the induction of apoptosis by Fas. Interestingly it has been shown that FADD knockout mice have reduced T-cell proliferation and that FADD deletion causes embryonic death. This indicates that Fas also has a role in cellular signaling and further studies have shown it is required for thymic development and T cell homeostasis (Zhang et al. 2005).

1.7.1.2.3 TRAIL Receptor signaling

Unlike Fas, which is limited to natural killer cells and activated T-cells, the TRAIL receptor is expressed constitutively in many different cell types. Unlike Fas, it also, does not appear to require FADD to induce caspase mediated apoptosis. Interestingly, there are four receptors for the TRAIL ligand that can either induce apoptosis or compete for binding of the TRAIL ligand and hence protect from TRAIL-induced apoptosis. The TRAIL receptors DR4 and DR5, when stimulated with a TRAIL ligand, can rapidly induce apoptosis. How they do this is of some debate. It has been shown that DR4 induced cell death is dependent on FADD (Chaudhary et al. 1997), while other papers show non-binding TRAIL receptors (or decoy receptors) DcR1 and DcR2 bind to the TRAIL ligand but do not induce apoptosis. The DcR1 receptor lacks the cytoplasmic death receptor and its expression desensitizes cells to TRAIL induced apoptosis. Conversely, its removal by treatment of the cell with a phospholipase increases the cell’s sensitivity to TRAIL-induced apoptosis. The DcR2 receptor acts like the DcR1 receptor in that it reduces sensitivity of the cells to TRAIL-induced death. The DcR2 receptor does have a cytoplasmic domain, however it lacks between four to six amino acids that are vital for propagation of the cell death signal. Even removal of the entire cytoplasmic domain does not affect its inhibitory function (Merino et al. 2006). Interestingly, the polyphenol quercetin has been shown to increase TRAIL-induced apoptosis by inducing the accumulation of lipid rafts and hence increasing the TRAIL signaling (Psahoulia et al. 2007).
1.7.2. Mitochondrial mediated cell death

The mitochondria are key cell organelles with an important role in energy production, as well as being involved in multiple cell signaling pathways. They are also the organelle at the heart of the second major apoptotic pathway, the mitochondrial mediated or intrinsic cell death pathway. The mitochondrial mediated cell death pathway can be activated by numerous stimuli, including cross-activation by caspase 8 triggered by the receptor-mediated pathway (Afshar et al. 2006), genotoxic factors (Kaina 2003), UV light (Bivik et al. 2006), growth factor withdrawal (Cornelis et al. 2005) and excessive oxidative stress (Mao et al. 2006). Perhaps the most important event in this pathway is the permeabilization of the mitochondrial membrane. When the mitochondrial membrane is permeabilized, various small molecules including cytochrome c, endonuclease G and apoptosis inducing factor (AIF), leak out of the membrane transition pores into the cytoplasm. Once in the cytoplasm these mitochondrial proteins can induce apoptosis by several different mechanisms. The release of these mitochondrial proteins from the mitochondria and hence the control of the mitochondrial transition pores is primarily governed by the B-cell lymphoma 2 (Bcl-2) family of proteins. The Bcl-2 family of proteins consists of anti-apoptotic proteins that contain four Bcl-2 homology (BH) domains, and pro-apoptotic proteins, which contain between 1 and 3 BH domains.

1.7.2.1 Bcl family of Proteins

The Bcl family of proteins controls the release of mitochondrial proteins by regulating the membrane transition pore, which comprises of the voltage-dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocator (ANT) in the inner membrane (Shimizu et al. 1999). The Bcl proteins are up-stream regulators of the mitochondrial cell death pathway and can be either pro- or anti-apoptotic. They can be divided into different classes, depending on this end result. The anti-apoptotic class includes Bcl-2, B-cell lymphoma-extra large (Bcl-xL) and Bcl-2-like protein 2 (Bcl-W) and the pro-apoptotic class includes Bcl-2–associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), BH3 interacting domain
death agonist (Bid), Bcl-2-associated death promoter (Bad), and Bcl-2-like protein 11 (Bim) (Kim et al. 2006).

**1.7.2.1 Bax and Bak**

The pro-apoptotic proteins Bax and Bak are present in cells in an inactive state, until stimulation by a cell death signal causes them to undergo oligomer dimerisation. This causes them to be activated, internalized into the membrane of the mitochondria and mediates the release of mitochondrial components. Exactly how they regulate the efflux of the mitochondrial components is still not clear, but there are several theories. Bax and Bak oligomers may form pores that are large enough for the escape of mitochondrial proteins, or Bax and Bak may form complexes with the mitochondrial membrane proteins, adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC), which allows for the release of the mitochondrial proteins. Both of these possible mechanisms can be backed up with evidence; the former, with the fact that the oligomers of Bax and Bak are similar in structure to pore forming bacterial toxins and the latter by the fact that the blocking of the VDAC pore with antibodies can stop the efflux of cytochrome c and stop apoptosis (Kim et al. 2006). Given that similarities in protein structure do not guarantee the same function and that apoptosis has been shown to be prevented by blocking of VDAC, the evidence would lean towards the regulation of the VDAC being the mechanism by which Bax and Bak have their effect. Recently it has been shown that Bax complexes can be used to generate pores in artificial membranes through which cytochrome c can be released (Pavlov et al. 2001). Although this does not prove that Bax complexes are the major means by which cytochrome c is released, it may indicate that a Bax generated pore might release a small amount of mitochondrial proteins at a sub-apoptotic level. Other pro-apoptotic members of the Bcl-2 family, such as Bad and Bim, have a different mechanism of action.

**1.7.2.1.2 Bad, Bim and Bid**

Bad, Bim and Bid structures differ from those of Bax and Bak in that they only have one domain (the BH-3 domain) in common with the anti-apoptotic family, whereas,
Bax and Bak have 3 of the four BH domains in common (Kim et al. 2006). They, along with other members of the BH-3 family, BMF, Noxa, Puma and Bcl-G, affect their pro-apoptotic role by a different mechanism than that of Bax and Bak. Although the exact mechanism by which this family works is not fully understood, they are thought to be not as important as Bax and Bak, as a Bax/Bak knock-out cell does not release cytochrome c from the mitochondria. The role of the BH-3 family appears to depend on the specific protein and there are three potential functions which BH-3 proteins can perform; (i) they can activate Bax and Bak, once they are located in the mitochondria, ii) induce the dissociation of Bax and Bak from Bcl-2 and Bcl-xL protein, there by inducing pro-apoptotic function iii) or bind to the anti-apoptotic proteins, such as Bcl-2, before these proteins can bind to Bax and Bak and prevent their activation (Willis & Adams 2005).

1.7.2.2 P53

Protein 53 (P53) is a key anti-tumor transcription factor that is activated in response to multiple cell insults including oncogene expression, oxidative stress and DNA damage. It can activate DNA repair enzymes, cause cell cycle arrest at G1/S phase and can also induce apoptotic cell death. P53 induces apoptosis via multiple mechanisms and it appears to do so by acting as both a transcriptional factor and by direct activation of signaling pathways. P53 has been shown, in transformed baby rat kidney cells, to inactivate Bcl-2 through activation of the c-Jun N-terminal kinase 1 (JNK1) pathways via up-regulation of the gene encoding for cdc42, a small G protein (Thomas et al. 2000). P53 also upregulates the expression of various other pro-apoptotic genes, including members of the Bcl-2 family and members of the cell death receptor family. The evidence for P53 acting directly as a mediator of cell death includes its ability to activate Bax (a pro-apoptotic protein) in the absence of other members of the Bcl-2 family that act to permeabilize the membrane of the mitochondria (Chipuk et al. 2004). P53 has also been shown to bind to several anti–apoptotic members of the Bcl-2 family, Bcl-2 itself and Bcl-xL, which may allow for the subsequent activation of the now unbound pro-apoptotic proteins such as Bax (Mihara et al. 2003).
Much is still not known about the exact mechanisms by which many of the proteins involved in the mitochondrial cell death pathway function, particularly how they interact with each other and how they result in the loss of mitochondrial membrane integrity. Given the level of uncertainty around how certain areas of this pathway function it is best to focus on the regulation of core proteins that have an important and more defined role in inducing apoptosis, such as the Bax and Bak proteins. As with the receptor-mediated cell death pathway the downstream effector molecules are the caspases, a family of cysteine proteases.

### 1.7.3 The caspase cascade

Previously it has been mentioned that pro-caspase 8 and an inhibitory protein c-Flip can be bound to the complete signaling complex at the cytosolic side of a death receptor (Zhang & Fang 2005). The exact result of the binding of caspase 8 to the receptor depends on the type of cell the process occurs in. In type 1 cells, which includes lymphoid cell lines, there is a strong activation of caspase 8 that leads to the cleavage of pro-caspase 3 into active caspase 3 (Budd et al. 2006). In type 2 cells there is not a strong activation of caspase 8 and hence caspase 3 is not activated. However in these type 2 cells the activation of caspase results in the activation of Bid which can lead to the activation of the mitochondrial cell death pathway. Caspase 10 also possesses a DED-containing pro-domain so can also be activated in response to death receptor binding. Caspase 10 activation is found primarily in lymphoid cells and does not require caspase 8 to initiate apoptosis (Wang et al. 2001a). It is also possible that caspase 8 and 10 have different downstream substrates and hence may have differential effects on cellular signaling (Cowling & Downward 2002). The last of the caspase family that can bind to the death receptors is caspase 2, which requires the binding of adaptor molecules, such as the receptor interacting protein (RIP) and ICH-1, a RIP-associated homologous protein. Very little is known about the signaling targets of caspase 2, although it has been postulated that caspase 2 may also be an effector molecule for the mitochondrial based cell death pathway and may function independently of Apoptotic protease activating factor 1 (APAF-1) (Read et al. 2002). Receptor mediated activation of the caspases is outlined in Figure 1.
1.7.3.1 Cell receptor mediated caspase activation.

Previously it has been mentioned that pro-caspase 8 and an inhibitory protein c-FLIP can be bound to the complete signaling complex at the cytosolic side of a death receptor (Zhang & Fang 2005). The exact result of the binding of caspase 8 to the receptor depends on the type of cell the process occurs in. In type 1 cells, which includes lymphoid cell lines, there is a strong activation of caspase 8 that leads to the cleavage of pro-caspase 3 into active caspase 3 (Budd et al. 2006). In type 2 cells there is not a strong activation of caspase 8 and hence caspase 3 is not activated. However in these type 2 cells the activation of caspase results in the activation of Bid which can lead to the activation of the mitochondrial cell death pathway. Caspase 10 also possesses a DED-containing pro-domain so can also be activated in response to death receptor binding. Caspase 10 activation is found primarily in lymphoid cells and does not require caspase 8 to initiate apoptosis (Wang et al. 2001a). It is also possible that caspase 8 and 10 have different downstream substrates and hence may have differential effects on cellular signaling (Cowling & Downward 2002). The last of the caspase family that can bind to the death receptors is caspase 2, which requires the binding of adaptor molecules, such as the receptor interacting protein (RIP) and ICH-1, a RIP-associated homologous protein. Very little is known about the signaling targets of caspase 2, although it has been postulated that caspase 2 may also be an effector molecule for the mitochondrial based cell death pathway and may function independently of Apoptotic protease activating factor 1 (APAF-1) (Read et al. 2002). Receptor mediated activation of the caspases is outlined in Figure 1.
The mitochondrial cell death pathway can activate multiple caspases (Figure 2). This activation of caspases occurs after the Bcl-2 family of proteins induces the opening of the mitochondrial pore. The release of mitochondrial cytochrome c allows for the formation of the apoptosome, which is a large complex of cytochrome c, APAF-1 and pro-caspase 9. This results in the formation of active caspase 9 that can activate the effector caspases 3 and 7. Active caspase 3 can in turn activate pro-caspase 9 to create
a positive feedback loop (Jiang & Wang 2000). Pro-caspase 8 can also be activated via the mitochondrial cell death pathway. The release of cytochrome c from the mitochondria results in the activation of caspase 6 from pro-caspase 6. Active caspase 6 can activate pro-caspase 8 without any signaling from the receptor mediated cell death pathway and hence caspase 8 activation can occur without the stimulation of death receptors (Cowling & Downward 2002).
1.7.3.3 Substrates of the effector caspases.

The effector caspases consist of caspase 3, 6 and 7, all of which are highly homologous, likely have redundant function and share many of the same downstream substrates. Caspase activation leads to a number of downstream effects that can be related to the specific substrate being cleaved. Cell shrinkage, membrane blebbing and other morphological changes that occur during apoptosis result from the cleavage of a series of proteins such as lamin A, actin, growth arrest-specific protein 2 (GAS2)
and fodrin, which are important in nucleo- and cyto-skeleton formation in the cell. The cleavage of lamin A, an intermediate filament protein that lines the inner surface of the nucleus, can lead to the breakdown of the nuclear membrane, while the cleavage of fodrin can result in membrane blebbing and the formation of apoptotic bodies. DNA damage is mediated through the dissociation of the inhibitor of caspase activated deoxyribonuclease (ICAD) from caspase-activated deoxyribonuclease (CAD), which results in the breakage of double-stranded DNA into single-stranded DNA. The effector caspases also cause the cleavage of various DNA repair mechanisms, such as poly ADP ribose polymerase (PARP), a protein that repairs single strand DNA nicks, and DNA-dependent protein kinase catalytic subunit (DNA-PK), a nuclear serine/threonine protein kinase required for repairing DNA double-strand breaks. The effector caspases also cleave topoisomerase 1, a protein that allows for the correct formation of the DNA double helix by cutting and rejoining the DNA. Active caspase 3 also activates pro-caspase 3, 8, 9 and 10, resulting in propagation of the caspase signaling cascade (Sattar et al. 2003).

Although the caspase family of proteins is perhaps the most important family in the process of apoptosis and have key roles in the receptor and mitochondrial mediated cell death pathways, they also have roles in apoptosis induced by the other pathways such as the ER base cell death pathway and the lysosomal cell death pathway (Guicciardi et al. 2004; Momoi 2004).

1.7.4 Autophagy mediated cell death

The concept of autophagy mediated cell death is relatively new and not well understood. Autophagy is the process by which long-lived organelles are degraded by a lysosomal pathway (Ferraro & Cecconi 2007). Autophagy is interesting in that may be responsible for both the resistance to and initiation of cell death, as the degradation of unneeded or surplus cell organelles may provide energy for a stressed cell to survive, but it may also lead to the loss of too many organelles for the cell to continue to function (Ferraro & Cecconi 2007). Autophagy can be identified by the formation of autophagy vacuoles within the cell, and it has an interesting role in the neuronal cell death seen in aging and in neurological diseases such as Alzheimer’s disease. The
involvement of autophagy mediated cell death in Alzheimer’s disease is interesting in that the cells undergoing autophagy can take an extended amount of time to die. During this time the cells develop many autophagy vacuoles and it is thought that this may be the cause of eventual cell death (Bursch & Ellinger 2005).

Autophagy mediated cell death can act as a redundant form of cell death when cells are unable to die via other mechanisms. Mice fibroblasts that lack the Bax and Bak proteins will undergo autophagy when induced with a stimulus that would normally trigger apoptosis (Shimizu et al. 2004). Also, autophagy mediated cell death can be induced in L929 cells when caspase activation is inhibited (Yu et al. 2004). Although autophagy mediated cell death is not likely to be important in most experimental systems it is important to understand that compounds that block a stage in the apoptotic cell death pathway may cause the cell to die by autophagy mediated cell death. Autophagy is governed by both growth factors and cellular energy levels, and is summarized in Figure 3.
1.7.5 Necrotic cell death

The molecular mechanisms of necrosis or un-programmed cell death are yet to be fully characterized. One theory is that necrosis results from insufficient energy levels within the cell. Since the process of apoptosis places huge energy demands on the cell, a lack in reserves to undergo apoptosis might trigger a switch to necrosis. Multiple cellular events occur during necrosis that are related to the generation of cellular adenosine-5'-triphosphate (ATP). During necrosis the internalization of glucose receptors limits the supply of glucose for the generation of ATP (Ducluzeau et al. 2002). Decreases in ATP production from the mitochondria lead to both oxidative stress and an influx of calcium ions that results in the activation of phospholipase A2 which degrades the mitochondrial membrane (Malis & Bonventre 1988). Calcium influx during necrotic cell death is additionally associated with the

Figure 3: The general signaling pathway for autophagy.
activation of calpains that are themselves associated with lysosome destabilisation though a mitochondrial mediated mechanism (Vanlangenakker et al. 2008).

As with autophagy mediated cell death, perhaps the most important aspect of necrosis, is that the inhibition of certain pathways, the relative levels of a death-inducing agent and cell density can all cause a cell to undergo necrosis instead of apoptosis. Examples of this can be seen in multiple cell types. Jurkat cells stimulated with low concentrations of H₂O₂ undergo apoptosis, however, as H₂O₂ concentrations are increased, the cells switch to necrosis (Saito et al. 2006). Embryonic rat cortical neurons under starvation conditions undergo apoptosis when at a high density (Fujita et al. 2001), but necrosis at a low density (Fujita & Ueda 2003). In addition, A3.01 T-cells change from dying by apoptosis to dying via necrosis if caspase activation is blocked, after stimulation by the Fas ligand (Scheller et al. 2006).

1.7.6 Lysosomal mediated cell death

Lysosomes are the cell organelles responsible for digesting unwanted cellular components whilst simultaneously protecting the rest of the cell from the damaging digestive enzymes they contain. Lysosomes have several roles within the cell, including the endocytosis of cell surface receptors, the digestion of foreign matter that has been phagocytosed by immune cells and the mediation of two forms of programmed cell death; autophagy and apoptosis.

1.7.6.1 The role of lysosomes in apoptosis

Much like the role of mitochondria in apoptosis, lysosomes are also involved in the loss of membrane integrity and the leakage of inter-lysosomal proteins into the cytoplasm. Several different mechanisms can induce the leaking of the lysosomal membrane, such as oxidative stress, increases in extra-lysosomal sphingosine and the action of the Bcl-2 family of proteins (Terman et al. 2006). It is worth noting that both the generation of sphingosine and the activity of the Bcl-2 family of proteins are related to the receptor mediated and mitochondrial mediated apoptosis pathways respectively and represent a point of cross-talk between these pathways. Oxidative
stress can lead to the destabilization of the lysosome membrane and the subsequent release of lysosomal components. There are several possible mechanisms by which oxidative stress triggers the release of these enzymes. i). Lysosomal oxidative stress has been shown to be mediated, at least in part, by the actions of intra-lysosomal iron. A potent iron chelator, desferrioxamine, was added to cells in both a conjugated form that localizes in the lysosome and a free form that distributes throughout the cell. It was shown that the cells, with both the intra-lysosomal as well as the cytosolic iron chelator, were then resistant to lysosome rupture by the oxidant generator H$_2$O$_2$ (Persson et al. 2003). Interestingly, the polyphenol resveratrol has been shown to have the ability to regulate iron homeostasis and also protect cells from H$_2$O$_2$-induced apoptosis suggesting a possible mechanism of action (Jang & Surh 2001; Mokni et al. 2007). ii). Exposure to low dosages of H$_2$O$_2$ over several hours has been shown to induce cell death via the destabilization of both the lysosomal and mitochondrial membranes in both a murine histiocytic lymphoma cell line and also a human foreskin fibroblast cell line; this destabilization has been shown to be associated with the oxidative activation of phospholipase A2 (PLA2). Further, direct micro-injection of a secreted form of PLA2, sPLA2, was shown to induce lysosomal destabilization and apoptosis. It is also worth noting that the over expression of Bcl-2 by a lymphoma cell line was shown to prevent the oxidant induced activation of PLA2 and prevent apoptosis (Zhao et al. 2001). iii). Lysosomal enzymes that have escaped due to rupture of the lysosome can trigger membrane leakage from the mitochondria, which in turn induces more damage to the lysosomal membrane and hence creates a positive feedback loop (Zhao et al. 2003). In addition, the release of cathepsins, a family of serine proteases, has been shown to induce apoptosis in several cell types, and has been shown to have a role in both caspase dependent and caspase independent cell death. Interestingly, their release from the lysosome does not necessarily require the loss of lysosomal integrity, implying some sort of active transport process (Bidere et al. 2003).

The lysosomal mediated cell death pathway is interesting in that it has potential cross talk with the two major caspase-mediated apoptotic pathways, the receptor and mitochondrial mediated pathways, and hence maybe activated by either of these pathways. The endoplasmic reticulum mediated cell death pathway, a pathway that
can lead to caspase-independent cell death, can also activate the lysosomal cell death pathway. A basic summary diagram of the lysosome’s role in cell death is shown in Figure 4.
1.7.7 Endoplasmic reticulum mediated cell death

The ER is another cell organelle that plays an important role in cell death. The ER’s role in the cell is primarily to allow for the correct folding of proteins and also to allow for either the secretion of proteins or the transport of proteins to the correct part of the cell. The ER’s role in cell death is related to two different pathways; calcium...
homeostasis and protein misfolding. The ER has a significant role in calcium homeostasis as it contains both calcium binding proteins as well as calcium channels for the release of calcium into the cytosol. Once calcium is released into the cytosol, the calpain family of cysteine proteases are activated (Wang 2000). There are two classes of calpains and they are distinguished from each other based on the amount of calcium they require to be activated. The \( \mu \)-calpains are activated by \( \mu \)M amounts of calcium, where as the \( m \)-calpains are activated by mM amounts of calcium (Croall & DeMartino 1991). Calpains are produced as pro-enzymes, which are cleaved into their active states, and although they do not appear to directly induce apoptosis they may play a role in the activation of proteases that do. The pro-apoptotic actions of \( m \)-calpains have been shown to be mediated in part through caspase 12 activation (Nakagawa & Yuan 2000). Caspase 12 is localized on the cytoplasmic side of the ER, it has been seen to undergo activation during ER-stress and is thought to induce apoptosis by either direct nuclear translocation (Fujita et al. 2002) or by activating other caspases (Wootz et al. 2004). However, recently there have been doubts cast as to whether caspase 12 is the true inducer of ER mediated apoptosis. i) The suppression of caspase 12 only slightly suppresses beta-amyloid induced ER mediated apoptosis (Nakagawa et al. 2000), although this could merely indicate a secondary redundant system for activation of ER mediated cell death. ii) Caspase 12 is truncated in most of the human population and is only present in its full length form in some sub-Saharan Africans and a small proportion of Semites (Kachapati et al. 2006). This means that the work done on animal cell lines is not relevant to the human population as a whole.

As with most of the previous cell death pathways the ER mediated cell death pathway can cross-talk with other cell death pathways, such as the lysosomal and the mitochondrial. Also like many of the other cell death pathways, it can be activated by excess oxidative stress. Excess oxidative stress does not just induce cell death by damaging membranes, as is seen in the mitochondrial, lysosomal and ER mediated pathways, but can also act to trigger oxidant sensitive proteins that in themselves can trigger cell signaling pathways, as is the case with the c-Jun N-terminal kinase (JNK) cell signaling pathway. Below is a summary diagram for ER mediated cell death in Figure 5.
1.7.8 JNK, the oxidant regulated cell signaling pathway

JNK is a member of the mitogen activated protein kinase family that is often triggered in response to oxidative stress. The downstream targets of JNK include p53, c-Myc, a proliferation regulating transcription factor, AP-1 and the Bcl-2 family of proteins. Given its wide variety of targets it is hardly surprising that the JNK pathway is
involved in many cellular functions, such as, apoptosis, differentiation and proliferation. The stimulation of JNK activation generally occurs in response to oxidative stress, however it can also be activated by the cell death ligands FasL and TNFα, indicating activation by the receptor mediated cell death pathway, and also by UV radiation, which induces the mitochondrial cell death pathway, suggesting possible cross-talk between these pathways (Liu & Lin 2005).

1.7.8.1 JNK activation by oxidative stress.

JNK can be activated by both exogenous and endogenous oxidative stress (Martindale & Holbrook 2002; Ueda et al. 2002). H$_2$O$_2$ can be used as an exogenous activator of the JNK pathway (Torres 2003), as can menadione, a generator of oxygen radicals (Ma et al. 2002). These oxidative stressors can activate several different proteins, including apoptosis signal-regulating kinase 1 (ASK-1). ASK-1 activation has been shown to be regulated by two redox sensitive proteins, thioredoxin (Trx) and glutaredoxin (Saitoh et al. 1998; Song et al. 2002). Trx and glutaredoxin bind to and inhibit the ASK-1 mediated activation of JNK mediated signaling. However, increases in oxidative stress lead to the dissociation of thioredoxin and glutaredoxin from ASK-1 and hence the activation of JNK (Song & Lee 2003).

Src kinase is also another redox sensitive protein that can activate JNK (Yoshizumi et al. 2000). Src kinase has been shown to be a specific repressor of JNK activation and has been shown to be a regulator of JNK in H$_2$O$_2$ treated endothelial cells (Chen et al. 2001). It is currently not clear how Scr regulates the activation of JNK, however, it has been shown that the GRB2-associated-binding protein 1 (GAB1) protein may play a role by binding to Scr and allowing for the activation of JNK signaling (Ingham et al. 2001). JNK is also regulated by glutathione s-transferase Pi (GSTπ). GSTπ binds directly to suppress its activity (Wang et al. 2001b); this binding is, however, removed by the addition of H$_2$O$_2$ that generates oxidative stress (Adler et al. 1999).

JNK mediates cell death by both apoptosis and necrosis. Apoptotic cell death appears to be regulated primarily through the mitochondrial cell death pathway. Primary murine embryonic fibroblasts, which were a double knock-out for JNK 1 and 2, were
not killed by UV light induced stress due to a failure of the mitochondrial cell death pathway (Tournier et al. 2000). Further, JNK activation has been shown to be able to both inhibit the anti-apoptotic function of Bcl-2 (Yamamoto et al. 1999), and also enhance the action of Bax (Tsuruta et al. 2004). Interestingly, polyphenols have been shown to regulate JNK activation. EGCG has been shown to increase metalloproteinase-7 production in human colorectal cancer cells via activation of the JNK pathway (Kim et al. 2005) and isothiocyanate induced JNK in a human prostate cancer cell line (PC-3) cells (Xu et al. 2006). A summary diagram of the JNK-mediated cell death pathway is shown in Figure 6.
1.8 Regulation of direct and indirect antioxidant actions

Although the modulation of cell death signaling has been shown to be a mechanism by which phytochemicals can regulate cell death, it is possible that much of their protective effects are due, in part, to either direct chemical or indirect enzymatic antioxidant action. Direct chemical antioxidant action, via the direct chemical
quenching of oxidants, has long been thought of as the major mechanism by which phytochemicals mediate their protective effects (Torel 1986). During direct chemical antioxidant action phytochemicals are thought to work in a similar fashion to the antioxidant vitamins, and act to delocalize free radicals and hence prevent cellular damage. Indirect antioxidant action, via the upregulation of endogenous antioxidant enzymes, such as catalase, SOD and thioredoxin (Trx) (Hintze et al. 2005; Villa-Cruz et al. 2009) is thought to be due to an adaptive response by the cell in response to phytochemical treatment (Mattson & Cheng 2006). These endogenous antioxidants act to process oxidative stress within the cell and can directly reduce oxidants by enzymatic processing, rather than by acting as chemical antioxidants. Over recent years a growing body of evidence suggests that the majority of antioxidant action in a living organism is due to indirect enzymatic antioxidant action, rather than direct chemical antioxidant action (Stevenson & Hurst 2007).

1.8.1 Direct chemical antioxidant capacity

As discussed previously, oxidative stress is caused by the relative increase of oxidants to antioxidants within the cell or body and is associated with both the cause and progression of many disease states. In plants many phytochemicals, in particular phenolic acids and polyphenols, are present in great abundance and are certain to have significant chemical antioxidant effects. Further, their production is often induced in response to stress conditions, such as UV light for anthocyanins and fungal infection for resveratrol (Lindoo & Caldwell 1978; Roldan et al. 2003). Given this, there is certainly an important antioxidant role for phytochemicals in plants and it would seem likely that these dietary compounds could potentially have the same effect in animals. In vitro work has indeed shown certain phytochemicals to have high levels of chemical antioxidant action, with quercetin having a redox potential that allows for the reduction of many radicals (Bors et al. 1995). However, recently doubt had been cast as to whether the dietary consumption of phytochemicals can contribute to increased chemical antioxidant capacity of blood and also, whether increasing the chemical antioxidant capacity of blood has long term health benefits. Many phytochemicals, in particular polyphenols, do not appear to have a biologically important effect on blood antioxidant levels (Stevenson & Hurst 2007). Most of the
blood antioxidant capacity appears to be due to other circulating chemical antioxidants, such as uric acid (Stevenson & Hurst 2007). Further, when an increase in the chemical antioxidant capacity of the blood is detected, it has often been shown to be due to changes in non-phytochemical endogenous chemical antioxidants (Stevenson & Hurst 2007). Perhaps more importantly, there is growing evidence that increased direct antioxidant capacity of blood is not beneficial to long term health. Several large epidemiological studies have shown that direct antioxidant vitamins do not offer protection from smoking induced diseases and a more recent meta-analysis showed no beneficial effect for vitamin A, C or E supplementation (Goodman et al. 2004; Miller et al. 2005; Bjelakovic et al. 2006). A growing body of evidence points towards the regulation of indirect antioxidant actions as a potential mechanism for dietary phytochemical mediated protection from disease and damage (Stevenson & Hurst 2007).

1.8.2 Indirect endogenous enzymes mediated antioxidant action

Endogenous antioxidant enzymes are involved in the processing of many different oxidants within the body. Some enzymes are potentially more important than others, specifically catalase, SOD, and thioredoxin reductase (TrxR), which represent enzymes with specialised function, as well as general markers of oxidative status within the cell. They often function together and potentially interact with chemical antioxidants, to remove excessive oxidative stress.

Catalase is a potent enzyme that catalyses the decomposition of H$_2$O$_2$ into water and molecular oxygen. It is present in almost all forms of life and has one of the highest turnover rates of any enzyme, allowing it to process a large amount of H$_2$O$_2$ relative to the amount of catalase present. Elevation of H$_2$O$_2$ through increased endogenous generation, as well as exogenous addition, causes a variety of cellular effects in vitro, ranging from modulation of oxidative sensitive cell signalling through to induction of apoptosis and necrosis (Bhat & Zhang 1999; Teramoto et al. 1999). Cytotoxic effects of H$_2$O$_2$ are mediated through its ability to induce oxidative damage to cells, which results in lipid peroxidation, formation of protein carbonyls and direct DNA damage (Imlay et al. 1988; Sohal et al. 1995; Bechoua et al. 1999). Although H$_2$O$_2$ itself is not
generally associated with human disease, oxidative stress, which excessive H$_2$O$_2$ levels can generate, is associated with both the development and progression of a multitude of diseases ranging from cancer to Alzheimer's disease and general age related neurological decline (Markesbery 1997; Droge & Schipper 2007). Given this strong association between oxidative stress and the aetiology of diseases, the potential exists to mitigate either the progression or initial development of many diseases by modulating the body’s capacity to enzymatically remove excess H$_2$O$_2$.

Phytochemicals, such as quercetin and resveratrol, have previously been shown to be cytoprotective against H$_2$O$_2$ induced cellular damage in multiple cell lines (Duthie et al. 1997; Wang & Joseph 1999; Jang & Surh 2001). Conventionally, this protective effect was assigned to a direct chemical antioxidant quenching of the oxidative stress generated by the H$_2$O$_2$ treatment. Recent work, however, has focused on examining an adaptive response by which endogenous antioxidant activities are increased in response to phytochemical pre-treatment (Surh et al. 2008). Catalase can, in conjunction with SOD act to process both H$_2$O$_2$ and *O$_2$-.

SOD is a class of enzymes that oxidises *O$_2$- radicals into H$_2$O$_2$ and molecular oxygen. It is present in all mammals and exists as three distinct families that are classified based on their metal cofactor. Copper/Zinc SOD, Iron/Magnesium SOD and Nickel SOD. Copper/Zinc SOD is found primarily in eukaryotes, Iron/Magnesium SOD in bacterial and animal liver respectively and Nickel SOD in prokaryotes. Within humans, three types of SOD exist, SOD1, SOD2 and SOD3 and they are classified based on their cellular location, SOD1 being cytoplasmic, SOD2 being mitochondrial and SOD3 being extracellular. SOD1 and 3 contain a zinc metal cofactor, where as SOD 2 contains a manganese cofactor. Despite the different metal cofactor and indeed different genes and protein structure for each of these proteins, they all share the same enzymatic function and as such, their overall activity can be assessed by an enzymatic assay that evaluates *O$_2$- clearance. Enzymatically, the requirement for SOD is due to the potentially damaging nature of the *O$_2$-. Although, *O$_2$- can spontaneously decompose, the reaction of *O$_2$- with biological molecules, such as NO, is more rapid and in the absence of SOD would result in the formation of the highly active and biologically damaging ONOO- (Squadrito & Pryor 1998).
ONOO- is highly reactive and even moderate fluxes in levels can, over time, induce serious cellular damage, ranging from disruption of cellular signalling pathways through to cell death (Troy et al. 1996; Levonen et al. 2001; Salvemini et al. 2006). ONOO- reacts with proteins via reactions with transition metal centres, cysteine oxidation and tyrosine nitration, with lipids via lipid peroxidation of membranes, liposomes and lipoproteins, and with DNA acids via oxidation of both the nucleic bases and also the sugar-phosphate backbone (Radi et al. 1991; Gow et al. 1996; Burney et al. 1999). ONOO- is associated with multiple disease states, including cardiovascular disease, arthritis, inflammatory bowel disease and cancer (Maeda & Akaike 1998; McCafferty 2000; Wattanapitayakul & Bauer 2001). Hence processing of $\cdot O_2^-$ into the less active H$_2$O$_2$ and the associated prevention of ONOO- formation, represent a potential mechanisms underlying potential health benefits of increased SOD activity.

SOD has previously been shown to be regulated both in vitro and in vivo by phytochemicals (Murakami et al. 2002; Yeh et al. 2009). Whereas both catalase and SOD act as directed specific enzymatic antioxidants, processing H$_2$O$_2$ and $\cdot O_2^-$ respectively, other endogenous antioxidants, such as GSH, have a more general function.

Assessment of general oxidative status within a cell can be done by measuring the concentration of GSH. GSH is a tripeptide thiol based antioxidant that protects cells from oxidative stresses originating from a wide variety of oxidants. Generally speaking, cellular thiols, such as GSH, are kept in a reduced state where they can act as antioxidants to mitigate damage from increases in oxidative stress. Although GSH is not an enzymatic antioxidant, it is regenerated by endogenous antioxidant enzymes and has multiple cellular effects, including direct antioxidant effects and indirect regulator effects (Jones 2002). Specifically, GSH can act directly by both directly quenching oxidants and regenerating antioxidant chemicals such as vitamin C and E (Winkler 1987; Jones 2002), as well as indirectly by modulating various aspect of the immune system, such as, antigen presentation and immune cell response (Peterson et al. 1998). GSH can also affect biochemical reactions like DNA synthesis and repair (Suthanthiran et al. 1990). GSH deficiency or depletion occurs in a multitude of
diseases including human immunodeficiency virus (HIV), cancer, Alzheimer’s disease, Parkinson’s disease as well as aging and malnutrition (Jackson 1986; Sian et al. 1994; Herzenberg et al. 1997; Liu et al. 2004). Although it appears increases in GSH levels would be beneficial, direct supplementation with oral GSH is not practical due to poor oral bioavailability (Olsson et al. 1988). Even though direct oral supplementation is not possible, oral supplementation with some non-GSH compounds have been shown to either restore or increase cellular GSH levels (Burgunder et al. 1989). Oral consumption of NAC as well as supplementation with un-denatured whey protein has both been shown to raise GSH levels (Burgunder et al. 1989; Micke et al. 2001). In addition, several phytochemicals have also been shown to increase GSH levels in vitro, including EGCG (Chen et al. 2004), and effectively boosting GSH levels may be a mechanism by which cyanidin supplementation mediates its health benefits.

Endogenous antioxidants, such as GSH, act to regulate cellular oxidative state, however, they can become oxidized and need to be regenerated by reduced nicotinamide adenine dinucleotide phosphate (NADPH) driven enzymes. Perhaps the most important of these NADPH driven enzymes is TrxR.

Regulation of cellular redox state is vital to both the maintenance of homeostasis and also the resistance to disease. Maintenance of redox balance within the cell is significantly governed by the selenium dependent protein TrxR, which regulates redox balance via direct regeneration of the dithiol active site of the redox protein Trx, in addition to having a wide variety of cellular specificities itself (Schallreuter & Wood 2001; Holmgren et al. 2005). Alterations in TrxR concentration or activity are associated with a variety of different human diseases. TrxR has been shown to be an important factor in the selenium induced suppression of HIV transcription (Kalantari et al. 2008). Additionally, TrxR has been shown to be associated with increased longevity in a TrxR1 over expressing mouse model (Murata et al. 2002). Previously it has been shown that dietary compounds, including several phytochemicals, notably sulforaphane and cinnamaldehyde, can upregulate cellular TrxR activity, either individually or synergistically, and with the addition of selenium, (Zhang et al. 2003; Liao et al. 2008). Given the potential health benefits of increasing TrxR activity,
coupled with previously observed induction by phytochemicals, it offers an excellent target to examine for potential benefits from other phytochemical metabolite compounds.

A detailed evaluation of the potential \textit{in vitro} and \textit{in vivo} health and antioxidant benefits of the phytochemical metabolites examined here is described within the introduction of the relevant chapters.

1.9 Questions

Much is known about the mechanisms of cell death and the capacity of phytochemicals to modulate it, both via protection from and potentiation of cytotoxicity. However, several vital questions relating to phytochemicals, their role in protection from oxidative stress, and how this may affect health \textit{in vivo} remain to be answered. The experiments conducted in this thesis will attempt to further the field of phytochemical research by answering the following questions:

1. Do phytochemical metabolites offer protection from oxidative stress \textit{in vitro} and can a combination of phytochemical metabolites give synergistic protection from oxidative stress induced cell death \textit{in vitro}?
2. If so, what mechanism(s) underlie the protection that phytochemical metabolites confer \textit{in vitro}?
3. Are these potential protective effects conserved across multiple cell types?
4. Can \textit{in vitro} screening using phytochemical metabolites be used to better predict \textit{in vivo} efficacy?
Chapter 2
Materials and methods

This chapter describes the general materials and methods that apply to much of the work conducted throughout this thesis. Any chapter specific methods will be described within the relevant chapter.

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All phytochemicals were made up in dimethyl sulfoxide (DMSO).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aranofin</td>
<td>Alexis Biochemicals (Lausen, Switzerland).</td>
</tr>
<tr>
<td>2,3 DHBA (99%)</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
</tr>
<tr>
<td>2,4 DHBA (98%)</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
</tr>
<tr>
<td>2,5 DHBA (99%)</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
</tr>
<tr>
<td>3,4-DHBA (98%)</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
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<tr>
<td>DMSO (99.9%)</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
</tr>
<tr>
<td>TPTZ (99%)</td>
<td>Sigma-Aldrich (St. Louis, MO) (Fluka)</td>
</tr>
<tr>
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</tr>
<tr>
<td>Heps base (99.5%)</td>
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</tr>
<tr>
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<td>Sigma-Aldrich (St. Louis, MO)</td>
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<td>Sodium acetate anhydrous (99%)</td>
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<tr>
<td>Ferric Chloride (99.9%)</td>
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<td>AAPH (98%)</td>
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<td>Monopotassium phosphate (98%)</td>
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<td>Disodium Hydrogen Phosphate (98.5%)</td>
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<tr>
<td>DTNB (98%)</td>
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<tr>
<td>SOD Kit</td>
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<tr>
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<td>BD Biosciences, San Diego, CA</td>
</tr>
<tr>
<td>Cell death kit</td>
<td>BD Biosciences, San Diego, CA</td>
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</tbody>
</table>
2.1 Cell lines and cell culture

All cell lines were maintained in media supplemented with 10% fetal bovine serum (FBS), 100 units per mL penicillin and 100 µg per mL streptomycin (complete medias) and were grown at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cell growth and viability was determined by counting trypan blue stained cells on a hemocytometer.

Collection of cells for resuspension at different density cultures and for plating for experiments was conducted using a in a Heraeus Multifuge 1 S-R centrifuge (Buckinghamshire, England.) at 300 x g for 5 mins.

All cell lines were treated/exposed to no more than 0.2% DMSO during experiments. Unless described specifically, all cells used for enzymatic assays were lysed in lysis buffer containing complete ethylenediaminetetraacetic acid (ETDA)-free protease inhibitor (Roche, Basel, Switzerland) to inhibit the enzymatic breakdown of cellular proteins. Cells were subjected to three freeze-thaw cycles to release intracellular proteins, then were centrifuged at 16 000 g for 15 min (Eppendorf centrifuge 5415D) (Eppendorf, AG, Hamburg)

2.1.1 SH-SY5Y

SH-SY5Y cells are a human neuroblastoma cell line that was originally sub-cloned from SK-N-SH cells. SH-SY5Y cells are known to be reactive to dopamine beta hydroxylase and are acetylcholinergic, adenosinergic and glutamatergic. SH-SY5Y cells are semi-adherent, with 80% of the culture being adherent and 20% being in suspension. The morphology of SH-SY5Y cells resembles neuronal cells that extend neuritis, although they do form large undifferentiated masses. This cell line is genetically female and the original SK-N-SH cells were established in 1970 from a bone marrow biopsy of a metastatic neuroblastoma site. Within this thesis the SH-SY5Y cells are being used as an example of cells that are sensitive to oxidative stress and are not being used for any neuronal cell specific measures.
Neuroblastoma derived SH-SY5Y cells (CRL-2266™) were obtained from ATCC (American Type Culture Collection; Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F12) (Invitrogen, Carlsbad, CA.) (cat. no. 11320; Gibco Invitrogen). SH-SY5Y subculturing was carried out as follows:

1. Culture medium was aspirated and discarded.
2. The cell layer was briefly washed with phosphate buffered saline (PBS) that was pre-warmed to 37 °C. 2.0 mL of trypsin (0.25%)-EDTA solution (Invitrogen, Carlsbad, CA.) (cat. no. 25200-056; Gibco Invitrogen) was then added to the cells.
3. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air to facilitate detachment and dispersal.
4. 8.0 mL of DMEM/F12 medium was added and cells were aspirated gently by pipetting.
5. Cells were then subcultured at a ratio of between 1:20 and 1:50.

For experiments SH-SY5Y cells were seeded at densities of either 2 x 10⁴ cells per well in 96-well plates or 2 x 10⁵ cells per well in 24-well plates in complete DMEM/F12 and allowed to attach for 24 h.

2.1.2 Jurkat

Jurkat cells are an immortalized line of T lymphocyte cells that express numerous chemokine receptors, are susceptible to viral infection and can produce interleukin 2. They are often used in experiments that examine T cell leukemia, T cell signaling and HIV infection. They were originally isolated from the peripheral blood of a 14 year old boy with T cell leukemia in 1970 and several different subclones are now available. The E6-1 clone being used in the experiments conducted in this thesis is noted to produce large amounts of IL-2 after stimulation with phorbol esters. Jurkat
cells are being used in this thesis as a model cell line for examining the effects of oxidative stress on immune cells and no T lymphocyte specific effects are being examined.

Acute T cell leukaemia derived Jurkat cells, clone E6-1 (TIB-152<sup>tm</sup>) were obtained from ATCC and were cultured in Roswell Park Memorial Institute (RPMI) medium containing L-glutamine and phenol (cat.no 11330; Gibco Invitrogen). Jurkat cells are not adherent, and subculturing was carried out as follows:

1. Jurkat cells were collected then pelleted by centrifugation, 300 g for 5 min.
2. Culture medium was aspirated and discarded.
3. 8.0 mL of RPMI medium was added and cells were aspirated gently by pipetting.
4. Cells were then subcultured in RPMI medium so that the cell culture density was maintained between 1 x 10<sup>5</sup> and 3 x 10<sup>6</sup> cells per mL.

For experiments, Jurkat cells were seeded at densities of either 2 x 10<sup>4</sup> cells per well in 96-well plates or 2 x 10<sup>5</sup> cells per well in 24-well plates in complete RPMI and allowed to grow for 24 h, unless otherwise stated.

**2.1.3 Transformed embryonic kidney (HEK)**

HEK cells belong to a transformed cell line that was originally generated in early the 1970s by the transformation of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA. They are often used in cell biology research as they are very easy to grow and transfec readily. Recently it has been suggested that HEK cells should not be used as an in vitro model for kidney cell function or studies involving kidney cells (Shaw et al. 2002). As the HEK cells are being used here as a model for an internal organ-based cell and no kidney cell specific measurements are being examined, any issue regarding how representative of true kidney cells HEK cells are, is unlikely to affect the results presented.
HEK cells (CRL-1573tm) were obtained from ATCC and cultured in Modified Eagle Medium (MEM) containing glucose, L-glutamine and phenol red (cat. no. 21063; Gibco Invitrogen). HEK cells are adherent, and subculturing was carried out as follows:

1. Culture medium was aspirated and discarded.
2. The cell layer was briefly washed with PBS that was pre-warmed to 37 °C. 2.0 mL of trypsin (0.25%)-EDTA solution was then added to the cells.
3. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air to facilitate detachment and dispersal.
4. 8.0 mL of MEM medium was added and cells were aspirated gently by pipetting.
5. Cells were then subcultured at a ratio of between 1:10 and 1:20.

For experiments, HEK cells were seeded at densities of either 2 x 10⁴ cells per well in 96-well plates or 2 x 10⁵ cells per well in 24-well plates in complete MEM and allowed to attach for 24 h.

2.1.4 HT-29

HT-29 cells are human intestinal epithelial cells that produce both immunoglobulin A and carcinoembryonic antigen. This cell line is often used for tumorigenicity studies involving phytochemical treatments. They are being used in this thesis as a model of the response of intestinal cells to phytochemical exposure. No transport or receptor specific measurements are being examined.

Colorectal adenocarcinoma derived HT-29 cells (HTB-38™) were obtained from ATCC and cultured in McCoy's 5a medium containing L-glutamine and phenol red (cat. no. 116600; Gibco Invitrogen) (McCoy’s media). HT-29 cells are adherent, and were subcultured as follows:
1. Culture medium was aspirated and discarded.
2. The cell layer was briefly washed with PBS that was pre-warmed to 37 °C. 2.0 mL of trypsin (0.25%)-EDTA solution was then added to the cells.
3. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air to facilitate detachment and dispersal.
4. 8.0 mL of McCoy’s medium was added and cells were aspirated gently by pipetting.
5. Cells were then subcultured at a ratio of between 1:3 and 1:8.

For experiments HT-29 cells were seeded at densities of either 2 x 10⁴ cells per well in 96-well plates or 2 x 10⁵ cells per well in 24-well plates in complete medium and allowed to attach for 24 h prior to the commencement of the experiment.

### 2.2 Measurement of antioxidant capacity (FRAP)

The ferric reducing ability of plasma (FRAP) assay uses a ferric tripyridyltriazine (TPTZ) complex to detect electron donor potential of compounds. In acidic conditions the iron atom within the TPTZ complex is reduced from ferric (3+) to ferrous (2+), which results in the formation of a blue colour. In assay conditions the formation of the blue ferrous TPTZ complex is produced if an electron donor compound is present (Eq. 1). Given that the presence of iron is a key factor in colour development, iron is supplied in excess to prevent iron limitations affecting colour development. Given that iron is in excess, the colour development is directly related to the compounds ability to function as an electron donor and reduce ferric (3+) to ferrous (2+). The FRAP assay being used has been modified from the one described by Benzie et al (Benzie & Szeto 1999).

\[
\text{Fe(TPTZ)}_2(\text{III}) + \text{ElectronDonor} \rightarrow \text{Fe(TPTZ)}_2(\text{II})(\text{Blue}) + \text{ElectronDonor}^*\cdot
\]

Previously, the FRAP assay has been used to evaluate the chemical electron donor potential of phytochemicals and has been shown to induce linear dose dependent
results for electron donor compounds, including uric acid, ascorbic acid, α-tocopherol and Trolox (Benzie & Strain 1996).

There are several potential issue with the FRAP assay. One potential issue with the FRAP assay is that coloured compounds that absorb at 593 nM cause interference. Additional limitation are that the FRAP assay does not have a biological target and as such, cannot technically be classified as a measure of antioxidant power (as there is nothing to protect from oxidation). The FRAP assay has a fast reaction time (approximately 5 minutes) so will not detect electron donors that have a slower reaction rate.

2.2.1 Solutions for FRAP assay

*Acetate buffer: 300 mM, pH 3.6*

Na acetate anhydrous 1.8687 g
Glacial acetic acid (1.05 g/mL) 16 mL
Add salt to 1 L MQ-H₂O, add acid.

*Tro-Tripyridyltriazine (TPTZ): 10 mM*

TPTZ 0.156 g
MQ-H₂O 50 mL
HCl to 40 mM (172 µL concentrated HCl to 50 mL TPTZ solution)

*Ferric Chloride: 20 mM*

FeCl₃.6H₂O 0.54 g
MQ-H₂O 100 mL
Store in a dark or foil wrapped bottle

*Trolox Standard solution*
0.250 mg Trolox
1 mL MQ-H₂O

2.2.2 FRAP assay method

Solutions were prepared as indicated in 2.2.1. FRAP reagent was made by preparing a 10:1:1 mixture of Acetate buffer: TPTZ: Ferric Chloride, and was stored in the fridge and used on the day it was made. Trolox standards were made by adding 0, 1, 2, 3, 4, 5, 7.5 and 10 µL of Trolox standard solution to wells in a 96 well plate, with volume of lower concentrations being made up to 10 µL with MQ-H₂O. Dihydroxybenzoic acid (DHBA) samples were dissolved in MQ-H₂O and added at equivalent concentrations to the Trolox standard. 10 µL FRAP reagent and 30 µL of MQ-H₂O was added to each sample or control well. The plate was then incubated for 20 min at 23 °C, then the absorbance was read at 593 nM using a BioTek Synergy HT plate reader (Winooski, United States).
2.3 Measurement of antioxidant capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) assay is a method of measuring antioxidant capacities in biological samples \textit{in vitro}. This method is based on that of Prior \textit{et al} 2002, and Huang \textit{et al} 2003. (Huang \textit{et al}. 2002; Prior \textit{et al}. 2003), and has been modified for a 96-well plate format.

The ORAC assay is based on the inhibition of ROO$^*$-induced oxidation initiated by thermal decomposition of an azo-compound, in this case 2,2$'$-azobis(2-amidino-propane) dihydrochloride (AAPH). The ROO$^*$, generated by AAPH in the ORAC assay, is a biologically relevant radical source and the antioxidant activity of test compounds are examined for both how rapidly they sequester oxidation and also what their kinetic profile of antioxidant activity is.

ROS generated from the decomposition of AAPH oxidise the fluorescent probe fluorescein and therefore quench the fluorescent signal. Antioxidant action from a control compound or an antioxidant phytochemical reduces the rate of fluorescent signal degradation, with signal stability being used to evaluate the antioxidant capacity of the test compound. The data points are measured over time by plate reader software and are compared to the standard, Trolox®, and are expressed as Trolox® equivalents (TE) per gram.

Potential issues with this assay are: i) the ROS generated is specifically the ROO$^*$, so the effectiveness of a compound in this assay depends on its capacity to scavenge the ROO$^*$ and ii) the relationship between high ORAC values and health benefits are not proven.

2.3.1 Solutions for ORAC Assay

\textit{10x Phosphate Buffer, 75 M, pH 7.0}
0.75 M KH$_2$PO$_4$: Dissolve 10.2 g of KH$_2$PO$_4$ in 100 mL MQ-H$_2$O.

0.75 M Na$_2$HPO$_4$: Dissolve 10.6 g of Na$_2$HPO$_4$ in 100 mL MQ-H$_2$O.

The two solutions above were combined at a ratio of 40:60 (KH$_2$PO$_4$: Na$_2$HPO$_4$), and the pH was checked. If necessary, the above solutions were used to adjust the pH to 7.0.

This buffer was diluted 1:10 for the working solution that was used to make the fluorescein, 2,2′-azobis-2-methyl-propanimidamide,dihydrochloride (AAPH) and sample working solutions. The working buffer solution (PB) was stored at 4 ºC for up to one month.

**Fluorescein**

A stock solution of 6.6 mg of fluorescein in 50 mL PB was prepared and stored at 4 ºC, protected from light. Working solutions for the assay were prepared by diluting 15 μL in 25 mL PB, and then 2.5 mL of this working solution was diluted in 22.5 mL PB. Diluted fluorescein was kept at room temperature for one day, provided it was protected from light.

**Trolox and samples**

An 8 mM stock solution was prepared by dissolving 100 mg of Trolox in 50 mL of acetone. A working solution was prepared by diluting the stock 1:40 with PB (i.e. 25 μL in 975 μL), then 1:1 with PB, to provide a concentration of 100 μM.

DHBA samples were prepared in the same manner as the Trolox to make a concentration of 100 μM.

**AAPH**

AAPH (0.414 g) was completely dissolved in 10 mL of PB.
2.3.2 Methods

Trolox standards of 25, 50, 75 and 100 μM were prepared by serial dilution of Trolox stock solution with assay buffer. DHBA samples were dissolved and prepared as for the Trolox standards. Each plate contained a fluorescein control (containing 25 μL and 175 μL of buffer only), and blank (containing 25 μL fluorescein, 25 μL buffer, and 150 μL AAPH). To each well 25 μL of fluorescein, 25 μL of sample/standard/buffer, and 150 μL of AAPH was added (excluding the fluorescein control wells). The samples and fluorescein were loaded on to the plate and the plate was warmed to 37 °C. Once warmed, AAPH was added to each well to start the reaction. Results were measured kinetically on a BioTek Synergy HT plate reader with an excitation frequency of 485/20 nm and an emission of 528/20 nm. Optical position was set to read from top, sensitivity was set to 60. The reaction was measured for 1 h with readings being taken at an interval of 1 min 18s. The plate was gently shaken before each reading. ORAC value was calculated as described in Cao et al 1993. Briefly, the net area under the fluorescence curve of the samples and standards was calculated. The standard curve was generated by plotting Trolox concentrations against fluorescence measurements for each concentration. ORAC values were calculated using a regression equation between Trolox concentrations and the net fluorescence. The net fluorescence is obtained by subtracting the fluorescence of the blank from that of a sample. The relative Trolox equivalent ORAC value was calculated as relative ORAC value by the below equation:

\[
\frac{(\text{Fluorescence of the sample} - \text{fluorescence of the blank})}{(\text{Fluorescence of the Trolox} - \text{fluorescence of the blank})}
\]

2.4 Assessment of cell death

Apoptosis is a physiological process that occurs during the maintenance of tissue homeostasis, in addition to occurring when cells are exposed to a cytotoxic insult.
Apoptosis is characterized by morphological changes to the cells, including loss of plasma membrane rigidity, then later integrity, and by inter-nucleosomal cleavage of DNA. The loss of plasma membrane rigidity occurs early in apoptosis and results in the exposure of the membrane phospholipid phosphatidylserine. The exposure of phosphatidylserine can be detected using a phospholipid binding protein, annexin 5. As phosphatidylserine exposure occurs early in the apoptotic process, although after caspase activation, identification of phosphatidylserine exposure can identify early stage apoptosis. Later in the apoptotic process, the plasma membrane integrity is lost and non cell-soluble DNA stains, such as propidium iodide (PI), can enter the cell and stain the DNA. The staining of the cellular DNA by PI indicates late stage apoptosis or necrosis. By combining annexin 5 and PI staining, early phase and late phase apoptosis can be identified. Although in the case of the experiments presented in this thesis, cells will only be classified as either viable or non-viable.

Cell viability was assessed by flow cytometry using co-staining with fluorescein isothiocyanate (FITC) - annexin 5 (BD Biosciences, San Diego, CA) and PI. The method used was a variation on the protocol described by Vermes et al. (Vermes et al. 1995). Briefly, SH-SY5Y cells were pre-treated with a phytochemical or a vehicle-only control for 24 h prior to the addition of 100 µM H₂O₂ for 18 h, or Jurkat cells were treated with only 50 µM H₂O₂ for 18 h (No phytochemical treatment). Cells were then washed twice with PBS at 4 °C then re-suspended in 100 µL annexin 5 binding buffer contain FITC-annexin 5 according to the manufacturer’s instructions and incubated in the dark at room temperature for 20 min. A 200 µL aliquot of annexin 5 binding buffer containing PI was then added and incubated for another 20 min in the dark at room temperature. A further 200 µL of annexin 5 binding buffer was added immediately prior to analysis by flow cytometry. Flow cytometric analysis was performed within 1 h using a Cytomics FC500MPL (Beckman Coulter, Miami, FL). Cells were excited by a 485 nM wavelength laser and the emission was measured using a 530 nM filter for FITC-annexin V and a 575 nM filter for PI. Total cell count was set to 35 000 cells.

An example flow cytometer plot is shown below, and more detailed methods have been presented by Zhang et al (Zhang et al. 2006). Prior to analysis of experimental
samples, an un-induced stained control was run. Gating was set using this control so that the viable cells fell within the middle of the A3 section (as below), cells that were either FITC-annexin 5 positive (A4), PI positive (A1) or FITC-annexin 5 and PI positive (A2) were considered non-viable. Cytotoxicity of phytochemical compounds was also assessed in the same manner.

![Graph of Annexin V - FITC](image)

### 2.5 Measurement of catalase activity

Catalase is an enzyme that catalyses the conversion of H$_2$O$_2$ to oxygen and H$_2$O, and is expressed in all living organisms. Catalase activity, the measurement of the enzymatic function of catalase, can be determined in cell and tissue lysates by evaluating the rate of processing of H$_2$O$_2$. This evaluation can be quantified with an assay that uses the combination of H$_2$O$_2$, Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) and horseradish peroxidase (HRP). Amplex Red, in the presence of the peroxidase HRP, reacts with H$_2$O$_2$ with 1:1 stoichiometry to produce the red compound Resorufin, which can then be measured by spectrophotometry. The more H$_2$O$_2$ that is in the sample that is mixed with Amplex red, the higher the concentration of Resorufin that will form, and hence a more intense red colour will form (Eq. 2). By incubating tissue and cell lysates with a known concentration of H$_2$O$_2$, then reacting the remaining H$_2$O$_2$ in the lysate with HRP and Amplex red, the catalase activity of the sample can be determined.
Catalase activity was assessed using the Amplex Red™ catalase detection kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. This assay uses H$_2$O$_2$ as a substrate and tests the capacity of the lysate sample to process a known amount of H$_2$O$_2$ vs a catalase standard. The assay determines the capacity of the cell lysate to enzymatically process H$_2$O$_2$ and as such, determines the total hydrogen peroxidase activity of the lysate, with results expressed as catalase equivalents. It is important to note that this assay does not specifically measure the activity of catalase protein as the activity of all hydrogen peroxidases in the sample is measured. Results determined from this assay are expressed as catalase activity throughout this thesis.

These experiments were conducted using the DHBAs, a family of phytochemicals of which some have been shown to be present in human blood as breakdown metabolites of other phytochemicals. They are used in these experiments to either, improve the biological relevance of the experiments or to allow for structure function comparisons that may help determine mechanism(s) of action.

Briefly, cells were pre-treated with either a DHBA or vehicle-only control for 24 h (all cell lines) or not pretreated with phytochemicals (Jurkat cells, experiments from chapter 3). Cells were then washed with PBS and resuspended in a lysis solution containing 0.2% Triton X-100, 10 µM Tris HCl (pH 7.4), supplemented with complete ETDA-free protease inhibitor tablets (Roche, Basel, Switzerland) to inhibit the enzymatic breakdown of cellular proteins. Cells were subjected to three freeze-thaw cycles to release intracellular proteins, then were centrifuged at 16 000 g for 15 min (Eppendorf centrifuge 5415D). An aliquot of the supernatant (25 µL) was taken and analyzed against catalase standards as described in the kit protocol. Cells were counted prior to resuspension in lysis solution, and suspended at a final concentration of 1 x 10$^6$ cells per mL. Activity from cell lysates were compared to a standard curve.
generated using a catalase standard, and results were expressed as units of catalase activity per $1 \times 10^6$ cells (catalase equivalents)

2.6 Catalase and glutathione peroxidase

The catalase and glutathione peroxidase (GPx) proteins are two of the hydrogen peroxidases that exist within cells and tissues. Both catalase and GPx possesses catalase activity, the capacity to enzymatically process $\text{H}_2\text{O}_2$. Given this enzymatic capacity, the aforementioned catalase activity assay can be used to measure their enzymatic activity. However, as they both have the same enzymatic function, this assay cannot identify how much each specific protein contributes to the total catalase activity. By using the above mentioned catalase activity assay, combined with specific inhibitors of both catalase and GPx, the contribution of each of these proteins to total catalase activity can be determined. The amount of catalase activity that can be inhibited by either a catalase or GPx inhibitor, relative to a non-inhibitor treated control, represents a measurement of the proportion of catalase activity that is being mediated by that specific protein.

These experiment were conducted using the DHBAs, a family of phytochemicals of which some have been shown to be present in human blood as breakdown metabolites of other phytochemicals. They are used in these experiments to either, improve the biological relevance of the experiments or to allow for structure function comparisons that may help determine mechanism(s) of action.

Catalase activity was assessed using the Amplex Red™ catalase detection kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions, and as described above. As this assay measures all hydrogen peroxidase activity of the lysate, specific activities attributable to either the catalase or the glutathione peroxidase proteins were determined by examining the effect of relevant specific inhibitors. The amount of catalase activity in cells treated with either the catalase or glutathione peroxidase inhibitor was compared with a control sample that was treated with either 2,4-DHBA or 3,4-DHBA. The difference between the control and the inhibitor treated sample was then attributed to the actions of the inhibited protein.
Briefly, cells were pre-treated with either DHBA or the vehicle only for 24 h and grown at 37 °C, 5% CO₂ in air. Prior to harvesting cells were treated with either a specific catalase inhibitor, 3-amino-1,2,4-triazole (3-AT, 10 mM) or a specific glutathione peroxidase inhibitor, mercaptosuccinic acid (MS, 1 mM), or the vehicles only for 2 h. The concentrations of 3-AT and MS used were determined by dose response experiments measuring catalase activity, and represented concentrations that mediate maximum possible inhibition of catalase activity in SH-SY5Y cells (data not shown). Cells were then washed with PBS and resuspended in a lysis solution containing 0.1% Triton X-100, 10 μM Tris pH 7.4 supplemented with Roche complete ETDA-free protease inhibitor tablets (Roche, Germany) to prevent the enzymatic breakdown of cellular proteins. Cells were subjected to three freeze-thaw cycles to release intracellular proteins, then centrifuged at 16 000 g for 15 min (Eppendorf centrifuge 5415D). The supernatant was collected and activity compared with catalase standards as described in the kit protocol. Cells were counted prior to re-suspension in lysis buffer and were suspended at a final concentration of cells 1 x 10⁶ cells per mL. Cell lysates were compared to a standard curve generated using a catalase standard and results were expressed as units of catalase activity per 1 x 10⁶ cells (catalase equivalences).

2.7 Measurement of Trx concentration

Trx measurement was based on the method developed by Holmgren et al (Holmgren 1979). This assay measures the Trx catalysed reduction of insulin disulfides that are generated by dithiothreitol (DTT). The reduction of insulin by DTT is greatly accelerated by the catalytic action of Trx.

Trx proteins contain an active cysteine disulfide that can be reduced to the dithiol form. In vivo the reduction of this oxidation-reduction sensitive active cysteine disulfide is mediated by the action of the NADPH-dependent redox protein TrxR. In vitro, this same reduction can be mediated by the chemical DTT. When reduced, Trx is a potent disulfate reductase that can catalyse the reduction of the disulfide bound of the insulin protein. When the disulfide bonds of insulin are reduced, the free β chain insulin forms a white precipitate that can be measured by spectrometry. As the
configuration of the insulin protein is vital to Trx measurement, it must be prepared by adjusting the pH of the solution to below 3 and then up to 7.5 as described below. The rate of insulin precipitation can be used to determine that concentration of Trx protein present. The reaction is described below (Eq. 3).

Eq. 3

\[
\text{DTT-(SH)}_\text{2} + \text{Insulin-S}_\text{2} \xrightarrow{\text{Thioredoxin}} \text{Insulin-(SH)}_\text{2} \text{ (white precipitate)} + \text{DTT-S}_\text{2}
\]

2.7.1 Solutions for Trx measurement

*Sodium phosphate buffer 100 mM, pH 7.0 at 25 °C*

Sodium Phosphate, Monobasic, Anhydrous,
Prepared in 100 mL MQ-H\text{2}O
Adjust to pH 7.0 at 25 °C with 1 M NaOH

*EDTA 100 mM*

EDTA, Disodium Salt, Dihydrate
Prepared in 10 mL MQ-H\text{2}O

*DTT 100 mM*

DTT
Prepared in 1 mL MQ-H\text{2}O

*Tris HCl Buffer 50 mM, pH 7.5 at 25 °C*

Tris Base
Prepared in 10 mL MQ-H\text{2}O
Adjusted to pH 7.5 at 25 °C with 1 M HCl
1% (w/v) Insulin Solution (Insulin)

10 mg of Insulin
Prepared as described below

Trx Solution

0.025 - 0.1 mg/mL of Trx protein in MQ-H₂O
Prepared immediately before use.

2.7.2 Procedure

SH-SY5Y cells were plated into 24 well plates, and then pre-treated with either 3,4-DHBA or vehicle only for 24 h and grown at 37 °C, 5% CO₂ in air. Cells were then washed with PBS and resuspended in a lysis solution containing 0.1% Triton X-100, and 10 µM Tris pH 7.4 supplemented with Roche complete ETDA-free protease inhibitor tablets (Roche, Germany) to prevent the enzymatic breakdown of cellular proteins. Cells were subjected to three freeze-thaw cycles to release intracellular proteins then centrifuged at 16 000 g for 15 min (Eppendorf centrifuge 5415D).

A 1% (w/v) insulin solution was prepared by dissolving insulin in a 50 mM Tris HCl Buffer, pH 7.5 at 25 °C. This produced a milky solution. The pH was adjusted to below 3 at 25 °C with 5 N HCl to redissolve the precipitate. Once redissolved, the 1% insulin solution was adjusted to pH 7.5 with 5 N NaOH, initially resulting in the formation of a precipitate which redissolved when the pH approached 7.5. 1.20 µL of 1% insulin solution was mixed with 0.24 µL of 100 mM EDTA solution, and 7.56 µL of 100 mM phosphate buffer, to make the reaction mixture. 75 µL of reaction mixture was mixed with either 24 µL of sample or Trx control and 1 µL of 100 mM DTT. Samples were mixed and incubated at 25 °C for 20 min. Changes in absorbance at 650 nM were then measured for 15 minutes with a BioTek Synergy HT plate reader.
2.8 Measurement of TrxR activity

TrxR is a redox protein that acts to regenerate many direct and indirect antioxidant compounds, as well as having specificity for enzymatically processing some oxidants itself. The capacity for TrxR to regenerate other compounds forms the basis for the assay being used to determine its activity. TrxR will, in the presence of NADPH, reduce inactive Trx back to its active form. Trx can react with the colourless 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to form the coloured 5-thio-2-nitrobenzoic acid (TNB) (Eq. 4). The activity of TrxR can be determined by measuring the rate of TNB formation, from added DTNB, in a cell or tissue lysate when treated with NADPH. Given any Trx present in the lysate, along with other thiol containing compounds, may react with DTNB to form TNB in absence of TrxR activity, the lysate should be incubated with DTNB, before the addition of NADPH, to allow for any thiols to react. When NADPH is added, TrxR will regenerate Trx then Trx will begin to convert DTNB to TNB. Measuring the rate of the change in TNB formation, can be used to determine the TrxR activity of the lysate. Importantly, the action of other reductase enzymes, primarily glutathione reductase, can also influence the conversion of DTNB to TNB. Therefore, to specifically determine TrxR activity, two lysate samples should be run in parallel, one containing a TrxR specific inhibitor and one without the inhibitor. The amount of TNB production prevented by the TrxR inhibitor represents the TrxR activity of the lysate.

Eq. 4:

\[
\text{Thioredoxin-S} + \text{DTNB} \xrightarrow{\text{Thioredoxin Reductase}} \text{TNB} + \text{Thioredoxin-(SH)2}
\]

2.8.1 Solutions for TrxR assay

*NADPH Solution 2 mM*

NADPH, anhydrous

Prepared in 1 mL MQ-H₂O immediately before use
DTNB Solution 10 mM

DTNB (98% pure)
Prepared in 10 mL MQ-H$_2$O

Auranofin Solution

Auranofin
Prepared in DMSO

2.8.2 Procedure

TrxR activity was measured as described by Cox et al. and expressed as relative TrxR activity (Cox et al. 2008). Briefly, cells were pre-treated with either DHBA or vehicle only for 24 h and grown at 37 °C, 5% CO$_2$ in air. Cells were then washed with PBS and resuspended in a lysis solution containing 0.1% Triton X-100, 10 µM Tris pH 7.4 supplemented with Roche complete ETDA-free protease inhibitor tablets (Roche, Germany) to prevent the enzymatic breakdown of cellular proteins. Cells were freeze thawed three times to release intracellular proteins then centrifuged at 16,000 g for 15 min (Eppendorf centrifuge 5415D).

Supernatants were collected, transferred to a 96 well plate and treated with 100 µL of a 10 mM DTNB, which forms the yellow TNB when exposed to thiol groups. TNB formation was measured using a BioTek Synergy HT plate reader with absorbance at 612 nM. Once a steady state of TNB formation was reached (after 5 minutes), 10 µL of 2 mM NADPH solution was added with or without the addition of the TrxR inhibitor Auranofin (Alexis Biochemicals, Switzerland) (10 µM), and absorbance was measured at 30 s intervals for 20 min. NADPH-induced increases in the conversion of DTNB to TNB represents the combined activity of glutathione reductase and TrxR. The inhibition of NADPH-mediated increases in TNB formation by Auranofin represents the proportion of this activity that is due to TrxR, and hence the TrxR activity. A similar methodology was also used to generate a standard curve for TrxR inhibition with Auranofin, in the SH-SY5Y cells. Auranofin was added to SH-SY5Y
at concentration of up to 4 µM for 6 h then TrxR activity was determined as described above. The results were fitted with an inhibition curve.

2.9 Assessment of SOD activity

SOD is a biologically important family of enzymes that process \( \cdot \text{O}_2 \) into \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \). Their importance is covered in detail in the introduction.

The SOD assay used in this thesis evaluates SOD activity by using an oxidant generator to produce \( \cdot \text{O}_2^- \) and a colourimetric dye that develops a colour in the presence of \( \cdot \text{O}_2^- \). \( \cdot \text{O}_2^- \) is produced by the reaction of xanthine with \( \text{O}_2 \) in the presence of xanthine oxidase and also forms uric acid and \( \text{H}_2\text{O} \). \( \cdot \text{O}_2^- \) then either reacts with SOD and is turned into \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) or reacts with a Tetrazolium salt to form the coloured Formazan.

2.9.1 Solutions for SOD assay

SOD activity was assessed using a SOD kit (Cayman Chemicals, item number 706002, Michigan, USA) according to the manufacturer’s instructions. This assay measures the cells’ enzymatic processing capacity of \( \cdot \text{O}_2^- \), compared with a SOD standard. Briefly, cells were pre-treated with either DHBA or vehicle only for either 6 or 24 h, cultured at 37 °C, 5% CO\(_2\) in air. Cells were then washed with PBS and
resuspended in a lysis solution containing 0.1% Triton X-100, 1 mM ethylene glycol tetraacetic acid (EGTA), 210 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl pH 7.2, supplemented with complete ETDA free protease inhibitor tablets (Roche, Germany) to prevent the enzymatic breakdown of cellular proteins. Cells were freeze-thawed three times to release intracellular proteins, and then centrifuged at 16 000 g for 15 min (Eppendorf centrifuge 5415D). The supernatant was collected and analysed by spectrophotometry at 440 nM, as described in the kit protocol. Cells were counted prior to resuspension in lysis buffer and were resuspended to a final concentration of 1 x 10⁶ cells per mL. Cell lysates were compared to a standard curve generated using a SOD standard and expressed as units SOD per 1 x 10⁶ cells. This method measures total cellular SOD activity, it does not discriminate between mitochondrial and cytoplasmic SOD.

2.10 Measurement of GSH levels

GSH levels were measured using a glutathione assay kit (Cayman Chemicals, item number 703002) according to the manufacturer’s instructions. This assay measures the GSH mediated conversion of DTNB to TNB which occurs with 1:1 stoichiometry. The method is similar to what is described in section 2.8. Briefly, cells were pre-treated with either DHBA or vehicle only for either 6 or 24 h, cultured at 37 °C, 5% CO₂ in air. Cells were then washed with PBS and lysed in a solution containing 2 mM EDTA and 100 mM phosphate buffer (pH 6) by freeze thawing three times. Samples were compared to a GSH standard.

2.11 Assessment of MAPKinase activation

MAPKinase activation was measured by flow cytometry using a BD multiplexed bead-based cytometric bead array kit (BD Biosciences, CA, USA). This assay uses beads that are coated with various antibodies that each has specificity for a specific MAPKinase protein. The antibodies have specific fluorochromes that, when excited, fluoresce at different wavelengths. This allows for the analysis of multiple proteins simultaneously. The experiment was conducted as described in the manufacturer’s protocol.
Briefly, all reagents for the set up of the flow cytometer were warmed to room temperature (23°C) before use. A master buffer solution was prepared as described in the manufacturer’s protocol and used to make the standards for flow cytometer calibration. Detection antibodies and standards were prepared by dilution with master buffer. Flow cytometer (FC 500 MLP) was calibrated using cytometric set up beads as described by the manufacturer’s protocol. Briefly, FC 500 MLP was turned on and allowed to initialise. The side scatter and forward scatter parameters were set to log mode. Cytometer set up beads A were run and the side scatter voltage and forward scatter threshold were adjusted so that the set up beads appeared as a localised distinct population in the upper right of the forward scatter side scatter window. The set up beads were then gated. A FL1 log / FL3 log window was created and the photomultiplier tube values were adjusted until the beads separated into distinct localised populations with the greatest possible difference between them. These populations were then gate. This process was repeated for other combinations of detectors. Set up beads B and C were used to adjust compensation settings that were adjusted so that the median value was equal for the distinct bead populations. This process was repeated for the other detectors. Calibration of the flow cytometer was verified using control standards and detection antibodies. Total count number was set to 15,000 per sample.

Briefly, SH-SY5Y cells were treated with either 3,4-DHBA or a vehicle only for 4 h or H2O2 for 1 h prior to cell harvesting. Time points were chosen based on previous results showing oxidative stress being induced at a 4 h time point by 3,4-DHBA. A 1 h treatment time with H2O2 was chosen as cellular oxidative stress is induced within this time, and hence this provides a positive control for oxidative stress mediated activation of MAPKinase signalling. Concentrations of 3,4-DHBA were chosen to represent levels that have effects on cell death and antioxidant enzyme expression, as determined by earlier catalase activity experiments, as well a higher concentration which, based on previously published work, should induce activation of MAPKinase signalling.
Cells were prepared as described in the BD™ Cytometric Bead Array Cell Signaling Master Buffer Kit manual (catalogue number 560005). Briefly, cells were aspirated of medium, washed with 50 µL 1 x denaturing buffer containing Invitrogen complete phosphatase inhibitor tablets, and scraped with a cut 200µL pipette tip. Samples were collected and denatured by heating to 100 °C for 5 min in a Thermoline™ dry block heater. DNA within the samples was sheared by passing thought a 26 gauge needle until viscosity was considerably reduced. Protein content was determined using the Bio-rad protein assay kit (catalogue number, 500-0112) and samples were diluted 1:5 with Assay Diluent buffer prior to transfer to antibody coated beads that bind to the phosphorylated forms of AKT1, ERK1/2, JNK1/2, MEK1/2, p38 and STAT1. All beads were prepared as described by manufacturer’s protocol. 50µL of mixed captured beads was mixed with 50µL of sample or standard, and incubated at room temperature for 3 h in darkness. 50µL of mixed detection reagent was added and the mixture incubated for 1 h at room temperature in darkness. Samples were then washed with 1 mL of wash buffer and centrifuged at 200 g for 5 min (Heraeus Multifuge 1 S-R centrifuge). The supernatant was carefully aspirated and the pellet was re-suspended in 300 µL of wash buffer. Samples were then analysed by fluorescence-activated cell sorting (FACS). Results are given as MAPKinase activation relative to the control cells.

2.12 Data analysis

Data are expressed as means ± standard error of the mean (S.E.M). Statistical analyses were performed on GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Experiments were compared by one-way analysis of variance (ANOVA) with Tukey pairwise posthoc test. P-values are annotated in the figures as; * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001. Unless otherwise stated.
Chapter 3: Cell density, culture conditions and oxidative stress

3.1 Summary

H$_2$O$_2$ is routinely used in cell culture experiments as an inducer of oxidative stress, primarily due to its ability to generate the biologically relevant hydroxyl radicals. However, there are major inconsistencies in the published literature in both the amount of cell death and type of damage induced by exposure of cells to H$_2$O$_2$. Whilst exposure time and concentration of H$_2$O$_2$ are generally considered, there is typically no mention of prior cell culture density. This chapter presents an investigation into the effects of both the absolute assay cell concentration, and the prior long-term cell culture density on H$_2$O$_2$ induced cell death in Jurkat cells. Paradoxically, while absolute assay cell density shows a negative correlation with induction of cell death, prior long-term cell culture density is positively correlated with cell death in cells treated with H$_2$O$_2$. Furthermore, this enhanced sensitivity to H$_2$O$_2$-induced cell death in long term high density cultures, persists for several days before returning to the relationship seen in standardised culture conditions (4 x 10$^5$ cells per mL). To evaluate the potential mechanisms related to these observations, changes in catalase activity, ATP levels and intracellular oxidative stress markers were measured. Prior cell culture density was found to be inversely related to all three measures in Jurkat cells. We conclude that prior cell culture density impacts on the cells basal levels of intracellular oxidative stress and therefore there is a need for culture conditions to be standardised before exposure to external inducers of oxidative stress.

3.2 Introduction

•OH can cause oxidative damage, which has been implicated in multiple acute and chronic disease states including; coronary heart disease, stroke, cancer, arthritis and Alzheimer’s disease (Toyokuni 1998; Dhalla et al. 2000; Perry et al. 2002; Faraci &
H₂O₂ is routinely used in cell culture experiments as an inducer of oxidative stress, as it can generate the biologically relevant •OH produced in the body at various stages of oxygen metabolism (Puppo & Halliwell 1988), but also due to its low cost, stability and ease of use. It is an excellent and relevant compound for the identification of protective effects of putative antioxidant factors against oxidant-induced cell death. Treatment of Jurkat cells in vitro with H₂O₂ generally induces a concentration-dependent induction of cell death, with lower dosages (0-50 μM) primarily inducing apoptosis while higher dosages (50-100 μM) induce necrotic cell death (Hampton & Orrenius 1997). The type of cell death induced by H₂O₂ treatment is of great importance when testing for either the ability of a compound to protect from cell death or determining activation of signalling pathways during an oxidative assault. However, it is evident from the literature that H₂O₂ can generate inconsistent results using the same reported assay conditions. For example, a 6 h exposure to 500 μM of H₂O₂ was required to induce 25% cell death in Jurkat cells by Hampton et al. (Hampton & Orrenius 1997), whereas only 25 μM was required in another study using the same cell line and similar culture conditions (Zhang et al. 2006). Furthermore, in our laboratory varying levels of cell death have been induced using a consistent protocol for final cell density at the start of the experiment, concentration of and exposure time to H₂O₂ as well as flow cytometer settings for analysis of cell death (data not shown).

Certain compounds, such as Z-DEVD-Fluor methyl ketone (Z-DEVD-FMK), a caspase 3 inhibitor, can prevent the activation of specific caspase signalling pathways that have a causative role in inducing apoptosis (Vantieghem et al. 1998). The inhibition of this caspase signalling pathway can prevent apoptotic cell death. However, Z-DEVD-FMK does not offer protection against necrotic cell death as the caspase signalling pathway is not activated in this process (Vantieghem et al. 1998). Conversely, the upregulation of heat shock proteins (HSP) 72 and 27 have been shown to protect against necrotic but not apoptotic cell death in vascular smooth muscle cells when treated with serum deprivation and heat shock respectively (Champagne et al. 1999). Absolute cell density has also been shown to affect the quantity and type of cell death induced, as well as regulating cellular stress pathways both before and after exposure to oxidative stress. Embryonic rat cortical neurons
have been shown to undergo apoptosis when stressed by serum deprivation if they are grown at a high density, but necrotic cell death results when the same cells are grown at low density, due to differences in ATP levels within the cells (Fujita et al. 2001). Conversely, long-term treatment of murine pre-B-cells with weakly genotoxic dosages of cadmium induced apoptosis at densities lower than $5 \times 10^5$ cell per mL and necrosis at densities over $5 \times 10^5$ cells per mL (Banfalvi et al. 2007). Further, regulation of signaling pathways and protein phosphorylation have been shown to differ between high and low-density cultures (Li & Goldstein 1996; Petridou et al. 2000). Higher density cell cultures are also unable to localize AMP kinase, a cellular energy sensor, into the nucleus in response to extracellular signal-regulated kinases (ERK)1/2 signaling in comparison with low density cultures treated the same way (Kodiha et al. 2007).

The effect of cell density is often overlooked in in vitro experiments and can potentially be dictated by requirements of experimental kits. Given the relevance of in vitro experiments is directly related to their ability to reflect the in vivo situation, one might question whether the cell density that is being used in many in vitro assays is suitable. How might changing the density of in vitro cell cultures to more accurately reflect in vivo conditions effect factors ranging from the localization of membrane proteins within the cell to signaling pathway activation and levels and types of cell death?

The data presented in this chapter examines the possible influence of prior cell density during the growth of cells and experimental cell density on the susceptibility of Jurkat cells to H$_2$O$_2$ mediated oxidative stress induced cell death and attempts to define the molecular mechanisms responsible for cell death. Given that both variations in cell culture density and concentration of H$_2$O$_2$ have been shown to affect the type and amount of cell death induced, the initial experiments are based on these variables (Whittemore et al. 1995; Hampton & Orrenius 1997).
3.3 Specific materials and methods

3.3.1 Cell culture conditions and treatment

Jurkat cells were initially taken from a culture grown to a density of $3 \times 10^6$ cells per mL and tested for susceptibility to H$_2$O$_2$-induced cell death by treating from $1 \times 10^5$ - $3 \times 10^6$ cells per mL with H$_2$O$_2$ for 18 h. The original culture was then diluted into 5 cultures at cell densities ranging from $5 \times 10^4$ to $8 \times 10^5$ cells per mL and were maintained at 5 density levels; (1) $5 \times 10^4$ - $2 \times 10^5$, (2) $1 \times 10^5$ - $4 \times 10^5$, (3) $2 \times 10^5$ - $8 \times 10^5$, (4) $4 \times 10^5$ - $1.6 \times 10^6$ and (5) $8 \times 10^5$ - $3 \times 10^6$ cells per mL for 2 weeks by sub-culturing every 2 days. After 2 weeks cells from the different density level cultures were standardised to a density of $4 \times 10^5$ cells per mL and maintained at this density for 2 days. They were spun down resuspended at $4 \times 10^5$ cells per mL and individual cultures were tested on days 0, 1 and 2 for their susceptibility to H$_2$O$_2$-induced cell death, caspase activation after H$_2$O$_2$ treatment and catalase activity, ATP levels and level of intracellular oxidative stress. H$_2$O$_2$ was diluted in MQ-H$_2$O to a 100 x stock and 10 μL added to the bottom of a 24 well plate well before the addition of 1 mL of cells. Control wells had 10 μL of MQ-H$_2$O added before the addition of cells. After H$_2$O$_2$ treatment cells were incubated for 18 h in a 5% CO$_2$ in air incubator at 37°C. Experiments were performed three times using duplicate wells for each sample. Viability and cell densities were determined by haemocytometer on cells stained with trypan blue.

3.3.2 Caspase 9 activation

Caspase 9 activation was measured as a marker of mitochondrial mediated apoptotic cell death. Caspase 9 activation occurs early in apoptotic cell death and can be used to classify cell death as apoptotic (Budihardjo et al. 1999). Activation of caspase 9 was measured by flow cytometry using an excitation at 485 nm and emission at 580nm. Flow cytometric analysis was performed using a Cytomics FC500MPL (Beckman Coulter, Miami, FL) Staining was conducted using FLICA caspase detection kits (Immunochemistry Technologies, Bloomington, MN, USA) according to the
manufacturer’s instructions. Briefly, 4 x 10^5 Jurkat cells were exposed to 50 µM of H_2O_2 for 18 h after being cultured at densities ranges of either 0.5 x 10^5 - 2 x 10^5, 1 x 10^5 - 4 x 10^5, 2 x 10^5 - 8 x 10^5, 4 x 10^5 - 1.6 x 10^6 or 8 x 10^5 – 3 x 10^6 cells per mL for 2 weeks. Caspase activation was measured immediately prior to the addition of 50 µM H_2O_2 to generate a baseline of activation as well as 18 h after the addition of 50 µM H_2O_2 to allow for maximal activation of caspases. A caspase 9 fluorescent inhibitor, which is up taken by the cells, was added and cells were incubated at 37°C in 5% CO_2 in air for 2 h with gentle mixing to ensure even distribution. Cells were then washed three times with apoptosis wash buffer (Immunochemistry Technologies, Bloomington, MN, USA) prior to analysis. Total cells analysed was set to 15,000 cells.

3.3.3 Measurement of ATP levels

Cells were taken from long-term cultures (2 weeks) grown at a particular density. Cell number was standardised (1 x 10^5 cells per mL) and ATP content was measured by luminescence. Cells were allowed to grow normally with ATP levels being measured every day.

Cellular ATP was determined using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Corporation, WI, USA) as per manufacturer’s instructions. Briefly, Jurkat cells were culture as illustrated in Figure 7. 100 µL of Jurkat cells (4 x 10^5 cells per mL) was added to an opaque-walled multiwell plate and allowed to equilibrate at room temperature for approximately 30 minutes. 100 µL of CellTiter-Glo® reagent was added to each experimental well and mixed on an IKA MTS orbital shaker for 2 minutes at 300 rpm to facilitate cell lysis. Samples were then incubated at room temperature for 10 minutes to stabilize the luminescent signal. ATP content was measured via luminescence on a BioTek Synergy HT plate reader with an integration time 1 second per well.

The ATP assay CellTiter-Glo® Luminescent Cell Viability Assay uses highly sensitive beetle luciferin to measure ATP levels of the cells. The assay involves adding a single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum
supplemented medium. Cell washing or removal of medium is not required and hence was not performed for this assay. The reaction that generates luminescence from ATP is as described below.

\[
\text{Beetle luciferin} + \text{ATP} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{PP}_i + \text{CO}_2 + \text{light}.
\]

### 3.3.4 Measurement of ROS

The method was based on a modified version of the protocol used in Krejša et al. (Krejša et al. 1997). Jurkat cells were cultured under previously described density conditions in complete RPMI prior to treatment with the oxidation sensitive dye 2’,7’-dichlorodihydrofluorescein (DCFDA). Briefly, cells were washed in pre-warmed (37°C) PBS containing 1% FBS then treated with 25 µM DCFDA in PBS for 30 min at 37°C in a 5% CO₂ in air. Cells were then washed twice with PBS containing 1% FBS warmed to 37°C, in order to remove excess DCFDA dye and incubated for 1 h at 37°C to allow for oxidation of the DCFDA dye. DCFDA fluorescence was measured by flow cytometry, using a Cytomics FC500MPL (Beckman Coulter, Miami, FL) with excitation measured at 485 nM and emission measured at 530 nM as recommended in manufacturers’ protocol. Non-viable cells were removed from the analysis by gating based on light-scattering properties. Prior to gating, non-viable cells could be seen as a population of low fluorescing cells. Total cells analysed was set to 15,000 cells.
Jurkat cells were taken from an original culture grown up to a density of $3 \times 10^6$ cells per mL and tested for susceptibility to $\text{H}_2\text{O}_2$-induced cell death. The original Jurkat culture was then split into 5 cultures that were grown for 2 weeks over different density ranges. After 2 weeks cells from the different density cultures were diluted/concentrated to a standard density of $4 \times 10^5$ cells per mL and cultured for 2 days during which time they were tested daily for susceptibility to $\text{H}_2\text{O}_2$-induced cell death, caspase activation after $\text{H}_2\text{O}_2$ treatment and catalase activity determined. Jurkat cells were also sub-cultured to $1 \times 10^5$ and ATP levels were measured daily over 5 days of culture growth. Intracellular oxidative stress...
levels were measured in cells originating from both the highest and lowest immediately after the 2 weeks of culture density specific growth.

### 3.4 Results

#### 3.4.1. Effect of cell number exposed to H$_2$O$_2$ on cell death

H$_2$O$_2$ is known to induce cell death in various cells including Jurkat cells. To determine the effect of varying the concentration of cells on H$_2$O$_2$-induced cell death a predetermined concentration of H$_2$O$_2$ was used to induce cell death in cells that had been grown up to a density of $3 \times 10^6$ cells per mL and then diluted before exposure to H$_2$O$_2$. Cell death, as measured by DNA staining with PI using flow cytometry (described in chapter 2.4), was inversely related to cell concentration. At the highest two concentrations ($3 \times 10^6$ cells and $1.6 \times 10^6$ cells per mL) cells were 98.5% viable. At lower concentrations, $8 \times 10^5$, $4 \times 10^5$ and $2 \times 10^5$ cells per mL there were 80, 49 and 29% viable cells respectively, while at the lowest concentration, $1 \times 10^5$ cells per mL, only 1% were viable (Figure 8).
Figure 8: Effect of Jurkat cell concentration on H$_2$O$_2$-induced cell death.

Jurkat cells taken from a single culture of density 3 x 10$^6$ cells per mL were resuspended in fresh complete RPMI media at different concentrations and treated with 50 μM H$_2$O$_2$ for 18 h. After this time samples were taken and Annexin 5 PI staining was used to assess cell viability (described in chapter 2.4). The results of 3 separate experiments with 2 replicate cultures expressed as means ± S.E.M Significant difference is denoted by * (P > 0.05), ** (P > 0.01) and *** (P < 0.001).

3.4.2. Cell death and caspase activation after long term cell culture density treatments

Cell viability and caspase activation was assessed in Jurkat cells that had been cultured as illustrated in Figure 7 for two weeks. Jurkat cell concentration was then standardised to 4 x 10$^5$ cells per mL and cells were tested for susceptibility to H$_2$O$_2$ induced cell death and caspase 9 activation after 0, 1 and 2 days.

Jurkat cells taken from the lowest density culture of 0.5 – 2.0 x 10$^5$ cells per mL were significantly more resistant to H$_2$O$_2$-induced cell death, compared with the cells taken from the highest density culture (8 x 10$^5$ - 3 x 10$^6$ cells per mL), when tested immediately after the cell concentrations were standardised to 4 x 10$^5$ cells per mL, with 82% vs 37% viability (P < 0.001). The lowest density culture cells were also more resistant to H$_2$O$_2$-induced cell death than cells originating from all the other
culture densities (Figure 9a). The increased resistance to H$_2$O$_2$-induced cell death conferred by culturing at the lower density was maintained, albeit to a lesser extent for the following 2 days. Differences in viability between cultures from the highest and lowest densities were 55% vs 35% (P < 0.001) on day 1 (Figure 9b) and 59% vs 51% (P < 0.05) on day 2 (Figure 9c).

Caspase 9 activity was measured to assess whether the mechanism of cell death was primarily apoptotic or necrotic. Caspase 9 activation is a marker of apoptotic cell death and is induced in Jurkat cells which are undergoing apoptosis in response to H$_2$O$_2$ treatment. Caspase 9 activation was shown to increase in a cell density dependent manner, from 28% in cells from the lowest density culture up to 42% in cells from the second highest density culture (1.6 x 10$^6$ cells per mL), when cells were treated with H$_2$O$_2$ immediately after culture concentrations were standardised (P < 0.05). Interestingly, caspase 9 activation was lower in cells from the highest density culture compared to cells from the second highest density culture despite a higher rate of cell death (P < 0.05), signifying a greater proportion of necrotic cell death (Figure 9d). There were no significant differences in caspase 9 activation seen in the cultures during day 1 and 2 (Figure 9e and f).
(a) 

H$_2$O$_2$-induced cell death vs long term culture densities (day 0)

% Cell Viability

Parent culture cell density

(b) 

H$_2$O$_2$-induced cell death vs long term culture density (day 1)

% Cell Viability

Parent culture cell density

(c) 

H$_2$O$_2$-induced cell death vs long term culture density (day 2)

% Cell Viability

Parent culture cell density
Figure 9: Effect of culturing Jurkat cells at different densities on H$_2$O$_2$-induced cell death & caspase 9 activation.

Cells taken from cultures that had been cultured for 2 weeks at different cell densities then adjusted to a standard concentration were treated with H$_2$O$_2$ immediately or 1 or 2 days later. Annexin V PI staining was used to assess cell viability (described in chapter 2.4) and caspase 9 activation was used as a marker of apoptotic cell death after H$_2$O$_2$ treatment. (a), H$_2$O$_2$-induced cell death of Jurkat cell treated with H$_2$O$_2$ immediately after culture density was standardised to 4 x 10$^5$ cells per mL (b), H$_2$O$_2$-induced cell death of Jurkat cell treated with H$_2$O$_2$ 1 day after culture density was standardised to 4 x 10$^5$ cells per mL (c), H$_2$O$_2$-induced cell death of Jurkat cell treated with H$_2$O$_2$ 2 day after culture density was standardised to 4 x 10$^5$ cells per mL (d), H$_2$O$_2$-induced caspase 9 activation in Jurkat cell treated with H$_2$O$_2$ immediately after culture density was standardised to 4 x 10$^5$ cells per mL (e), H$_2$O$_2$-induced caspase 9 activation in Jurkat cell treated with H$_2$O$_2$ 1 day after culture density was standardised to 4 x 10$^5$ cells per mL (f), H$_2$O$_2$-induced caspase 9 activation in Jurkat cell treated with H$_2$O$_2$ 2 days after culture density was standardised to 4 x 10$^5$ cells per mL. The results of 3 separate experiments with 2 replicate cultures expressed as means ± S.E.M. Significant difference is denoted by * (P > 0.05), ** (P > 0.01) and *** (P < 0.001).

3.4.3. ATP levels

As apoptosis is a highly energy dependent process, insufficient cellular energy levels can potentially cause a shift from apoptosis to necrosis. ATP levels were measured to
support the observation that decreased caspase 9 activation in cells from the highest density culture may have been related to a switch in mode of cell death from apoptosis to necrosis. ATP levels were found to be inversely associated with prior cell culture density until one day after the cells were plated at the same concentration. Cells from the highest density culture contained 60% less ATP than those from the lowest density at day 0 (P < 0.001). ATP levels in all cultures were not significantly different after 24 h and no further differences were detected. In general, cellular ATP levels were found to decrease over time as the cultures became denser (Figure 10).
Cells were taken from long term cultures grown at various densities and cell concentrations were standardised on Day 0. Measurements of intercellular ATP levels were taken at the time of standardisation and also after 1-5 days of growth. (a) The change in cellular ATP levels over the 5 days subsequent to cell culture conditions being standardised. (b) Differences in ATP levels of between different density cultures immediately prior to cell concentrations being standardised. The results of 3 separate experiments with 2 replicate cultures expressed as means ± S.E.M. The significant difference between levels ATP in cells from 2 x 10^5 and 3 x 10^6 cell density cultures is shown by ***(P < 0.001). Significant difference is denoted by * (P > 0.05), ** (P > 0.01) and *** (P < 0.001)
3.4.4. Catalase activity in long term cell cultures grown at different densities

Catalase activity was assessed (described in chapter 2.5) to determine whether the difference in susceptibility to H₂O₂-induced cell death was due to an increased ability of the cells to process H₂O₂ and hence lessen the exposure of the cell to the oxidant. Catalase activity was significantly increased in cells that were grown at lower densities. Catalase activity was 2-fold higher in cells from the lowest density culture as compared with that of the highest (4.2 vs 2.1, P< 0.001) and was generally inversely proportional to long-term culture density (Figure 11 a). Catalase activity was still increased in cells grown at the lowest density compared with those at the highest on day 2 after cell concentrations were standardised (P < 0.001) (Figure 11 b). There were no significant differences between the different cultures on day 3 (Figure 11 c).
Catalase Activity vs culture density (day 0)

Catalase Activity vs culture density (day 1)
Figure 11: Effect of cell culture density on cellular catalase activity.

Cells from long term cultures grown at various densities were diluted to a standard concentration of $4 \times 10^5$. Measurements of intercellular catalase activity were determined (as described in chapter 2.5) at (a) the time of standardisation and after, (b) 1 and (c) 2 days of growth. The results of 3 separate experiments with 2 replicate cultures expressed as means ± S.E.M. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).

3.4.5. ROS levels

Expression of antioxidant enzymes such as catalase has previously been shown to be induced by oxidative stress conditions. Intracellular oxidative stress levels were measured using the dye DCFDA to determine if an increase in intracellular oxidative stress is a possible mechanism for inducing the increase seen in catalase activity. Cells from the highest and lowest density cultures were taken immediately after 2 weeks of continuous culturing and intracellular oxidative stress levels were determined. Cells from the lower density culture was shown to have significantly higher (P<0.05) levels of intracellular oxidative stress (Figure 12).
Figure 12: Intracellular oxidative stress levels in Jurkat cells from high and low density cultures. Cells were taken from 2 week long cultures grown at high density (8 x 10^5 - 3.0 x 10^6 cells per mL) and a low density (0.5 x 10^5 - 2 x 10^5 cells per mL) and concentration of intracellular reactive oxygen species was determined immediately. The results of 3 separate experiments with 2 replicate cultures expressed as means ± S.E.M. Significant difference is denoted by * (P > 0.05).

3.5 Discussion

The results presented in this chapter have clearly demonstrated that both cell concentration and prior long-term cell culture density have an impact on results of in vitro oxidative stress experiments.

Resistance to cell death was observed both in Jurkat cell exposed to H_2O_2 at relatively high cell concentrations and in cells that were grown for 2 weeks at a low culture density. The viability of Jurkat cells at a concentration of both 3 x 10^6 and 1.6 x 10^6 cells per mL was unaffected by 50 µM H_2O_2 treatment (Figure 8). Cells at the lower concentrations showed a concentration dependent decrease in viability with cells at 1 x 10^5 having the lowest viability (Figure 8). As cell concentration at the time of H_2O_2 exposure clearly had a strong effect on the susceptibility of Jurkat cells to H_2O_2-
induced cell death, further experiments were conducted to evaluate whether long-term culture density affected this susceptibility to cell death if the cell concentration was standardised during the actual exposure to H$_2$O$_2$.

Given Jurkat cells at a concentration of 4 x 10$^5$ cells per mL were approximately 60% viable (Figure 8), this concentration was used in further experiment to test the effect of long term culture densities. Jurkat cells from cultures grown for 2 weeks at set density ranges showed an inverse relationship between susceptibility of H$_2$O$_2$-induced cell death and culture density after cell concentrations were standardised to a density of 4 x 10$^5$ cells per mL (Figure 9 a). Despite both high cell concentration and low culture density conferring protection from H$_2$O$_2$-induced cell death, the mechanisms behind the protection conferred to cells by each of these conditions appears to be fundamentally different. The protection from cell death by absolute high density of the cells is potentially due to the combination of two factors. Firstly, due to the ability of the cells as a whole to enzymatically process both H$_2$O$_2$, and/or any of the radicals it produces, by natural antioxidant enzymes, primarily catalase. Secondly, the division of the introduced H$_2$O$_2$ amongst a greater number of cells which results in less H$_2$O$_2$ exposure per individual cell. Interestingly, although the ability of the culture as a whole to metabolize H$_2$O$_2$ may be greater in higher density cultures, this does not indicate that the expression of catalase or other antioxidant enzymes is greater per cell, nor does it imply that the cells are more resistant to H$_2$O$_2$ on a per cell basis. It has been previously shown that high-density cultures of *Rhizobium leguminosarum* cells have a lower expression of catalase per cell (AJ Crockford 1995), which is related to the accumulation of compounds released by these cells into the growth media (Crockford. et al. 1995). The same changes in per cell expression of catalase may also be responsible for the relative sensitivity of Jurkat cells originating from a high-density culture to H$_2$O$_2$ induced cell death. In order to determine the possible mechanisms responsible for the observed correlation between the protection from cell death and culture density, changes in apoptosis, necrosis, catalase levels, cellular ATP levels and ROS levels were evaluated.
3.5.1 Density effect on apoptosis and necrosis induced by H$_2$O$_2$

The data presented in this chapter shows that culturing Jurkat cells at various densities causes a density dependent decrease in cellular ATP levels that was corrected after the cell culture density was normalized for 24 h (Figure 10). Further, the treatment of cells from the highest density culture with H$_2$O$_2$ immediately after sub-culturing, resulted in increased cell death but reduced caspase 9 activation (Figure 9 d). Increased cell death in the absence of caspase activation indicates necrotic cell death (Kroemer et al. 1998). The elevated proportion of necrosis in the cells from the highest density culture was gone after 24 h of growth, the same amount of time it took for ATP levels to stabilize, suggesting a related mechanism. Previously it has been shown that cell culture density can alter both the patterns of cell death observed after a cytotoxic insult and also cellular ATP levels. Fiorani et al (1994) showed decreases in cellular ATP levels in CHO cells related to increased density (Fiorani et al. 1994), while Fujita et al (2003) showed that density-induced changes in cellular ATP levels were responsible for a shift from apoptotic to necrotic cell death (Fujita & Ueda 2003). Given the previously reported association of decreased ATP levels with necrotic cell death, it is likely that this increase in necrosis is primarily due to the decreased ATP levels seen in these cells. Interestingly, it has also been shown by Cross et al (1978) that the maintenance of complete catalase activity in *Saccharomyces cerevisiae* was dependent on cellular energy levels, mostly from ATP (Cross & Ruis 1978). It is possible that decreased ATP may also have an effect on catalase levels in Jurkat cells grown at a high density.

3.5.2 Density effect on catalase levels and ROS levels

The data presented here has shown that Jurkat cells that were cultured at lower densities, possess significantly higher amounts of catalase activity (Figure 11) and show a striking similarity to those of absolute susceptibility to H$_2$O$_2$ induced cell death (Figure 9 a, 9 b and 9 c). Additionally, cells originating from the lowest density culture had significantly higher levels of basal oxidative stress compared to cells from the highest density (Figure 12).
Previously, cellular catalase activity has been shown to decrease in fungal cells in response to increasing culture density (Cross & Ruis 1978; Crockford et al. 1995). Additionally, Long et al. (2003), using mouse fibroblast L-cells, showed a positive correlation between lower culture density and an increased resistance to H$_2$O$_2$-induced cell death on a per cell basis (Long et al. 2003). Interestingly, they also observed increased spontaneous apoptosis in high density cultures. Bello et al. (2004) showed that ROS levels within Hela cells were inversely associated with culture density levels (Bello et al. 2004) and Limoli et al. (2004) demonstrated that culturing rat hippocampal neural precursor cells at lower densities led to a 4-fold increase in ROS levels compared to that seen in cells cultured at higher densities (Limoli et al. 2004). Further, exposure to lower levels of chronic oxidative stress can lead to increased expression of endogenous antioxidant enzymes. Hachiya et al. (2005) have shown that long-term treatment of HL-60 cells with H$_2$O$_2$ can induce a 13-fold increase in catalase levels (Hachiya & Akashi 2005). The results described here agree with previous reports on cell density dependent regulation of catalase, ROS and resistance to cell death. Further we show for the first time that changes in cell density affects all these measures in Jurkat cells and that the density dependent change in both catalase levels and resistance to cell death is transiently maintained after cell culture conditions are standardised.

Recent work has highlighted the importance of ROS as mediators of cellular signalling in response to oxidative stress (Touyz 2004; Winterbourn & Hampton 2008). Perhaps most importantly and unsurprisingly the presence of ROS is in itself a mechanism for upregulation of protection from ROS induced damage (Rushmore et al. 1991). The generation of excessive oxidative stress, both endogenously and exogenously, at a sub-lethal level, has been shown to induce several putative ROS defence mechanisms. These mechanisms include, induction of the antioxidant response element by the transcription factor NF-E2-related factor-2 (Nrf2) (Rushmore et al. 1991), the oxidation of the peroxiredoxin (Prx) family of proteins and the induction of the xenobiotic response element by MAPKinase activation (Kong et al. 2001; Fourquet et al. 2008). Here we have shown for the first time that prior cell culture density induces changes in both ROS and antioxidant enzyme activity in Jurkat cells. Mechanistically this increase in endogenous antioxidant enzyme activity,
which has previously been shown in other cell lines, has been linked to induction resulting from increased levels of intracellular oxidative stress. Reasons for increased ROS in low density cultures is currently unclear, however several possible mechanisms do exist. It is possible that intercellular signalling, which suppresses the generation of oxidative stress is deficient in low density culture or that the generation of excessive ATP causes an increase in ROS within the cells. Until a more complete mechanistic study is completed the exact causes will not be known, however, this work does highlight the importance of cell culture density in in vitro work.

Catalase expression, and its ability to modulate ROS, also acts to modulate cell growth. In the catalase over-expressing clone of HL-60, HL-100, Hachiya et al (2005). showed that increased catalase levels were responsible for increased growth rates and that inhibiting the catalase overexpression suppressed growth rates within these cells (Hachiya & Akashi 2005). This data shows that Jurkat cells that came from the highest density culture, which possessed the lowest catalase activity, had a slower growth rate after sub-culturing than cells from the other densities (data not shown).

In summary, the long-term culturing of Jurkat cells at a low cell density leads to increased levels of basal oxidative stress, higher catalase activity, increased levels of ATP and an increased resistance to H$_2$O$_2$-induced cell death on a per cell basis.

We conclude that the likely mechanism for both the initial resistance as well as the transient protection from H$_2$O$_2$ is due to increased basal levels of oxidative stress leading to an upregulation in catalase expression. This work highlights the importance cell culture density can have in in vitro assays, particularly ones involving oxidative stress, and demonstrates the importance of maintaining consistent cell culture conditions. We recommend that stringent long term culture density condition are standardised, maintained and reported for all in vitro assays using cell lines grown as suspension cultures. This will help to improve inter-lab variation in sensitivity to both H$_2$O$_2$ and potentially other important factors.
Chapter 4
Phytochemical screening

4.1 Summary

Phytochemicals are well documented to protect cells from oxidative insults and to prevent oxidative stress induced cell death in vitro. However most phytochemicals, particularly the more potently antioxidant ones, have very limited bioavailability in vivo and hence results seen in vitro are difficult to relate to the real physiological effects one might expect upon consumption of these compounds. Here, several bioavailable phytochemicals and bioavailable phytochemical metabolites, along with non-bioavailable positive control phytochemicals, were tested for their ability to prevent H2O2-induced cell death in the oxidative stress-sensitive cell line SH-SY5Y. In general, the phytochemical metabolites had little cytoprotective effect when compared to the positive control phytochemicals; quercetin and (+) catechin. Of the more bioavailable phytochemicals, EGCG and sulforaphane were shown to be cytotoxic even at low concentrations in vitro and only the phytochemical metabolite 3,4-DHBA was found to offer protection from cell death without inducing toxicity at the concentrations tested. Further, it was shown that even the most potent phytochemical metabolite tested here offers far less protection from cell death than the non-bioavailable phytochemical control compounds tested. The relatively limited biological effect of the phytochemical metabolites tested may in part explain why phytochemical supplementation is generally less effective in vivo than what would be expected from in vitro evidence. This also likely reflects the bodies attempt to reduce the toxicity and bioactivity of these xenobiotic compounds by metabolising them into less active metabolites.
4.2 Introduction

*In vitro* phytochemical screening is a common experimental technique used to determine the bioactivity of dietary compounds. Phytochemicals are often highly active *in vitro* with compounds such as quercetin showing potent activity in various measures of inflammation, antioxidant action, immune response and cytoprotection (Wang et al.; Rice-Evans et al. 1996; Zhang et al. 2006; Ruiz et al. 2007). Most dietary phytochemicals are however substantially modified by either detoxifying enzymes or are broken down prior to their release into the blood or have very low absorption (Scalbert & Williamson 2000), and hence the use of dietary phytochemicals in *in vitro* assays may not be representative of their *in vivo* effects. While the *in vitro* effects of many phytochemicals are potent, replicating such effects in *in vivo* experiments has been difficult. For example, quercetin has been shown to directly enhance the expression of the HDL-associated enzyme paraoxonase 1 (PON1) in liver cells and when fed to mice, however no effect was seen in a human intervention trial (Boesch-Saadatmandi et al.). Additionally, quercetin has been shown to offer significant protection from oxidative stress to cells *in vitro* without showing any such effect in several human intervention trials (Kim et al.; Shanely et al.; Egert et al. 2008). The differences in bioactivity seen between cellular, animal and human trials are likely due, in part, to the specific processing and differential bioavailability of phytochemicals in humans. Recently, a substantial quantity of research has examined both the enzymatic systems that process dietary phytochemicals and also the metabolites that are present in the blood after phytochemical consumption. Dietary phytochemicals have been shown to be either, enzymatically conjugated with sulfate and glucuronide moieties, broken down into simpler phenolics, including hydroxy and dihydroxy derivatives, or excreted back into the gut lumen by efflux pumps (Seeram et al. 2001; Silberberg et al. 2006). Additionally, some phytochemicals have been shown to be absorbed unmodified in humans, although this has been reported to be variable and may be related to the genetic makeup of the subject (Chow et al. 2001; Lee et al. 2002). Potentially, it is possible to chemically modify dietary phytochemicals to represent their *in vivo* metabolites; however, it is difficult to entirely remove the dietary precursor making interpretation of *in vitro* results using these compound mixtures difficult (Stevenson et al. 2008). Given the inherent
difficulty in enzymatically conjugating phytochemicals to accurately represent their *in vivo* metabolites, the use of unconjugated hydroxy and dihydroxy metabolites offers a simple and arguably, more relevant method of assessing phytochemical effects *in vitro*.

Cytoprotective assays are often conducted using co-treatment of the phytochemical and the cytotoxic compound (Zhang et al. 2006). Although this methodology has previously shown significant protective effects and potentially measures chemical antioxidant potential, it can be argued that chemical antioxidant action is unlikely to significantly contribute to health effects seen *in vivo*. *In vivo* actions of phytochemicals are potentially mediated by the induction of an adaptive response, the upregulation of cellular defence mechanisms, that protect from subsequent oxidative insult and further, this response may be more important *in vivo* than their chemical antioxidant activity (Stevenson & Hurst 2007). Previously, phytochemical induced adaptive responses have been measured *in vitro* using phytochemical pre-treatments, with longer pre-treatments inducing greater upregulation of endogenous protective mechanisms (Bacon et al. 2007). Studies examining the upregulation of endogenous protective mechanisms *in vitro* have typically used non-orally bioavailable phytochemicals, such as curcumin, and as such, have limited relevance to the *in vivo* situation (Bacon et al. 2007; Xu et al. 2007). Examining the effect of phytochemical blood metabolite pre-treatment on protection from cell death *in vitro* provides a more relevant method of identifying potential effects of the parent compound *in vivo* and may help to explain the disconnect between *in vitro* results using dietary phytochemicals and *in vivo* studies.

Here the capacity of pre-treatments with several hydroxy and dihydroxy-based phytochemical metabolites and orally bioavailable phytochemicals, to protect from H$_2$O$_2$-induced cell death in the oxidative stress sensitive cell line SH-SY5Y has been assessed. The compounds tested were selected based on either; known *in vitro* bioactivity (controls), or presence in blood after oral phytochemical consumption (test compounds).
SH-SY5Y cells were used for the experiments presented in this and the next chapter for 2 reasons. i) They are sensitive to H$_2$O$_2$-induced cell death and this cell death has been prevented by phytochemical treatment in many previous papers (Gao et al. 2001; Jang & Surh 2001). ii) Unlike the Jurkat cells that are a suspension cell line and have been shown to have a highly variable responses to H$_2$O$_2$ treatment, the SH-SY5Y cell line is adherent, has more stringent requirements for culturing condition and has proven to give more consistent levels of cell death in preliminary experiments.

4.3 Phytochemical selection

Phytochemical compounds were selected based on previous reports of bioavailability in the literature or as control compounds with known *in vitro* bioactivity. Phytochemical positive controls included quercetin and (+) catechin. Previous experiments (data not shown) indicated that a 24 h pre-treatment with quercetin was not able to offer protection from subsequent oxidative insult and therefore a two hour pre-treatment was used for cells treated with quercetin, as opposed to 24 h for the other phytochemicals tested. The phytochemical metabolites hydroxybenzoic acids (HBAs) and DHBAs were chosen as they appear in blood after the consumption of many different phytochemicals, particularly anthocyanins (Azzini et al.; Vitaglione et al. 2007).

The anthocyanin precursors of the DHBAs and HBAs are, due to the changed oxygen in their A ring, inherently unstable at physiological pH and will ‘break’ across this oxygen. In theory, all anthocyanins will undergo spontaneous degradation at physiological pH to form a benzoic acid and an unstable aldehyde (Laleh et al. 2006). The specific benzoic acid isomer formed after anthocyanin degradation depends on the substitutions present on the C ring of the parental anthocyanin molecule. For example cyanidin, which has an hydroxyl substitution at the 3’ and 4’ position of the C-ring, forms 3,4-DHBA while pelargonidin, which has a 4’ hydroxyl substitution, will form 4-HBA (Azzini et al 2010.; Vitaglione et al. 2007). The formation of these anthocyanin degradation products is reasonably well characterise, with benzoic acid breakdown products being observed in both *in vitro* studies of anthocyanin stability as well as several human bioavailability studies (Azzini et al 2010.; Vitaglione et al. 2007).
EGCG is found in the blood of certain individuals following its dietary consumption and sulforaphane is present un-modified in the blood for a brief period after oral consumption in most people (Lee et al. 2002; Vermeulen et al. 2008). Additionally, hippuric acid, an end point excretion product was also chosen (Mulder et al. 2005). The structure of the various compounds tested is illustrated in Table 2.

Although it would have been preferable to use the dietary anthocyanin precursors of 3,4-DHBA in the experiments conducted in this thesis, these precursors have previously been shown to be unstable and to breakdown in tissue culture media. Further, their in vitro breakdown products vary with temperature and pH, both of which are difficult to control when sub-culturing cells for experiments. For this reason, the dietary precursors of 3,4-DHBA were not examined in the experiments conducted in this thesis.
Table 2: Selected phytochemical compounds.

Phytochemical compounds were selected based on either their presence in blood after dietary phytochemical consumption or as positive controls with known in vitro bioactivity against oxidative stress. Where possible, maximum likely blood concentrations of the compounds are listed, as determined by either actual measurements of compounds in blood or derived values based on absorption of dietary precursor and reported rates of conversion into relevant metabolites. Additionally, the % bioavailability of orally consumed compounds is listed.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td><img src="structure1.png" alt="Structure" /></td>
<td>Non-bioavailable positive control (0 µM, 0%) (Morand et al. 1998)</td>
</tr>
<tr>
<td>(+) Catechin</td>
<td><img src="structure2.png" alt="Structure" /></td>
<td>Non-bioavailable positive control (0.002 µM, &gt; 2%) (Donovan et al. 1999)</td>
</tr>
<tr>
<td>3-HBA</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td>Phytochemical metabolite (Rios et al. 2003)</td>
</tr>
<tr>
<td>4-HBA</td>
<td><img src="structure4.png" alt="Structure" /></td>
<td>Phytochemical metabolite (Rios et al. 2003)</td>
</tr>
<tr>
<td>2,3-DHBA</td>
<td><img src="structure5.png" alt="Structure" /></td>
<td>Phytochemical metabolite (350 µM, from aspirin breakdown) (Kuhn et al. 1995)</td>
</tr>
<tr>
<td>2,4-DHBA</td>
<td><img src="structure6.png" alt="Structure" /></td>
<td>Phytochemical metabolite (Koskela et al. 2007)</td>
</tr>
<tr>
<td>Phytochemical</td>
<td>Metabolite (100 µM from aspirin breakdown) (Davison 1971; Pedersen &amp; FitzGerald 1984)</td>
<td></td>
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<tr>
<td>---------------</td>
<td>----------------------------------------------------------------------------------</td>
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<tr>
<td>2,5-DHBA</td>
<td>Phytochemical Metabolite (20+ µM) (Han et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>3,4-DHBA</td>
<td>Phytochemical end point urinary excretion product (2 mM) (Mulder et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>Hippuric Acid</td>
<td>Bioavailable dietary phytochemical (1 µM, 27.4%) (Chow et al. 2001)</td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>Bioavailable dietary phytochemical (&gt;0.1 µM, 37%) (Vermeulen et al. 2008)</td>
<td></td>
</tr>
</tbody>
</table>

### 4.4 Specific methods

#### 4.4.1 Cell culture conditions

Phytochemicals were dissolved in pure DMSO and added directly to warm (37°C) cell culture media (DMEM/F12) while it was being mixed in a grant bio VP-1 vortex mixer set to the maximum setting. This was done to ensure no point precipitation of
phytochemicals occurred. Media containing the dissolved phytochemical was then added immediately to cells and incubated as described in the methods chapter. Cells were pre-treated with phytochemicals for 24 h for all cell-based experiments, with the exception of experiments using quercetin where a 2 h pretreatment was used. For synergy testing, cells were either treated with both phytochemicals concurrently or if quercetin was used, the non-quercetin phytochemical was administered 24 h prior to the addition of H$_2$O$_2$ then quercetin 2 h before the addition of H$_2$O$_2$. Cell media was not changed before the addition of phytochemicals, this approach was taken in order to most accurately represent how phytochemical exposure in the body occurs.

4.4.2 Caspase 8 and 9 activation

Activation of caspase 8 and 9 was measured by flow cytometry using an excitation at 485 nm and emission at 580 nm for caspase 9 and an excitation at 485 nM and emission at 515 nM. Flow cytometric analysis was performed using a Cytomics FC500MPL (Beckman Coulter, Miami, FL). Staining was conducted using FLICA caspase detection kits (Immunochemistry Technologies, catalog numbers 912 and 930, Bloomington, MN, USA) according to the manufacturer’s instructions.

4.4.3 Synergy testing

Protection from H$_2$O$_2$-induced cell death by phytochemical synergies was determined as described in section 2.4. Expected values for phytochemical synergies were calculated by adding the individual observed protection against H$_2$O$_2$-induced cell death for each of the phytochemicals being tested.
4.5 Results

4.5.1 DMSO effects

Treatment of SH-SY5Y cells with various concentrations of DMSO (0.1%-1%) showed cytoprotection against subsequent H$_2$O$_2$-induced oxidative insult at the highest concentration of 1%. A non-significant trend towards protection was seen at 0.5% DMSO (Figure 13).

![Figure 13: Effect of DMSO on protection from H$_2$O$_2$-induced cell death in SH-SY5Y cells.](image)

Cells were treated with 100 µM H$_2$O$_2$, alone or in the presence of increasing percentages of DMSO (0.1-1%) for 18 h. The cell viability was determined by flow cytometry after staining with annexin 5 and PI as described in section 2.4. Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from cells treated with H$_2$O$_2$ alone. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).

4.5.2 Caspase activation

SH-SY5Y cells treated with H$_2$O$_2$ were assessed for activation of the apoptotic signalling molecules, capase 8 and capase 9. Capase 9 activation was seen to occur
after a 3 h exposure, with the number of capspase 9 positive cells reaching a maximum after 18-24 h. Caspase 8 activation occurred after 4 h with capspase 8 positive cells also reaching a maximum at 18-24 h. Basal caspase 8 and 9 activation were approximately 9% of total cells measured (Figure 14).
SH-SY5Y cells were treated with either H$_2$O$_2$ (100 μM) or a water control for up to 24 h. Samples were taken at 0, 1, 2, 3, 5, 18 and 24 h, and analysed for (a) caspase 9 and (b) caspase 8 activation by flow cytometry after treatment with a cell soluble caspase antibody. Significant difference is denoted by * (P>0.05). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M.
4.5.3 Phytochemical cytotoxicity

Phytochemicals were assessed for their cytotoxic effects (as described in section 2.4) against the SH-SY5Y cell line (Figure 15) The phytochemical metabolites DHBA and HBAs and the end stage phytochemical excretion product hippuric acid, showed no cellular toxicity towards SH-SY5Y cells when tested at concentrations as high as 100 µM. Quercetin was toxic at concentrations of 50 µM and above, with 100% cell death occurring at 100 µM. EGCG and sulforaphane were significantly toxic with 100% cell death occurring at 5 and 50 µM respectively (Figure 15).

![Figure 15: Cellular toxicity of phytochemical compounds.](image)

SH-SY5Y cells were treated with phytochemical compounds for 24 h. The cell viability was determined by flow cytometry after staining with annexin 5 and PI (as described in section 2.4). Significant difference is denoted by * (P>0.05). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M.
4.5.4 Phytochemical mediated cytoprotection

Quercetin, (+) catechin, 3,4-DHBA and EGCG all mediated protection from H₂O₂ induced cell death. Quercetin was the most potent cytoprotective agent, with 10 µM increasing cell viability to over 80% after an H₂O₂ oxidative insult. (+) catechin showed a dose dependent cytoprotective effect with maximal protection occurring at the highest concentration tested (100 µM). EGCG showed some protection at 5 and 10 µM, but was also cytotoxic and higher concentrations resulted in increased cell death. Of the DHBA and HBA metabolites tested only 3,4-DHBA offered significant protection from H₂O₂ oxidative insult, with 2,3-DHBA offering non-significant protection (P >0.06) (Figure 16 a and b).
16: Phytochemical mediated protection from $\text{H}_2\text{O}_2$-induced cell death in SH-SY5Y cells.

SH-SY5Y cells were treated with either control compounds (a) of (+) catechin, quercetin, EGCG and sulfurophane for 24 h (2 h for quercetin) or (b) phytochemical metabolite compounds of hippuric acid, 3-HBA, 4-HBA, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA or 3,4-DHBA for 24 h prior to being exposed to $\text{H}_2\text{O}_2$ (100 µM) for 18 h. The cell viability was determined by flow cytometry after staining with annexin 5 and PI (as described in section 2.4). Significant difference is denoted by * (P>0.05). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M.
4.5.5 Synergy testing

Phytochemicals were combined with the active metabolite 3,4-DHBA to determine if any synergistic cytoprotective effects occurred. The expected protective effects of the combination between 3,4-DHBA and quercetin, both at 10 µM, was 70.6% (60.5 for quercetin + 10.1 for 3,4-DHBA), the actual observed protection was 66.8%. The combination of 3,4-DHBA with quercetin resulted in additive protection with observed values not being significantly different from the expected additive protective effects. The combination of 3,4-DHBA with other compounds resulted in either, subadditive effects (+ catechin) or non-additive effects (other DHBAs and HBAs) (Figure 17).

Figure 17: Determination of synergistic, additive and subtractive effects of phytochemicals on H2O2-induced cell death in SH-SY5Y cells.

SH-SY5Y cells were treated with a combination of phytochemical compounds for 24 h prior to the addition of 100 µM H2O2 for 18 h. The cell viability was determined by flow cytometry after staining with annexin 5 and PI (as described in section 2.4). Ex = Expected protection based on the sum of their protective effects. Obs = Actual observed levels of protection seen when compounds were added. Synergistic protection was defined as observed protection that was significantly greater than expected protection. Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M.
4.6 Discussion

Research directed toward understanding the bioavailability of consumed phytochemicals has shown that ‘in blood’ metabolites of dietary polyphenols are primarily glucuronidated conjugates of dietary phytochemicals or their metabolic breakdown products, such as the HBAs and DHBAs (Silberberg et al. 2006; Vitaglione et al. 2007). Additionally, the dietary phenolic acids, such as the HBAs and DHBAs, are among the most bioavailable of the phenolic phytochemicals and as such, are both bioavailable dietary phytochemicals and breakdown products of other non-bioavailable phytochemicals (Lafay et al. 2006a; Lafay et al. 2006b). The use of phytochemical metabolites, be they phytochemical conjugates or blood breakdown products, in in vitro assays would greatly improve the relevance of these assays to the related in vivo effects, and as such, their use in in vitro assays is highly advantageous. Previously it has been reported that conjugated metabolites, such as quercetin and resveratrol derived conjugates, are significantly less bioactive than their non-conjugated precursors in an in vitro measure of antioxidant action (Day et al. 2000; Hoshino et al. 2010). Here we evaluate the cytoprotective action of some phytochemical blood metabolites, including the HBAs and DHBAs, in addition to several dietary compounds with little or no bioavailability but which are known to be active in vitro.

4.6.1 Protection from H$_2$O$_2$ induced cell death

H$_2$O$_2$ is routinely used as an inducer of oxidative insults and cell death in in vitro models of oxidative damage. H$_2$O$_2$ treatment of cells induces increased levels of intracellular oxidative stress (Carter et al. 1994). In vivo, oxidative stress is implicated in the pathology of many disease states and its modulation by dietary compounds may prevent or delay the onset of both chronic and acute diseases (Madamanchi et al. 2005; Andreadou et al. 2007).

H$_2$O$_2$ treatment of SH-SY5Y cells induced primarily apoptotic cell death after 18 h of exposure as indicated by caspase 8 and 9 activation (Figure 14). Protection from H$_2$O$_2$ induced cell death was seen to be dose dependent in cells pre-treated with either (+)
catechin or quercetin. Less potent protection from cell death was seen in cells pre-treated with EGCG, sulforaphane or 3,4-DHBA. None of the other phytochemical metabolites offered any significant protection from H$_2$O$_2$ induced cell death in SH-SY5Y cells. Generally, the blood metabolites had significantly less bioactivity than the non-bioavailable positive controls of (+) catechin and quercetin.

The relative lack of cytoprotection offered by the phytochemical metabolites tested here is not necessarily an unexpected result. It is generally accepted that the role of the body’s detoxifying systems, coupled with the role of the gut lumen efflux pumps, is to either prevent the absorption of, or to reduce the toxicity of, dietary compounds. This system acts to prevent the potential bioactivity of dietary compounds from entering the blood system and hence prevents unwanted affects on cellular homeostasis. Form an evolutionary standpoint, the absorption of highly bioactive compounds from dietary sources could be unfavourable and hence a system that robustly processes these into less bioactive metabolites is advantageous. Dietary compounds that are less active would, in theory, be less important substrates for detoxifying mechanisms and hence more likely to enter the bloodstream. Additionally, phytochemical breakdown products, which have limited bioactivity, would potentially not be bioactive enough to require a directed mechanism to either prevent their entry or remove them from the circulation. It is worth noting that this system is believed to be somewhat non-specific (Jack et al. 2000), in that it recognises general structures of chemicals that may potentially be toxic. The recognition of a compound by this system is not implicit in cellular toxicity nor does it suggest potent bioactivity, but it does however indicate a commonality of structure between a given compound and other potentially toxic compounds.

Previously, quercetin and (+) catechin have been reported to offer protection from exogenously induced cell death in the SH-SY5Y cell line. Quercetin mediated protection from H$_2$O$_2$ exposure has previously been shown to be via the regulation of SREB2 after a 2 h pre-treatment with 10 µM (Soundararajan et al. 2008). (+) catechin has been shown to protect from a variety of oxidative stress mediated toxic effects at concentration ranges of 0.1-100 µM (Zhang et al. 2006). Although reports of significant in vitro cytoprotection have been shown for both quercetin and (+)
catechin at a concentration of 0.1 µM generally, concentrations of 10 µM or greater are required and as such, the results here agree with the majority of previous findings (Conte et al. 2003; Soundararajan et al. 2008). EGCG has also previously been reported to be a potently cytoprotective agent in SH-SY5Y cells (Wang et al. 2009). Although previous reports have shown greater levels of cytoprotection, the results presented here are broadly in agreement with what is reported in the literature (Chung et al. 2007). Previous reports on the cytoprotective effects of the phytochemical metabolites measured here is somewhat limited, with results that do show cytoprotective effects being almost entirely derived from short term pre-treatments as opposed to the longer term pre-treatment used here (Shui et al. 2006; Liu et al. 2008). This distinction is important, as it was shown here that quercetin could protect after a 2 h pre-treatment but not after a 24 h pre-treatment, indicating that the protective effects can be dictated by pre-treatment times and potentially affect mechanisms of protection. Additionally, it has previously been shown that many phytochemicals are unstable and break down after a few hours in cell culture media (Seeram et al. 2001; Hong et al. 2002), indicating that protection seen after longer term pre-treatments is likely to be due to a more biologically relevant adaptive response and not a chemical antioxidant effect.

Previously, 3,4-DHBA has been shown to be cytoprotective in several studies, however, these studies have used shorter pre-treatment times and as such, are likely to examine direct chemical antioxidant effects and not an adaptive response (Shui et al. 2006; Liu et al. 2008). Hippuric acid, a major urine metabolite of most dietary phenolics, has not previously been evaluated for cytoprotective effects against oxidative stress in vitro. Given it is a major urinary excretion product of dietary phytochemicals and levels in excess of 500 mg are excreted daily in urine (Rechner et al. 2002), it could be suggested that a lack of bioactivity was not unexpected. 3- and 4-HBA, in vivo metabolites of anthocyanin rich fruits such as cranberries and strawberries, did not offer any significant protection from H₂O₂ induced cell death in SH-SY5Y cells. Although no reports evaluating the cytoprotective nature of these compounds have been published, paraben esters of 4-HBA have been suggested to induce oxidative stress and could therefore potentiate oxidative stress in vivo (Nishizawa et al. 2006). It is possible that both 3- and 4-HBA acid undergo similar
reaction to their paraben derivatives and hence may act to either magnify acute oxidative stress conditions or induce an adaptive response in vivo, although no such effect was seen here. 2,3 2,4 and 2,5 DHBA have not previously been examined for cytoprotection in the SH-SY5Y cell line and although 2,3 and 2,5 DHBA are used as markers of oxidative state in tissue injury (Santamaria et al. 2001), specific data on their innate protective effects in vivo or in cellular assays is limited. 2,5-DHBA has previously been shown to be protective against tissue plasminogen activator mediated deletion of antioxidant enzymes in mouse skin (Sharma et al. 2004). 2,3-DHBA is a chemical antioxidant and has been shown to act to suppress oxidative stress in several model systems (Lodovici et al. 2001; Okai & Higashi-Okai 2006), however, these experiments again used short or no pre-treatment times and as such are less likely to measure relevant adaptive responses. Generally the lack of effectiveness of these in vivo phytochemical metabolites, when compared to non-bioavailable control phytochemicals, in this in vitro model of oxidative stress supports the majority of current literature, which shows that dietary phytochemicals have potent in vitro effects but only minor in vivo effects.

4.6.2 Cellular toxicity of phytochemical compounds

Phytochemicals have been well documented to induce cellular toxicity at high concentrations in in vitro assays. Given the known toxicity of phytochemicals, compounds being used here were assessed for toxicity in the absence of H₂O₂ in the SH-SY5Y cell line. Concentrations tested where chosen based on previously reported approximate physiological concentrations of phenolics (0.1-10 µM) after consumption of phenolic rich food (Stevenson et al. 2008), in additional to two supra physiological concentration (50 and 100 µM). Quercetin, EGCG and sulforaphane were shown to be significantly cytotoxic to SH-SH5Y cells after a 24 h treatment. In vitro cellular toxicity of EGCG has previously been reported to be mediated though the actions of H₂O₂ (Nakagawa et al. 2004), which is generated in cell media when it is exposed to EGCG. The toxicity observed with quercetin was potentially mediated by its hydrophobic nature. Quercetin was observed to partly precipitate in tissue culture media at a concentration of greater than 50 µM, corresponding to concentrations that were cytotoxic. Hence quercetin toxicity may have been affected by its inherent
insolubility in the cell culture media. Although it would have been possible to use greater concentrations of DMSO to prevent this, DMSO was shown here to function as a cytoprotectant at concentrations greater than 0.5% (Figure 13) and previously to regulate many cell signalling pathways (Nakagawa et al. 2004), consequently the concentration of DMSO was kept to a minimum. Sulforaphane was shown to be highly toxic to this cell line with even low concentrations inducing cell death. Although no previous reports of sulforaphane toxicity in this cell line exists, reports of toxicity in other cell lines suggest the sensitivity to sulforaphane seen here is highly elevated (Sestili et al. 2010). Potentially, this elevated toxicity could have been due to the unique assay conditions or specific properties of this cell line. The hydroxy and dihydroxy acids showed no toxicity towards this cell line even at the supra physiological concentration of 100 µM.

4.6.3 Synergy screening of phytochemical compounds

Dietary phytochemicals are constituents of fruits or vegetables and as such, are typically consumed as complex mixtures of many compounds. Given the complex nature of dietary phytochemical mixtures, it is likely that their metabolites are also present in vivo as complex mixtures of phytochemical metabolites. Epidemiological data has shown that diets high in botanical diversity, hence high in a broad range of phytochemicals, are associated with greater health benefits than diets with low botanical diversity (Thompson et al. 2006). Potentially, beneficial interactions between different phytochemical metabolites may influence the beneficial health effects in these botanically diverse diets. Here we examined the effect of phytochemical metabolite mixtures on protection from H$_2$O$_2$-induced cell death and attempted to uncover any synergistic, additive and subtractive effects. Given the limited effectiveness of the phytochemical metabolites, metabolite compounds were also mixed with the control compounds of quercetin and (+) catechin. Although the mixture of phytochemical metabolites with non-bioavailable controls has limited biological relevance, it does test the theory that mixtures of phytochemical compounds can mediate synergistic protection against oxidative stress.
Combination of non-active DHBAs or HBAs with the active 3,4-DHBA did not result in any significant additive or synergistic effects. Given the similar chemical structures of the compounds being tested, it is possible that they may trigger a similar protective mechanism, which may have already been saturated by the 3,4-DHBA treatment. The combination of (+) catechin with 3,4-DHBA resulted in less cytoprotection than was expected given the individual protective effects of each compound. Although this reduction in observed cytoprotection was not significant, it does highlight the possibility that the combination of discrete phytochemicals may result in detrimental effects.

Protective synergies against H₂O₂ induced cell death by pure phytochemical compounds have not previously been reported. However, the regulation of the cytoprotective protein thioredoxin reductase has been shown to be synergistic in cells treated with selenium and sulforaphane (Zhang et al. 2003). Further, multiple reports of cytotoxic synergies have been reported between cancer drugs and phytochemicals and at least one report has shown a cytotoxic induction of cell death in cells treated with two phytochemical compounds (Liu 2004; Hsieh & Wu 2008). A non-cellular chemical antioxidant synergy has been shown using a heme-enhanced oxidative reaction when both curcumin and resveratrol are added together (Aftab & Vieira 2009). Additionally, it is worth noting that many traditional medicines, which are made from plant, are more potent as a crude extract rather than as a purified active compound (Kimura et al. 1992; Stermitz et al. 2000). Potentially, this again supports the concept that beneficial synergies can occur, however, the exact mechanisms behind these synergies may be complex and characterising them difficult. Although there are a growing number of reports of synergistic phytochemical effects being published, the lack of cellular protective synergies using pure phytochemical compounds would suggest that the results presented here are not in disagreement with the literature.

4.7 Conclusion

 Phytochemicals can act as modulator of disease models in vitro, offering protection in models of cancer, heart disease and neurological conditions such as Alzheimer’s and
Parkinson’s disease. However, *in vivo* trials have not shown such potent effects. Here we have examined the capacity of phytochemicals metabolites to protect from oxidative stress *in vitro*, and shown them to have very limited effectiveness when compared to their parent dietary phytochemicals, such as quercetin. The limited protective effect of phytochemical metabolites shown here may explain the reported discrepancies between *in vitro* and *in vivo* intervention trials with phytochemicals.

Although most of the phytochemical metabolites tested here had little bioactivity in the measure of cytoprotection used, 3,4-DHBA did show significant protective effects at concentrations that have previous be reported to occur in plasma of an animal after oral consumption of a phytochemical. The next chapter examines the mechanisms underlying this protection by 3,4-DHBA starting with the conventional hypothesis that it is via direct chemical antioxidant action and progressing to examine potential adaptive responses.
Chapter 5
Mechanisms of 3,4-Dihydroxybenzoic acid (DHBA) mediated protection from oxidative stress-induced cell death

5.1 Summary

Conventionally, the protection from oxidative stress offered by phytochemicals has been attributed to their chemical antioxidant capacity, i.e. their ability to chemically quench free radicals. Many phytochemicals do have potent chemical antioxidant capacity and can, in theory, act to quench the free radicals that cause oxidative stress. Cellular systems are however extremely complicated and multiple mechanisms of action may contribute to the overall cytoprotective effects of phytochemicals. Phytochemicals, unlike many drugs, have not been designed to have a specific effect and are likely to have multiple and diverse effects on cellular homeostasis. Although in the case of protection from H$_2$O$_2$-induced oxidative stress, one mechanism may be significantly more important than others, a detailed examination needs to be conducted into both the major putative mechanisms of protection, such as chemical and enzymatic antioxidant function, and any potential interactions which exists between cell death and cell survival signals.

To assess the mechanisms underlying the phytochemical protection from cell death seen in the previous chapter, four isomers of DHBA were chosen, that are blood metabolites of either the cyanidin derived anthocyanins (CDAs) or the phenolic acid; salicylic acid. An examination was conducted starting with the conventional hypothesis, that their chemical antioxidant properties are the principle mechanisms of protection. The ability of these four isomers of DHBA, which are present in plasma after the consumption of fruits or vegetables, to protect cells from H$_2$O$_2$-induced oxidative damage, was examined.
The chemical antioxidant capacity of four isomers of DHBA was determined by both the FRAP and ORAC assays. 2,3-DHBA and 2,5-DHBA were shown to be potent chemical antioxidants, with 3,4-DHBA and 2,4-DHBA being moderate and weak chemical antioxidants, respectively. Pretreatment of the neuroblastoma cell line SH-SY5Y with the isomer 3,4 DHBA up-regulated catalase activity and significantly reduced intracellular oxidative stress, caspase 8 and 9 activation and oxidative stress-induced apoptosis. The increased catalase activity, and the associated protection from cell death, was shown to be partially mediated by elevated thioredoxin reductase activity and was not associated with increases in either the catalase protein or glutathione peroxidase. In contrast, 2,3-DHBA offered only slight cytoprotection, whereas 2,4- DHBA and 2,5-DHBA offered no protection despite the latter being a potent chemical antioxidant.

These data suggest that protection from H$_2$O$_2$ by DHBA isomers is unrelated to their chemical antioxidant capacity and is significantly mediated by regulation of antioxidant enzyme expression. Given the previously reported health benefits of increased endogenous antioxidant expression, in addition to the 3,4-DHBA mediated upregulation of antioxidant enzymes presented in this thesis, it is likely that increasing the consumption of the dietary precursor to 3,4-DHBA, namely CDAs, may convey significant health benefits.

5.2 Introduction

Dietary consumption of fruits and vegetables is linked to many health benefits including the protection from or delay of heart disease, cancer and Alzheimer’s disease (see (Youdim & Joseph 2001; Ignarro et al. 2007; de Kok et al. 2008) for review). These health benefits are believed to be due to the cytoprotective effects of plant phytochemicals and many reports show in vitro data indicating that these dietary phytochemicals are able to quench oxidants, regulate protein expression and modulate signaling (Virgili & Marino 2008). However, phytochemical compounds are substantially modified by enzymatic conjugation and/or metabolic breakdown before, or if, they are eventually released into plasma (Baba et al. 2000; Day et al. 2001), (see...
(Manach et al. 2005) for review). For example, cyanidin, a primary anthocyanidin in the human diet, has been shown to be present at extremely low levels (0.5 nM) in human plasma following its consumption. In contrast, following a cyanidin-3-glucoside containing meal, its metabolite 3,4-DHBA is present in plasma at a concentration of 1 µM, over 2000 times higher than its dietary precursor (Vitaglione et al. 2007). Given the lack of bioavailability of many dietary phytochemicals in vivo and the significant levels of their absorbed metabolites, questions can be asked about the in vivo relevance of in vitro bioassays using non-metabolized dietary phytochemicals. Recently studies using phytochemical metabolites have highlighted the differences in activity of dietary polyphenols as compared to their in vivo metabolites. For example, Stevenson and co-workers have shown that the cytoprotective effects of polyphenols vary between dietary compounds and their conjugated metabolites, with the metabolites offering either less or more protection than their parent compound (Stevenson et al. 2008). It has further been shown that the quercetin metabolite, quercetin 3-glucuronide, can have significant effects in an in vitro model of neutrophil activity (and quercetin does not), despite its relatively lower anti-oxidant capacity (Suri et al. 2008). Conversely, quercetin has been shown to down-regulate expression of the inflammation induced adhesion molecules vascular cell adhesion protein 1 (VCAM-1) and inter-cellular adhesion molecule 1 (ICAM-1), whereas its metabolites do not (Tribolo et al. 2008). These results suggest that in vitro experiments using precursor dietary compounds at non-physiological concentrations can generate both false positive and false negative results.

The DHBAs are catechol based phytochemical metabolites and as such, are likely to exhibit similar properites to other catechol based compounds. Significant research has previously been conducted into both the chemical and biological properties of other catechol based compounds. For example, L-DOPA, a sychoactive drug found in certain kinds of food and herbs, possesss the same 3,4-catechol moeity as 3,4-DHBA and has been shown to both inhibit oxidaitve damage to phospholipids and conversly generate oxidaitve stress via reaction with various ferric containing compounds (Spencer et al 1996). Catechol based compounds have also been shown to cycle between hydroxy quinone, semi-quinone and quinone forms, and that both the kenetics of cycling and the stabiliy of the cycling products can be affected by the
steriochemistry of the substutied groups. For example, catechol estrogens, 2-hydroxyestrone and 4-hydroxyestrone, can both cycle to form quinone forms, however, the stability and reactivity of the quinone forms is greatly different. A quinone form of 2-hydroxyestrone has a half life of 47s, where as the quinone form of 4-hydroxyestrone is considerably longer lived with a half life of 12 minutes (Iverson et al. 1996). In addition to the differences in stability, biological activity can also be affected by the steroiochemistry of the substutied groups. Both 2-hydroxyestrone and 4-hydroxyestrone derived quinones can from DNA adducts via depurinating reactions with adenine and guanine. However, 4-hydroxyestrone forms these adducts via 1,6 Michael type addition (Cavalieri et al. 2000; Cavalieri et al, 2006), as opposed to the 1,4 type additions 2-hydroxyestrone causes (Stack et al. 1996; Debrauwer et al. 2003). Further, it appears that 4-hydroxyestrone is both more capable of forming these adducts and that specific adducts formed are also considered to be more likely to result in carcinogenesis (Zahid et al. 2006). Given both the notable biological reactivity of catechol base compounds and the potential diversiy of this activiy, the DHBAs being examined in this chapter may have a range of bioligical effects spanning from antioxidant through to pro-oxidant and this effect may vary with both time and concentration.

Here we examine the ability of the DHBAs, *in vivo* metabolites of anthocyanins, catechins and procyanidins (Tsuda et al. 1999; Gu et al. 2007), to protect the oxidatively sensitive human neuroblastoma cell line SH-SY5Y from H$_2$O$_2$ mediated oxidative insult. We show that the cytoprotection they convey appears to be mediated by the upregulation of endogenous cellular protective mechanisms and is not related to any direct effects on caspase mediated cell death signaling pathways or the chemical antioxidant capacity of the compounds.
5.3 Specific methods

5.3.2 Cell culture conditions

SH-SY5Y cells were treated as described in the methods section (2.1). Inhibitor treatment was carried out either, 22 h after DHBA treatment and 2 h prior to either exposure to H$_2$O$_2$ or measurement of catalase activity for catalase and glutathione inhibitors, or 18 h after DHBA treatment and 6 h before H$_2$O$_2$ treatment in the case of TrxR inhibition.

5.3.5 Quantification of intracellular oxidative stress

SH-SY5Y cells were exposed to the test DHBA or vehicle-only control for 24 h in complete DMEM/F12 prior to treatment with the oxidative sensitive dye DCFDA. The method was based on a modified version of the protocol used by Krejsa et al. 1997 (Krejsa et al. 1997). Briefly, cells were washed in PBS containing 1% FBS pre-warmed to 37°C then treated with 25 µM DCFDA in PBS for 30 min at 37°C in a 5% CO$_2$ in air incubator. Cells were then washed twice with PBS pre-warmed to 37°C containing 1% FBS to remove excess DCFDA dye and then were treated with 100 µM H$_2$O$_2$ for 30 min at 37°C.

Alternatively, SH-SY5Y cells were treated with various concentration of 3,4-DHBA for up to 5 h with or without the co-addition of 100 units bovine catalase. Cells were washed in PBS containing 1% FBS pre-warmed to 37°C then treated with 25 µM DCFDA in PBS for 30 min at 37 °C in a 5% CO$_2$/ air incubator. Cells were then washed twice with PBS pre-warmed to 37°C containing 1% FBS to remove excess DCFDA dye. DCFDA fluorescence was measured using flow cytometry. Flow cytometric analysis was performed using a Cytomics FC500MPL (Beckman Coulter, Miami, FL) with excitation at 485 nM and emission measured at 530 nM. Non-viable cells were removed from the analysis by gating based on light-scattering properties. 35,000 viable cells were counted from each culture.
5.3.6 Caspase activation assay

Activation of caspase 8 and 9 was measured by flow cytometry using excitation at 485 nM and emission at 520 nM and 580 nM, respectively. Flow cytometric analysis was performed using a Cytomics FC500MPL (Beckman Coulter, Miami, FL.) Staining was conducted using FLICA caspase detection kits (Immunochemistry Technologies, Bloomington, MN, USA) according to the manufacturer’s instructions. Briefly, SH-SY5Y cells were exposed to 100 µM H₂O₂ for up to 24 h after treatment with either DHBA or a vehicle-only control for 24 h. Caspase activation was measured immediately prior to, as well as 1, 2, 3, 6, 18 and 24 h after the addition of 100 µM H₂O₂. Cell soluble caspase 8 and 9 fluorescent inhibitors were added and cells were incubated at 37°C in 5% CO₂ in air for 2 h with gentle mixing (60 rpm). Cells were then washed three times with apoptosis wash buffer (Immunochemistry Technologies, Bloomington, MN, USA) prior to analysis. Total cell count was set to 35,000 cells.

5.4 Results

5.4.1 Chemical antioxidant capacity of the DHBAs

The chemical antioxidant capacity of 2,3-, 2,5-, 2,4- and 3,4-DHBA (Figure 18) was measured using both the FRAP (as described in section 2.2) and the ORAC (as described in section 2.3) assays and compared to a Trolox control. 2,3- and 2,5-DHBA showed significant chemical antioxidant capacity of 2.21 and 2.12 Trolox equivalent (activity relevant to the same concentration of Trolox) respectively, in the FRAP assay, with 3,4-DHBA having about 1.5 times the ferric ion reducing capacity of the Trolox standard (Figure 18 a). 2,4-DHBA did not have the same chemical antioxidant capacity as the other metabolites and was a very weak chemical antioxidant in both the FRAP and ORAC assays (Figure 18 a and b). The ORAC results were lower than the FRAP results for 2,5, 3,4-DHBA and 2,3-DHBA. 2,5-DHBA was found to be the strongest chemical antioxidant with 2,3, 3,4 and 2,4-DHBA having strong, moderate and weak antioxidant chemical antioxidant capacity, respectively.
Figure 18: Chemical antioxidant capacity of isomers of DHBA

4 isomers of DHBA were tested for chemical antioxidant action using the FRAP and ORAC assay (as described in section 2.2 and 2.3 respectively). DHBA sample were prepered at 100 µM and compared to a Trolox standard. (a). Chemical antioxidant capacity as measured in the FRAP assay. (b). Chemical antioxidant capacity as measured by ORAC assay. Results of 2 separate experiments with 2 replicate samples expressed as means ± S.D.
5.4.2 Protection from cell death and correlation with antioxidant capacity

The ability of DHBAs to protect against oxidative cell death was determined using the biologically relevant oxidative stressor H\textsubscript{2}O\textsubscript{2}. Predetermined concentrations and lengths of exposure were used that resulted in a significant amount of cell death as assessed using assays of annexin 5 binding, a marker of phosphatidylserine exposure during early stage apoptosis, and PI, a non-membrane-permeable DNA stain that indicates cell viability (Vermes et al. 1995) (as described in section 2.4). Cells that were annexin 5 positive were deemed to be undergoing early stage apoptosis. Cell that were both PI positive and annexin 5 positive were determined to be late apoptotic cells while cells that were only PI positive were determined to be necrotic. Cells that were either annexin 5 positive, PI positive or both annexin 5 and PI positive were defined as non-viable for the purposes of this assay. Treatment with 100 µM H\textsubscript{2}O\textsubscript{2} for 18 h decreased cell viability to 21.4% (Figure 19 a) with almost all cells becoming annexin 5 and PI positive. A 24 h pre-treatment with 100 µM 3,4-DHBA offered significant (P < 0.001) protection, improving cell viability to 41.5%. Pre-treatment of these cells with 2,4-DHBA and 2,5-DHBA had no significant effect on cell viability. 2,3-DHBA offered slight, but non-significant protection (P = 0.06) with a 100 µM pre-treatment improving cell viability from 21.4% to 27.9%. Cells treated with a 0.1% DMSO control and no H\textsubscript{2}O\textsubscript{2} retained over 90% viability (Figure 19 a). Treatment with 100 µM of any of the four DHBAs alone did not induce cell death (data not shown). Given only 3,4-DHBA was shown to offer protection from H\textsubscript{2}O\textsubscript{2}-induced cell death, further experiments examining the mechanisms underlying this protection were conducted using only 3,4-DHBA.
(a) Cell viability of different treatments compared to control. 

(b) Graph showing the chemical antioxidant capacity (FRAP) and % protection from cell death for various DHBA concentrations. The data suggests a weak linear relationship with $R^2 = 0.04$. 

- 2,3-DHBA
- 2,5-DHBA
- 3,4-DHBA
- 2,4-DHBA
Figure 19: Correlation between antioxidant capacity and protection from oxidative stress,
Cells were exposed to 100 µM H$_2$O$_2$ 18 h after a 24 h treatment with either, 2,3-, 2,4-, 2,5-, or 3,4-
DHBA. The cell viability was determined by flow cytometry after staining with annexin 5 and
propidium iodide (as described in section 2.4). Results from cytoprotection assay were compared to the
FRAP and ORAC results from Figure 18. (a) Protection from H$_2$O$_2$-induced cell death by DHBA pre-
treatment. (b) Correlation between chemical antioxidant capacity (FRAP) and protection from cell
death (c) Correlation between chemical antioxidant capacity (ORAC) and protection from cell death.
Results for cytoprotection are from 3 separate experiments with 2 replicate samples expressed as means
± S.E.M. * denotes significantly different from cells treated with H$_2$O$_2$ alone. Significant difference is
denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001). Correlations are shown only to highlight that
no correlation can be made and are not representative of significance.

5.4.3 Intracellular oxidative stress

Intracellular oxidative stress was measured using the intracellular oxidative state
sensitive dye DCFDA. As the SH-SY5Y cells are only semi-adherent, levels of
oxidative stress as determined by DCFDA fluorescence were measured by flow
cytometry rather than a 96-well-based fluorometric assay. Further, a relatively high
concentration (25 µM) of DCFDA was used to ensure that the dye was in excess
relative to the oxidative stress level of the cell and even small changes in internal
oxidative stress could be measured. This resulted in higher baseline fluorescence but
allowed for greater separation of the peaks on the flowcytometer. A 24 h pre-
treatment with 3,4-DHBA conveyed a concentration-dependent decrease in
intracellular oxidative stress with a small, non-significant, decrease being evident at 1
µM and significant, P < 0.01 and P < 0.001, decreases seen at 10 and 100 µM, respectively (Figure 20).

![Graph showing the reduction of H₂O₂-induced intracellular oxidative stress by pre-treatment of SH-SY5Y cells with 3,4-dihydroxybenzoic acid (DHBA).](image)

Figure 20: Reduction of H₂O₂-induced intracellular oxidative stress by pre-treatment of SH-SY5Y cells with 3,4-dihydroxybenzoic acid (DHBA).

Intracellular oxidative stress was assessed using the oxidative sensitive dye, DCFDA, which is fluorescent under oxidative conditions. Levels of intracellular oxidative stress were determined by flow cytometry. Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from cells treated with H₂O₂ alone. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).

5.4.4 Caspase activation

Baseline caspase 9 activation was seen in approximately 10 % of SH-SY5Y cells with no increase being detected until 3 h after H₂O₂ (100 µM) challenge. Caspase 9 activation levels continued to increase until reaching a maximal value at 18 h. Pre-treatment with 3,4-DHBA induced a statistically significant (P < 0.01) concentration-dependent decrease in caspase 9 activation (Figure 21 a). Basal levels of caspase 8 positive cells were also shown to be approximately 10 %, with no increase seen during the first 3 h after the treatment with 100 µM H₂O₂. Caspase 8 activation increased to 18% 6 h after the H₂O₂ insult, indicating activation of caspase 8 occurred
between 3 and 6 h after the addition of the H$_2$O$_2$, with maximal levels being reached within 18 h. Pre-treatment with 3,4-DHBA also induced a statistically significant (P < 0.01) concentration-dependent decrease in caspase 8 activation (Figure 21 b). Untreated control cells has approximatly 10% activation of both caspase 8 and 9.

![Graph](image)

**Figure 21: Suppression of caspase 8 and 9 activation by 3,4-DHBA in response to a H$_2$O$_2$ challenge.**

Pre-treatment of SH-SY5Y cells with 3,4-DHBA for 24 h prior to exposure to 100 µM H$_2$O$_2$. (a) Caspase 9 activation after the administration of H$_2$O$_2$. (b) Caspase 8 activation after administration of H$_2$O$_2$. Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from cells treated with H$_2$O$_2$ alone. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).
5.4.5 Catalase activity

Pre-treatment for 24 h with 3,4-DHBA significantly increased catalase activity (as described in section 2.5), with 100 µM of 3,4-DHBA inducing a 30% increase in catalase activity levels relative to control (Figure 22). To determine whether this increase was responsible for the protection from H₂O₂-induced cell death, catalase activity was inhibited using the specific and irreversible catalase inhibitor 3-amino-1,2,4-triazole (3-AT) (Price et al. 1961; Darr & Fridovich 1986) (Figure 23).

Figure 22: Effect of 3,4-DHBA treatment on catalase activity in SH-SY5Y cells.
SH-SY5Y cells were treated with various concentrations of 3,4-DHBA for 24 h prior to assessments of catalase activity by fluorescent plate reader (as described in section 2.5). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from untreated cells. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).
5.4.6 Catalase and glutathione peroxidase

Inhibition of catalase activity in 3,4-DHBA treated SH-SY5Y cells by the specific catalase and glutathione peroxidise (GPx) inhibitors 3-AT and MS (as described in section 2.6) did not reduce the 3,4-DHBA mediated increase, indicating the direct regulation of either catalase or glutathione peroxide may not be the most significant mechanism underlying 3,4-DHBA induced increases in catalase activity (Figure 23) GPx data not shown.

![Figure 23: Regulation of catalase activity by 3,4-DHBA.](image)

SH-SY5Y cells were treated with either 100 µM 3,4-DHBA or a vehicle only control or for 22 h then the catalase inhibitor 3-AT (10 mM) for 2 h prior to assessment of catalase activity by fluorescent plate reader (as described in section 2.6). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from 3,4-DHBA treated cells. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).
5.4.7 Determination of Trx concentration

Treatment of SH-SY5Y cells with 100 µM 3,4-DHBA for 24 h induced a significant increase in Trx concentration (as described in section 2.7). The 3,4-DHBA treatment induced a 10% increase in Trx concentration (Figure 24 a). The Trx standard curve is shown in (Figure 24 b).

(a) SH-SY5Y cells were treated with either 100 µM of 3,4-DHBA or a DMSO control for 24 h prior to assessment of Trx concentration by spectrophotometry (as described in section 2.7). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from 3,4-DHBA treated cells. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).

(b) Standard Trx curve.

Figure 24: Regulation of Trx concentration by 3,4-DHBA.

\[ r^2 = 0.95 \]
\[ Y = 11.49X + 0.22 \]
5.4.8 TrxR activity

Treatment of SH-SY5Y cells with 100 µM 3,4-DHBA induced a significant increase in TrxR activity after a 24 h treatment (as described in section 2.8). Inhibition of the NADPH induced conversion from DTNB to TNB was almost completely inhibited by the addition of the TrxR inhibitor Auranofin (Figure 25 b). 3,4-DHBA treatment was seen to increase TrxR activity in SH-SY5Y cells by approximately 60% after a 24 h pretreatment with 100 µM. Treatment of 3,4-DHBA exposed SH-SY5Y cells with 0.5 µM Auranofin reduced TrxR levels to basal levels (Figure 25 b). The TrxR standard curve is shown in (Figure 25 a).
Figure 25: Induction of TrxR activity by 3,4-DHBA and its inhibition by Auranofin.

SH-SY5Y cells were treated with either a 100 µM 3,4-DHBA or a DMSO control for 18 h prior to the addition of either 0.5 µM, 5 µM Auranofin or a DMSO control vehicle for 6 h before the assessment of TrxR activity by plate reader (as described in section 2.7). (a) Standard curve for inhibition of TrxR activity (expressed as % of control) by the gold based inhibitor Auranofin. (b) Effect of both 3,4-DHBA treatment and/or Auranofin on TrxR activity (expressed as % of control). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from 3,4-DHBA treated cells. Significant difference is denoted by * (P>0.05), ** (P>0.01) and *** (P<0.001).
5.4.9 3,4-DHBA mediated changes in intracellular oxidative stress

Treatment of SH-SY5Y cells with 100 µM 3,4-DHBA induced a time dependent change in intracellular oxidative stress levels. Oxidative load was seen to decrease during the first 3 h of exposure and increase over basal levels after 3 h, reaching a maximum at 4 h then returning to basal levels (Figure 26 a). Decreases in oxidative stress levels seen after short term exposure to 3,4-DHBA likely represent a chemical antioxidant mediated effect. Treatment of SH-SY5Y cells with 100 units of bovine catalase prevented the significant increase in intracellular oxidative stress seen at the 4 h time point (Figure 26 b). The significant increase in oxidative stress seen after 4 h hours of exposure to 3,4-DHBA is similar to previously published data using similar compounds.
Figure 26: Induction of intracellular oxidative stress by 3,4-DHBA treatment.

SH-SY5Y cells were treated with either 100 µM 3,4-DHBA alone or co-treated with 100 µM 3,4-DHBA and bovine catalase (10 units) for up to 5h before assessment of intracellular oxidative stress by DCFDA fluorescent. (a) Treatment of SH-SY5Y cells with 3,4-DHBA induced oxidative stress after 4h (b) Treatment of SH-SY5Y cells with 3,4-DHBA and bovine catalase (10 units) Black horizontal line represents untreated control cells. (c) SH-SY5Y cells were treated with either 10, 1 or 0.1 µM 3,4-DHBA for up to 5h before assessment of intracellular oxidative stress by DCFDA fluorescent. Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. * denotes significantly different from untreated cells (P>0.05).

5.4.10 3,4-DHBA activation of MAPKinases

Treatment of SH-SY5Y cells with 10 µM 3,4-DHBA induced significant increases in AKT1 activation that is associated with anti-apoptotic signalling (as described in section 2.11). Additionally increased MEK activity was seen after 10 µM 3,4-DHBA treatment. Decreases in ERK1/2 activation were seen after treatments with 100 or 1000 µM 3,4-DHBA for 24 h (Table 3).
Table 3: Activation of MAPKinase signalling by 3,4-DHBA treatment.

Treatment of SH-SY5Y cells with 3,4-DHBA for 4 h induced increased activation of ARK1 and decreased MEK1/2 after 10 µM exposure and decreased levels of ERK1/2 after 100 µM and 1 mM exposure (as described in section 2.11). Results are shown from one analysis using 2 pooled samples of 2 wells from 2 independent experiments expressed as means ± S.E.M. * denotes significantly different from untreated cells. Significant difference is denoted by * (P>0.05).

<table>
<thead>
<tr>
<th></th>
<th>(-) Control</th>
<th>(+) Control (H₂O₂)</th>
<th>10 µM 3,4-DHBA</th>
<th>100 µM 3,4 DHBA</th>
<th>1 mM 3,4-DHBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atk1/PKB</td>
<td>211.7 ± 0</td>
<td>218.2 ± 8.2</td>
<td><strong>316.9 ± 25.6</strong></td>
<td>241.9 ± 5.6</td>
<td>260.2 ± 13.2</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>103.0 ± 2.0</td>
<td>63.75 ± 15.0</td>
<td>85.95 ± 8.9</td>
<td><strong>48.90 ± 1.0</strong></td>
<td><strong>42.70 ± 3.6</strong></td>
</tr>
<tr>
<td>JNK1/2</td>
<td>133.6 ± 28.5</td>
<td>234.4 ± 36.9</td>
<td>228.8 ± 10.0</td>
<td>192.6 ± 21.3</td>
<td>210.5 ± 17.9</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>371.8 ± 17.4</td>
<td>276.8 ± 15.5</td>
<td><strong>260.8 ± 12.0</strong></td>
<td>203.1 ± 15.8</td>
<td>312.9 ± 35.9</td>
</tr>
<tr>
<td>P38</td>
<td>7.244 ± 1.8</td>
<td>4.791 ± 0</td>
<td>1.844 ± 1.32</td>
<td>3.976 ± 0.82</td>
<td>8.964 ± 8.44</td>
</tr>
<tr>
<td>Stat1</td>
<td>5.498 ± 2.4</td>
<td>34.52 ± 13.7</td>
<td>27.58 ± 1.37</td>
<td>17.18 ± 4.8</td>
<td>12.66 ± 1.8</td>
</tr>
</tbody>
</table>
5.4 Discussion

5.4.1 Chemical antioxidant capacity

The chemical antioxidant capacity of 2,3-, 2,5-, 2,4- and 3,4-DHBA (Figure 18) was measured using both the FRAP and the ORAC assays and compared to a Trolox control (as described in section 2.2 and 2.3). ORAC and FRAP are examples of the two major classes of chemical antioxidant assays, the hydrogen atom transfer (HAT) and the electron transfer (ET) assays respectively, and hence measure a different type of chemical antioxidant action (ORAC vs FRAP vs other antioxidant assays are reviewed by Huang and coworkers (Huang et al. 2005)). 2,3- and 2,5-DHBA showed significant chemical antioxidant capacity of 2.21 and 2.12 g/g Trolox equivalents, respectively, in the FRAP assay, with 3,4-DHBA having about 1.5 times the ferric ion reducing capacity of the Trolox standard (Figure 18 a). 2,4-DHBA did not have the same chemical antioxidant capacity as the other metabolites and was a very weak chemical antioxidant in both the FRAP and ORAC assays (Figure. 18 a and b). The ORAC results were lower than the FRAP results for 2,5, 3,4-DHBA and 2,3-DHBA. 2,5-DHBA was found to be the strongest chemical antioxidant with 2,3, 3,4 and 2,4-DHBA having strong, moderate and weak antioxidant chemical antioxidant capacity, respectively.

It is of course important to note that the specific chemistry of the ORAC and FRAP assays means that the results in these assays may not be directly related to other HAT an ET assays. The ORAC assay measures the capacity of compounds to quench ROO• hence only measures this aspect of antioxidant action. Additionally it may be affected by the reaction of the antioxidant radical, which forms from the reaction of ROO• with the test compound, with the fluorescent dye (Huang et al. 2002; Prior et al. 2003), hence it is possible that the formation of an antioxidant radical of one of the DHBAs reacted with the fluorescent dye and lowered the perceived antioxidant capacity. Given the chemical structure of the DHBAs being tested, the delocalisation of electrons would be less in the ortho substituted DHBAs (2,3-DHBA and 3,4-DHBA) (Kalyanaramans et al. 1989). Since both 3,4-DHBA and 2,3-DHBA had
significant antioxidant action in this assay, it would be unlikely that a secondary reaction of the antioxidant radical with the fluorescent dye occurred. Additionally, as these assays are of short duration, the longer term antioxidant/redox capacity of these compounds may not be reflected in these assays.

### 5.4.2 Protection from cell death

The ability of DHBAs to protect against oxidative cell death was determined using the biologically relevant oxidative stressor H\(\textsubscript{2}O\textsubscript{2}\) and analysed by annexin 5 and PI co-staining (as described in section 2.4). Cells that were either annexin 5 positive, PI positive or both annexin 5 and PI positive were considered to be non-viable. To recap the results, treatment with 100 µM H\(\textsubscript{2}O\textsubscript{2}\) for 18 h decreased cell viability to 21.4% (Figure 19 c) with almost all cells becoming annexin 5 and PI positive. A 24 h pre-treatment with 100 µM 3,4-DHBA offered significant (P < 0.001) protection, improving cell viability to 41.5%. Pre-treatment of these cells with 2,4-DHBA and 2,5-DHBA had no significant effect on cell viability. 2,3-DHBA offered slight, but non-significant protection (P = 0.06) with a 100 µM pre-treatment improving cell viability from 21.4% to 27.9%. Cells treated with a 0.1% DMSO control and no H\(\textsubscript{2}O\textsubscript{2}\) retained over 90% viability (Figure 19 c). Treatment with 100 µM of any of the four DHBAs alone did not induce cell death (refer to Figure 15 b).

### 5.4.3 Relationship between chemical antioxidant capacity and protection from cell death

To ascertain whether these cytoprotective effects are related to the chemical antioxidant potential of the compounds tested, the effects on cell viability were compared to the chemical antioxidant capacity of each compound. No association was seen between the protection from H\(\textsubscript{2}O\textsubscript{2}\)-induced cell death and chemical antioxidant capacity using either the FRAP or the ORAC assays (\(r^{2} = 0.005\) and 0.04, respectively) (Figures 19 b and c). Importantly, the most potent chemical antioxidants, 2,3 and 2,5-DHBA, offered little and no protection, respectively. 3,4-DHBA showed significant cytoprotection and had a moderate antioxidant capacity whilst 2,4-DHBA offered no protection and had little antioxidant capacity (Figures.
19 a, b and c). The slight, but non-significant (P = 0.06), protection from H$_2$O$_2$-induced cell death by 2,3-DHBA may possibly have been due to the compound’s ability to bind iron, which would limit the generation of hydroxyl radicals via the Fenton reaction. The presence of intracellular, but not extracellular, iron has been shown to be critical for H$_2$O$_2$-induced cell death in Jurkat cells, and further, the binding of iron by phytochemicals has been shown to offer protection from H$_2$O$_2$-induced cell death (Barbouti et al. 2001; Melidou et al. 2005). The significant (P < 0.001) protection from H$_2$O$_2$-induced cell death by 3,4-DHBA is unlikely to be due to either a direct antioxidant effect or an iron-binding effect as it had relatively low ORAC and FRAP values, and little potential iron-binding capability relative to the other phenolic acids.

These results suggest that chemical antioxidant capacity, at least in the case of these four important phytochemical metabolites, does not account for the effectiveness of a phenolic compound in an in vitro cytoprotective assay against oxidative stress. This finding agrees with those of Zhang et al. 2006 (Zhang et al. 2006) who showed that the antioxidant capacity, as measured by ORAC and lipid peroxidation assays, of three dietary polyphenols and three dietary phenolic acids did not correlate with their ability to protect from H$_2$O$_2$-induced cell death. Together, these results highlight the fact that protective mechanisms of action by blood metabolites may not relate to their chemical antioxidant capacity. Hence a different mechanism(s) must exist, other than direct chemical antioxidant quenching of H$_2$O$_2$ to explain how SH-SY5Y cells are protected from H$_2$O$_2$-induced cell death by DHBA compounds. To evaluate the possible mechanisms of protection, we focused on 3,4-DHBA, as it gave the greatest protection in the in vitro cell death assay.

5.4.4 Protection from intracellular oxidative stress by pre-treatment with 3,4-DHBA

The results presented in this chapter have shown that the chemical antioxidant capacities of the DHBA’s do not correlate with protection from cell death, suggesting direct chemical neutralization of the oxidant is not the mechanism of protection in this instance. The other possible mechanisms involved can be separated into two distinct
groups; 1) endogenous antioxidant mechanisms, i.e. the up-regulation of the cellular antioxidant machinery or 2) endogenous cytoprotective mechanisms i.e. modulation of survival signaling pathways. Phytochemicals have previously been shown to regulate both of these protective mechanisms. The isothiocyanate sulforaphane has been reported to increase expression of the endogenous antioxidant enzyme TrxR, allowing for greater processing of oxidants within the cell (Wang et al. 2005a). Cytoprotective mechanisms, such as the inhibition of caspase-mediated cell death signaling and increased cholesterol expression resulting in resistance to H2O2-induced lipid peroxidation have been shown by members of the catechin family of polyphenols and quercetin, respectively (Katunuma et al. 2006; Soundararajan et al. 2008). These potential intracellular protective mechanisms were evaluated, starting with the ability of the compounds to inhibit the production of intracellular oxidative stress.

Oxidative challenge of cells has been shown to lead to an increase in the oxidative stress within the cell (Olivieri et al. 2003). Given that the protective actions of 3,4 DHBA against oxidative stress is unlikely to be due to its own antioxidant potential, the suppression of internal oxidative stress by pre-treatment with 3,4-DHBA may imply an increase in cellular processing ability of H2O2, putatively via an up-regulation of endogenous antioxidant mechanisms. If no decrease in internal oxidative stress is detected following oxidative challenge and 3,4-DHBA treatment, it would imply an endogenous cytoprotective mechanism of action.

Intracellular oxidative stress levels were shown to increase in SH-SY5Y cells after H2O2 exposure. Pretreatment with 3,4-DHBA induced a dose dependent decrease in H2O2 induced oxidative stress, with significant decreases being seen after a 24 h pretreatment with both 10 and 100 µM 3,4-DHBA and a small non-significant, decrease being evident at 1 µM. Potentially, a longer pretreatment with 1 µM 3,4-DHBA may induce a significant decrease in oxidative stress levels. These results show that 3,4-DHBA pretreatment of SH-SY5Y cells supresses oxidant induced increases in intracellular oxidative stress. Such decreases in intracellular oxidative stress can be mediated by multiple cellular events including induction of antioxidant enzyme expression and caspase inhibition.
5.4.5 Prevention of caspase 8 and 9 activation

The reduction in intracellular oxidative stress offered by 3,4-DHBA pre-treatment implies an increase in the ability of these cells to process H$_2$O$_2$ and hence an increase in the activity of endogenous antioxidant mechanisms. However, since the measurement of intracellular oxidative stress was taken at a fixed time point of 30 min and not over time, as would have been possible for a plate-based assay, it is still uncertain if any cell death signaling pathways had been modulated prior to the suppression of oxidative stress.

Previously, it has been shown that polyphenols, primarily the catechin family, bind to the cell death signaling protease family of caspases using an allosteric binding site and thereby inhibit their action (Katunuma et al. 2006). Caspase activation is a marker of apoptotic cell death and caspase 8 and 9 act to propagate cell death by the extrinsic and intrinsic apoptotic cell death pathways, respectively (see (Li & Yuan 2008) for review). Further, the suppression of cell death processes by inhibiting caspase activation has been shown to decrease the intracellular oxidative state within multiple cell lines including neuronal cells (Kim et al. 2007a; Nakagawa et al. 2008) and the induction of caspase activation induces subsequent oxidative stress (Kagan et al. 2002). Hence it is possible that 3,4 DHBA may have inhibited caspase activation during the 30 min after H$_2$O$_2$ treatment and thereby have suppressed the oxidative stress that would have been generated subsequently. To determine whether this is the mechanism of protection by 3,4 DHBA, the activation of caspase 8 and caspase 9 were assessed.

Baseline caspase 9 activation was seen in approximately 10% of SH-SY5Y cells with no increase being detected until 3 h after H$_2$O$_2$ challenge. Caspase 9 activation levels continued to increase until reaching a maximal value at 18 h. Pre-treatment with 3,4-DHBA induced a statistically significant (P < 0.01) concentration-dependent decrease in caspase 9 activation (Figure 21 a). Basal levels of caspase 8 positive cells were also shown to be approximately 10%, with no increase seen during the first 3 h after the treatment with 100 µM H$_2$O$_2$. Caspase 8 activation increased to 18% 6 h after the
H₂O₂ insult, indicating activation of caspase 8 occurred between 3 and 6 h after the addition of the H₂O₂, with maximal levels being reached within 18 h. Pre-treatment with 3,4-DHBA also induced a statistically significant (P < 0.01) concentration-dependent decrease in caspase 8 activation (Figure 21 b).

These studies indicate that the prevention of cell death by a direct interaction of the 3,4-DHBA with the caspase signaling pathway was unlikely as caspase 9 activation did not occur until 3 h after addition of H₂O₂, whereas the suppression of oxidative stress was measured as early as 30 min after H₂O₂ treatment. This suggests that cytoprotection by 3,4-DHBA may be mediated via increases in endogenous antioxidant mechanisms. Furthermore, the activation of caspase 9 prior to caspase 8 suggests that H₂O₂-mediated apoptotic cell death of SH-SY5Y cells occurs through the intrinsic cell death signaling pathway.

5.4.6 Catalase activity and its relation to cytoprotection

The decrease in intracellular oxidative stress prior to caspase activation indicates an increase in H₂O₂ processing capacity of the cells. It has been previously shown in multiple cell lines that catalase levels within the cell are related to its ability to survive H₂O₂-mediated cell death (Lindau-Shepard & Shaffer 1993; Mann et al. 1997; Bai et al. 1999; Ma et al. 2006). In addition, genetically engineered mice lacking catalase are more sensitive to oxidative injury (Ho et al. 2004). It has also been shown that selective up-regulation of catalase levels is possible when cells are treated over time with H₂O₂. Given that H₂O₂ was used to induce the oxidative insult and that catalase is known to both protect from H₂O₂-induced cell death, and to be either inducible or suppressible within this cell line (Schmidt et al. 2008; Bar-Am et al. 2009), we evaluated the capacity of the cell to process H₂O₂ (catalase activity). It is worth noting that the catalase assay used here measured the enzymatic capacity of the cell to process H₂O₂ and not the levels or activity of the catalase protein per se. Many different cellular proteins contribute to overall H₂O₂ processing capacity of the cell, and as such, increases in catalase activity may represent an increase in one, many or all of these proteins.
Pre-treatment for 24 h with 3,4-DHBA significantly increased catalase activity, with 100 µM of 3,4-DHBA inducing a 30% increase in catalase activity levels relative to control (Figure. 22). To determine whether this increase was responsible for the protection from H₂O₂-induced cell death, we inhibited catalase activity using the specific and irreversible catalase inhibitor 3-AT (Price et al. 1961; Darr & Fridovich 1986) (Figure 23).

The inhibition of catalase removed the protection from cell death afforded by 3,4-DHBA pre-treatment. Treatment of control cells with 3-AT did not induce toxicity, but did make them more susceptible to H₂O₂-induced cell death. This suggest that the increases in catalase activity are responsible, at least in part, for the cytoprotection mediated by 3,4-DHBA and result in decreased oxidative stress, decreased caspase 8 and 9 activation and some protection from H₂O₂-induced cell death.

Regulation of catalase activity and the related increased ability to process H₂O₂ has many implications for human health. H₂O₂ has been shown to be produced by human skin cells in response to ultraviolet radiation B (UVB) exposure and hence increased catalase levels could protect from UVB-induced DNA damage. Catalase levels have been indirectly linked to protection from melanoma in human skin cells (Maresca et al. 2008). In addition, diet has been related to skin cancer prevalence and these results may suggest a potential food-derived mechanism by which this protection may be mediated. Catalase levels have also been linked to longevity in a Drosophila model (Aigaki et al. 2002) and are associated with protection from oxidative damage in many cell lines (Roos et al. 1980; Pietarinen et al. 1995). The ability of a dietary phytochemical metabolite known to be bioavailable in blood, to increase catalase activity may explain many of the health benefits that are seen in diets rich in the dietary precursor cyanidin.

Although catalase activity was associated with protection from cell death, no changes in 3,4-DHBA induced catalase activity were shown when catalase was inhibited with 3-AT. Given 3,4-DHBA was not seem to increase catalase’s activity specifically, but rather catalase-like activity, other cellular proteins that can effect catalase-like activity were assessed. Trx and TrxR are cellular proteins, which act to both regenerate
oxidised cellular antioxidants in addition to processing oxidants themselves. Both Trx levels and TrxR reductase activity were shown to be increased by 3,4-DHBA treatment of SH-SY5Y cells. Inhibition of 3,4-DHBA mediated increases in TrxR by the specific inhibitor Auranofin were shown to partially remove the increases in both catalase like activity and protection from cell death observed. These data suggests that 3,4-DHBA pretreatment of SH-SY5Y cells results in increased TrxR activity that partially mediates both the increased catalase-like activity and resistance to H₂O₂-induced cell death.

5.4.7 Induction of oxidative stress by 3,4-DHBA treatment

Generation of oxidative stress by phytochemicals as a mechanism for the induction of antioxidant enzyme expression has previously been reported (Rushmore et al. 1991). Further, it has been shown the increases in oxidative stress, generated with the addition of exogenous oxidants, can induce upregulation of endogenous antioxidant enzymes both in vitro and in vivo. Long term H₂O₂ treatment induced a 13-fold increase in catalase levels in human promyelocytic HL-60 cells (Hachiya & Akashi 2005). Additionally, exposure of rats to oxidative stress derived from cigarette smoke induced a two fold increase in testicular catalase activity and a 25% increase in testicular SOD activity (Ozyurt et al. 2006). Perhaps most importantly in the context of this thesis however, is the reported increase in antioxidant enzyme activity from the induction of oxidative stress in the SH-SY5Y related neuroblastoma cell line SK-N-BE (2C) after exposure to the phenolic compound meta-iodobenzylguanidine (Cornelissen et al. 1997).

Phytochemical-induced increases in oxidative stress have previously been shown to be involved in the induction of phytochemical induced apoptosis in cancer cell lines, and recently, have been suggested as a mechanism underlying upregulation of antioxidant expression. Sulforaphane, a potent antioxidant enzyme inducer, has been reported to both induce cell death by increasing redox signalling in addition to inducing antioxidant enzyme expression (Pham et al. 2004; Tanito et al. 2005; Brown et al. 2008). Further, the neurotransmitter dopamine, structurally similar to 3,4-
DHBA, has been shown to induce antioxidant enzyme expression mediated by increases in intracellular oxidative stress levels, specifically H$_2$O$_2$ (Shih et al. 2007).

Given these data, 3,4-DHBA was assessed for its capacity to induce intracellular oxidative stress and further determine if any stress generated could be suppressed by the removal of H$_2$O$_2$ using exogenous catalase. SH-SY5Y cells were treated with either 3,4-DHBA or 3,4-DHBA and exogenous catalase, which would process any H$_2$O$_2$ generated by the adding of 3,4-DHBA. 3,4-DHBA was found to generate intracellular oxidative stress that was substantially mediated by increases in H$_2$O$_2$ levels (Figure 26 a and b). Concentrations of 3,4-DHBA required to induce increases in oxidative stress were found to be 100 µM, as no increases were seen at 10 µM or lower (Figure 26 c). Although this is significantly greater than what is required for oxidative stress generation by many other phytochemicals, generation of oxidative stress by dopamine has previously been shown to occur at similar concentrations (Shih et al. 2007). Given both the structural and biological similarities between dopamine and 3,4-DHBA, it would seem likely they share a common mechanism of action during the generation of intracellular oxidative stress. In addition to the generation of intracellular oxidative stress, catechol type compounds have also been shown to directly bind to proteins. 3,4-dihydroxy-phenylanine (DOPA) forms long-lived redox-active products during free radical damage to proteins (Gieseg et al. 1993). Protein bond DOPA can react with other macromolecules, via its redox activity, causing damage and preventing normal protein function (Morin et al. 1998). DOPA has also been shown to be uptaken by cells in *in vitro* systems and results in a proportionally greater increase in protein bound DOPA within the cells (Pardoa et al. 2000). Although given DOPAs capacity to react with proteins in FBS, it is unlikely that this would occur in cell culture systems where FBS is used (Nelson et al. 2006). It is possible that 3,4-DHBA may also bind to proteins resulting in simialr redox active proteins and that these may be involved in either cellular damage or in antioxidant signaling systems. Given the potential long term detrimental effects of catechol integration into proteins, the extension of results presented here to human trials should be carefully considered.
Questions have been raised regarding potential *in vitro* artefacts relating to phytochemical reaction with cell culture media. Several reports associate the reaction of both vitamins and phytochemicals with cell culture media, which results in the subsequent generation of oxidative stress (Long et al. 2000). Phytochemicals, including quercetin, (+) catechin and EGCG, have all been shown to produce $H_2O_2$ when exposed to various media types (Long et al. 2000). Potentially, this could cast doubt upon the *in vivo* relevance of any results that show *in vitro* oxidative stress mediated up regulation of antioxidant enzymes (Clement et al. 2001). However, several mitigating factors could be considered before dismissing such *in vitro* results. Firstly, the generation of oxidative stress by media-phytochemical interactions depends on media type, and is potentially not as great for the DMEM/F12, as used in these SH-SY5Y cultures, as for some other media (Personal communication, Prof. Barry Halliwell). Secondly, the reaction of phytochemicals with blood also produces similar, albeit reduced, increases in oxidative stress levels (Personal communication, Prof. Barry Halliwell). Thirdly, in a limited number of studies, the regulation of antioxidant enzymes by phytochemical treatment has previously been shown to be consistent across *in vitro* and *in vivo* experiments (Tanito et al. 2005; Wang et al. 2005a). Finally, the specific structural/chemical features of phytochemical that facilitate the production of $H_2O_2$ in media are not known. Combined, these considerations suggest that while the pro-oxidant effects of phytochemicals may be potentiated in *in vitro* experiments, they are still relevant to *in vivo* effects and are still a valid research tool. Currently, development of cell culture medium that more closely mimics the effects of blood is underway and may potentially remove media specific effects and hence improve the relevance of *in vitro* assays further (Personal communication, Prof. Barry Halliwell).

### 5.4.8 Potential mechanisms underlying transcriptional events.

Pre-treatment of SH-SY5Y cells with 3,4-DHBA has been shown to offer protection from oxidant induced cell death. The data presented in this thesis suggests that an upregulation of endogenous antioxidant enzymes is an important mechanism underlying this cytoprotective effect. It does not, however, resolve any of the
transcriptional events which may link the two. The argument can be made that the transcriptional events are of less importance than functional outcomes, particularly when examining the effects of a dietary metabolite, as safety and overriding health benefits are already known. Further, as no additional development into the compound will occur, in contrast to what may occur with a drug, determining which transcriptional events are responsible may not necessarily lead to directly useful information. However, scientifically such an investigation would be interesting, and the identification of specific transcriptional events could, in theory, allow for a deeper understanding into how varieties of metabolites may interact. Although such an investigation has not been undertaken within this work, results, both pre- and post-transcriptional, in conjunction with published literature, suggests possible transcriptional mechanisms (Shih et al. 2007).

Activation of endogenous antioxidant expression by phytochemical compounds has been reported to occur via both, NRF2 mediated activation of the ARE and estrogen receptor mediated signalling (Rushmore et al. 1991; Montano et al. 2004). Induction of the ARE is regulated by the cytoplasmically located NRF2/Kelch-like ECH-associated protein 1 (KEAP1) complex. The disassociation of KEAP-1 from NFR2 occurs under several cellular conditions, including increased cellular redox state, sulfhydryl reduction of KEAP-1 and activation of MAPkinase signalling. Once dissociated from KEAP-1, NRF2 localises to the nucleus where it induces activation of the ARE that results in the expression of various endogenous antioxidant enzymes (Kwak et al. 2001). NRF2 mediated activation of the ARE has been shown to occur with multiple phytochemical treatments, including but not limited to, sulforaphane, curcumin, caffeic acid phenyl ester (CAPE), β-naphthoflavone, and EGCG (Venugopal & Jaiswal 1996; Balogun et al. 2003; Na & Surh 2008). The upregulation of the ARE by NRF2 nuclear localisation, occurs via several mechanisms. Activation of several MAPKinases, AKT1, p38, and ERK has been implicated in its activation, in addition to activation by oxidative stress independently of MAPKinase activation (Zipper & Mulcahy 2003; Martin et al. 2004; Hwang & Jeong 2008). Generally, most literature addressing phytochemical-induced activation of antioxidant enzymes, which examine transcriptional factors, suggests an NRF2 mediated mechanisms (Xu et al. 2006; Hwang & Jeong 2008; Na et al. 2008). Given, it has been shown that 3,4-
DHBA treatment of SH-SY5Y cells induces an increase in oxidative stress and also can, albeit from preliminary results, activate AKT1 signalling, the most likely transcriptional mechanism underlying 3,4-DHBA-mediated cytoprotection might be NRF2 nuclear translocation and subsequent activation of the ARE.

Interestingly, catechol-based oestrogens, which share a common 3,4 dihydroxy moiety with 3,4-DHBA, have also been shown to induce upregulation of antioxidant enzymes in vitro, suggesting a possible oestrogen receptor mediated mechanisms of activation (Montano et al. 2004). It is possible that 3,4-DHBA is activating antioxidant enzyme expression via the same mechanism as the catechol oestrogens. However, it has been shown that NRF2 knockout primary astrocytes have no antioxidant enzymes upregulation after oestrogen exposure (Lee et al. 2003), suggesting that catechol oestrogen mediated upregulation of antioxidant enzymes is also mediated via a NRF2 mediated pathway. Additionally, the enzymatic processing of the catechol oestrogen 17β-estradiol by cytochrome P450 A1 (CYP1A1) and cytochrome P450 B1 (CYP1B1) results in the formation of 2-OH-17β-estradiol. This molecule possesses the same 3,4 dihydroxy moiety as 3,4-DHBA, has been shown to activate NRF2, hence suggesting a possible related mechanism in antioxidant enzyme expression (Lee et al. 2003). It is interesting to note that post menopausal women, who have reduced catechol oestrogen levels, are at significantly greater risk of disease than pre-menopausal women (Harman et al. 2011) 3,4-DHBA is structurally similar to several compounds that have been shown to either, active endogenous antioxidant expression (catechol oestrogens) or induce increases in intracellular redox state (CAPE) (Lee et al. 2003; Chen et al. 2005a). Further, 3,4-DHBA is almost identical to dopamine, which has been shown to induce antioxidant enzyme expression via an oxidative stress dependent mechanism (Shih et al. 2007).

An examination of compounds which posses a similar 3,4-dihydroxy moiety as 3,4-DHBA (Table 4) suggests a possible mechanism for both the generation of oxidative stress and the activation of antioxidant enzyme expression. Compounds with this 3,4-catechol moiety have generally been shown to have significant biological effects (Ostlund & Fange 1962; Stokes et al. 1999). Two potential mechanisms to induce the activation of antioxidant enzymes centre on the ability of 3,4- catechol moiety to form
quinones and semi-quinones (LaVoie & Hastings 1999; Cavalieri & Rogan 2006). The formation of quinones and semi-quinones of 3,4-DHBA, via the actions of transition metals or the enzyme tyrosinase (Jozsef Balla 1992; Cavalieri et al. 2002), could result in both the generation of oxidative stress, primarily by the formation of H₂O₂, and also the reaction of these quinones with the sulfhydryl groups on KEAP-1, which will result in the induction of antioxidant enzymes (See Figures 27 and 28). Additionally, it is interesting to speculate whether the reaction of catechol quinones with cellular GSH, via a Michael type addition, could potentially result in the depletion of cellular GSH, which has previously been shown to also induce antioxidant enzyme expression (Zhou & Lim; Calkins et al. 2005). Although reactions to form 3,4-DHBA/GSH conjugates have not previously been shown, a metabolised conjugate of the similarly structured dopamine, a benzothiazine derivative, can undergo such reactions with GSH (Zhou & Lim 2010).
Table 4: Induction of antioxidant enzymes by compounds with a 3,4-ortho moiety

Compounds that share a common 3,4-ortho catechol moiety vs those that do not. It is suggested by the literature that the 3,4-ortho moiety is of significant importance for the induction of antioxidant enzyme expression. Multiple compounds which have this capacity possess the same 3,4 moieties (Balogun et al. 2003; Kraft et al. 2004; Yeh & Yen 2006b; Joung et al. 2007; Shih et al. 2007; Na & Surh 2008; Son et al. 2010). (Lee et al. 2003; Lim et al. 2007; Granado-Serrano et al. 2010)

<table>
<thead>
<tr>
<th>Non ortho catechol based inducers</th>
<th>3,4-ortho catechol induces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulforaphane</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Dopamine</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2-OH-17b-estradiol</td>
</tr>
<tr>
<td>EGCG</td>
<td>caffeic acid phenethyl ester (CAPE)</td>
</tr>
</tbody>
</table>
Plumbagin

Capsaicin

(-) epicatechin

Isoorientin

3,4-DHBA
Induction of antioxidant enzyme expression is potentially due to several mechanisms including the activation of the ARE by ROS mediated disassociation of KEAP1 from NRF2. 3,4-DHBA can potentially generate intracellular oxidative stress via the process of redox cycling. During this process, 3,4-DHBA is converted into its semi-quinone and quinone forms, via reactions with either transition metals or cellular enzymes such as tyrosinase. The 3,4-DHBA semi-quinone or quinone can then be reduced back to the dihydro form by the actions of cellular enzymes such as quinone reductase 2, which can then result in redox cycling and the potentiation of oxidative stress. Intracellular oxidative stress can mediate the dissociation of NRF2 from KEAP1 by several mechanisms, including, the direct reaction of ROS with the sulphydryl groups on KEAP1 or via the activation of the ROS sensitive MAPKinases, JNK and p38.

Figure 27: Possible mechanism 1. Oxidative stress induced activation.
In addition to ROS mediated activation of antioxidant enzyme expression, 3,4-DHBA may induce KEAP1 disassociation from NRF2 by direct reaction between 3,4-DHBA quinones and semi-quinones and the sulfhydryl groups on the antioxidant enzymes repressor KEAP1. A 3,4-DHBA semi-quinone could react with the sulfhydryl groups on the KEAP1/NRF2 complex resulting in dissociation of KEAP1 with NRF2. During this process, the 3,4-DHBA semi-quinone is regenerated back to its non-quinone form where it could react again to form a semi-quinone. A 3,4-DHBA quinone could also react with the sulfhydryl groups on KEAP1/NRF2 complex via a Michael type addition to form a conjugated KEAP1/3,4-DHBA diquinone complex and induce the dissociation of KEAP1 with NRF2. Once NRF2 is disassociated from its repressor KEAP1 it can then localise to the nucleolus and induce activation of the ARE, which controls antioxidant enzyme expression.

Although all of the DHBA isomers can in theory, undergo quinone formation, the literature often refers to the formation of quinone from compounds with the 3,4-catechol moiety i.e. 3,4-DHBA. 3,4-catechol based compounds have been shown to form a more stable semi-quinone than other dihydroxy substituted catechols.

**Figure 28: Possible mechanism 2. Sulfhydryl groups’ interactions mediated activation.**
It is likely that the more stable nature of the 3,4-catechol semi-quinone would allow for compounds that possess this moiety to reaction with cellular mechanisms, such as KEAP-1 and hence induce antioxidant enzyme regulation more effectively than compounds with different dihydroxy substitutions. Direct formation of quinones from catechol motifs has been shown to occur inside the cell after treatment with dopamine (Schipper et al. 1991). Additionally, the 3,4-catechol motif is arguably more biologically relevant than the other isomers of DHBA as it is found in multiple types of intercellular signalling molecules including, neurotransmitters, dopamine and adrenaline, and hormones, catechol oestrogens. Collectively, the more stable nature of the 3,4-catechol semi-quinones, coupled with the biological relevance of this motif and the data indicating the formation of intracellular catecholquinones suggest that the 3,4-catechol moiety of 3,4-DHBA is responsible for its increased biological activity.

Given the broad array of possible mechanisms for catechol induced antioxidant enzyme expression, there are several potential future experiments that may help to more fully characterise this process. Including, an examination of protein radical induced signalling, determination of the redox state of cellular thiols and also changes in gross oxidative state of the cellular environment. It has been theorised that the generation of protein bound catechol type radicals during oxidative damage triggers an oxidative defence mechanism though either specific modification of proteins or by altering gene expression (Nelson et al. 2006). To determine if 3,4-DHBA is forming protein bound catechols, 2 different approaches could be used. Firstly, a gross measure of oxidative damage to protein could be conducted, such as generation of protein carbonyls, which would indicate if any oxidative damage to proteins was occurring (He et al. 1999). Secondly, a more direct measure of protein bound catechols could be conducted using a modified version of Ito’s 1984 paper, which first identified protein bound DOPA using a HPLC based method (Ito et al. 1984). Changes in both redox state of the cells, as well as the generation of oxidative stress, can result in activation of antioxidant enzymes (Surh et al. 2008; Kang et al. 2005) and although these systems are closely linked, the methods of detection are somewhat discrete. 3,4-DHBA induced changes in redox state could be determined by measuring the relative amount of reduced to oxidised glutathione within the cell (the
GSH/GSSG ratio). This would have to be done over a time course, as changes in the GSH/GSSG ratio can subsequently result in an upregulation of GSH levels and hence an alteration in this ratio (Randle et al. 2008). If change in the GSH/GSSG ratio were found to occur, cellular levels could be increased by the exogenous addition of glutathione and hence the importance of thiol depletion in the 3,4-DHBA mediated activation of antioxidant enzymes could be determined. Measurement of changes in oxidative stress within the cell was done in this thesis using the dye DCFDA, and although it is a marker for general changes in oxidative stress, it is not very specific to the type of ROS being generated. A more detailed examination of the type of oxidative stressor generated after cellular exposure to 3,4-DHBA could be conducted. Specifically the \( \cdot \)O\(_2\)- dye MitoSOX™ could be used to determine both the kinetics and abundance of \( \cdot \)O\(_2\)- being generated by 3,4-DHBA exposure (Mukhopadhyay et al. 2007). Additionally, the upregulation of SOD by either exogenous addition or by the specific cellular induction in an over expressing cell line, could be used to determine the relative importance of \( \cdot \)O\(_2\)- generation in the induction of antioxidant enzymes.

Although it is suggested, by both the results presented here in addition to evidence from the literature, that an NRF2 mediated mechanism may underlie 3,4-DHBA induction of antioxidant enzymes, it is by no means proven and several other probable mechanisms exists. Regulation of several putative redox responsive signalling pathways, such as the Prx signalling pathway, may additionally be involved in either the activation of NRF-2 or the induction of antioxidant enzyme expression independently of NRF2.

For example, the Prxs, a family of thiol based peroxidases first discovered in yeast, have several key structural and enzymatic features that allude to potential functions additional to their peroxidase activity. A putative role in the sensing and propagation of signalling resulting from increases in redox state, specifically peroxide mediated, has been proposed based on their overtly complex structure and unique kinetic profile of peroxidase action. While detailed explanation of Prx activation is beyond the scope of this thesis, it is reviewed here (Cox et al. 2010). Although currently speculative, it is possible that mitochondrial and cytoplasmic redox stress initiates two distinct mechanisms, one being NRF2 mediated, while the other is Prx mediated. Evidence
supporting such a theory is as follows; mitochondrial forms of Trx and TrxR have been shown to function independently of their respective cytoplasmic forms (Hansen et al. 2006; Crosley et al. 2007). Additionally, there exists a putative recognition sequence for NRF2 on the promoter of cytoplasmic TrxR1 but the mitochondrial-localised TrxR2 is unaffected by NRF2 regulation (Sakurai et al. 2005; Suvorova et al. 2009). Further, ARE activation has been shown to induce expression of the cytoplasmically located Prx1 (Kim et al. 2007b), but no evidence exists for its regulation of mitochondrial Prxs, again suggesting specific regulation and function. One could suggest that a growing body of evidence indicates that mitochondrial and cytoplasmic antioxidant enzyme systems are at least partially isolated. Biologically, such a system would have clear advantages as mitochondrial and cytoplasmic stresses are often distinct in their origins and hence require different responses. Perhaps a future experiment comparing phenethyl isothiocyanate, which is shown to both oxidise mitochondrial Prx3 and to activate the ARE, to sulforaphane, which only activates the ARE, could highlight potential differences and commonalities between mitochondrial and cytoplasmically regulated responses to oxidative stress.

The addition of exogenous H$_2$O$_2$, as was carried out in these experiments, generates a gradient of oxidative stress, with much greater levels of H$_2$O$_2$ being present in the cytoplasm than the mitochondria (Antunes & Cadenas 2000). It would stand to reason that the activation of cytoplasmic enzymes, via an NRF2 mediated mechanism, would be most likely to mediate the protective effects seen here. It could be argued that the regulation of a mitochondrial response, which may potentially be possible via regulation of mitochondrial-located Prxs, may be more biologically relevant to mitochondria-generated oxidative stress. This would need to be examined using a mitochondrial model of oxidative stress, such as using the electron transport chain inhibitor rotenone that can induce mitochondrial oxidative stress (Li et al. 2003).

The data presented in this chapter shows that the CDA metabolite 3,4-DHBA can mediate anti-apoptotic action via the induction of endogenous antioxidant enzyme activity. It further shows that this protection is unrelated to the relative chemical antioxidant capacity of the DHBAs and is likely due, in part, to oxidant induced induction of TrxR, Trx and potentially other hydrogen peroxidases. Potentially, other
phytochemical metabolites, particularly ones that are derived or related to the DHBA, may promote health benefits by a similar mechanisms to what is shown here. The examination of such metabolites may help to uncover a conserved mechanism of action centred on antioxidant enzyme expression. The potential for multiple phytochemical metabolites to activate the same molecular adaptive mechanisms may allow for a cumulative response to metabolite exposure. Such a response could help explain why phytochemical consumption improves markers of human health, even when the blood levels of single metabolites are not sufficient to induce such responses.
Chapter 6
Regulation of antioxidant enzymes in multiple cell types by cyanidin-derived anthocyanin metabolites 2,4 and 3,4-dihydroxybenzoic acid

6.1 Summary

The previous chapter examined the effect of four isomers of DHBA on H_2O_2-induced cell death *in vitro*. The results show that, of the DHBAs, only the CDA metabolite 3,4-DHBA has significant protective effects, and further that these protective effects are mediated by upregulation of endogenous antioxidant enzymes. However, as only one cell line was tested in the previous chapter, it is possible that the regulation observed is unique to this particular cell line or cell type. Additionally, as only antioxidant enzymes that process H_2O_2 were examined, it is possible that other, non-H_2O_2-processing, antioxidant enzymes were also upregulated. The work described in this chapter examines the effect of the major and minor CDA metabolites, 3,4 and 2,4-DHBA, on the regulation of catalase, SOD and TrxR activity and GSH concentration in four cell lines that are derived from different tissue types. The results show that a 24 h treatment with 3,4-DHBA significantly increased endogenous antioxidant activities in all four cell lines tested, particularly in the neuronal cell line SH-SY5Y and the intestinal cell line HT-29, while treatment with 2,4-DHBA did not significantly alter any of these measures. The antioxidant activities of 3,4-DHBA offer insight into the potential mechanisms by which CDA consumption may affect human health. The greater activity of 3,4-DHBA over 2,4-DHBA suggests that directing the breakdown of CDAs in food products to 3,4 DHBA over 2,4-DHBA may potentially enhance CDA’s health benefits.
6.2 Introduction

High levels of oxidative stress both in human cell lines and in animal models can lead to lipid peroxidation, formation of protein carbonyls and cell death (Winterbourn et al. 2003; Maher & Hanneken 2005; Niki 2008; Mukhopadhyay et al. 2009). Health benefits conferred by diets high in fruits and vegetables, such as protection from heart disease, Alzheimer’s disease and certain cancers, have been suggested to be a result of increased cellular resistance to oxidative stress as a result of the bioactivity of plant-derived phytochemicals (Kim et al.; Borek 2004). This theory stems mainly from in vitro studies where various dietary phytochemicals have been shown to protect cells from oxidative stress (Tarozzi et al.; Aiyegoro & Okoh 2009). The protection is reported to potentially occur via three major mechanisms: 1) the direct chemical reduction of oxidants by antioxidant action (Aiyegoro & Okoh 2009), 2) the regulation of oxidant-induced cell signalling pathways (Katunuma et al. 2006) and 3) the upregulation of endogenous antioxidant enzymes (Yeh et al. 2009). All three mechanisms have been demonstrated to occur in vitro (Katunuma et al. 2006; Tanigawa et al. 2007; Aiyegoro & Okoh 2009). However, despite this volume of in vitro evidence, human intervention trials using a variety of dietary phytochemicals that offer protection in vitro often do not show the same protection in vivo (Beatty et al. 2000). There are several possible reasons for this discrepancy, which include: 1) one or more of the mechanisms that operate in vitro do not function in vivo, 2) chemical changes that occur to phytochemicals during the digestion and absorption process alter their bioactivity, or 3) the phytochemicals have poor bioavailability when consumed (Morand et al. 1998). Therefore, the use of either bioavailable dietary phytochemicals or blood metabolites of dietary phytochemicals for in vitro studies may provide greater relevance to in vivo bioactivities.

CDAs, such as cyanidin-3-glucoside, are present in fruits such as blackcurrant, blueberries and tart cherries and have been shown to have many potential health benefits using both in vitro bioassays and human intervention trials. Beneficial in vitro effects include chemical antioxidant scavenging, cytoprotection from oxidant-induced cell death (Delazar et al. 2010.; Tarozzi et al. 2005), regulation of cytokine release and modulation of adhesion molecules that play a role in diseases such as
atherosclerosis (Zhang et al. 2010.; Tsuda et al. 2002; Ruel et al. 2008). Results from animal trials have shown CDA-rich foods to protect against lipid peroxidation and to alleviate the symptoms of arthritis in rat models (Ramirez-Tortosa et al. 2001; He et al. 2005) Human studies convey that foods rich in CDAs have potential protection against oxidative stress, to reduce inflammation and to improve glucose clearance (Weisel et al. 2006; Lyall et al. 2009; Traustadottir et al. 2009).

CDAs have been shown to be unstable at physiological pH and to rapidly break down, both in vitro and in vivo, into isomers of DHBA, as well as into a highly unstable aldehyde (Vitaglione et al. 2007; Kay et al. 2009). An orally consumed CDA, cyanidin-3-glucoside has been shown to break down during the human digestive process primarily into 3,4-DHBA (Vitaglione et al. 2007). Further, CDAs from tart cherry have been shown to break down in cell culture media into both 3,4-DHBA and 2,4-DHBA (Figure 29). It is likely that these breakdown metabolites mediate some of the beneficial effects seen in vivo and, as such, they offer excellent targets for in vitro assays that attempt to decipher the mechanisms underlying the in vivo effects of oral CDA consumption. Since CDAs have been shown to break down in cell culture media as it does in blood, questions can be asked regarding the benefits of using the DHBA breakdown products as opposed to merely allowing their anthocyanin precursors to break down naturally. In vitro, CDAs have been shown to break down into 3,4 and 2,4-DHBA under basic and neutral conditions (Seeram et al. 2001). Interestingly, a recent detailed analysis of CDA breakdown in vitro using Caco-2 cells highlighted an important difference between the in vitro and in vivo process. It was shown that the in vitro breakdown of CDAs was found to take 2 minutes, with its complete conversion to 3,4-DHBA, via an open loop intermediate, taking 15 minutes. The resulting 3,4-DHBA was found to be mostly conjugated, with 20% being glucuronidated, 60% sulfated, and only 20% remaining un-conjugated (Kay et al. 2009). Conversely, in vivo, the analysis of CDA metabolites in humans demonstrated that 3,4-DHBA was in its native form and no conjugated metabolites were detected (Vitaglione et al. 2007). It has been shown that the glucuronidates and sulphates of other phytochemicals, such as quercetin and resveratrol, have greatly altered biological properties compared to the native compounds and hence the conjugates of 3,4-DHBA may not display representative bioactivity of CDA metabolites found in vivo (Day et al. 2000; Hoshino
et al. 2010). 2-(2,4,6-trihydroxyphenyl) acetaldehyde, the aldehyde formed during the *in vitro* breakdown of CDAs, has not been detected in blood and is likely to be unstable *in vivo*. Given the probable instability of 2-(2,4,6-trihydroxyphenyl) acetaldehyde together with evidence that CDA breakdown may occur in the gut (Vitaglione et al. 2007), it is perhaps incorrect to use 2-(2,4,6-trihydroxyphenyl) as a CDA blood metabolite in an *in vitro* assay. The use of 3,4-DHBA for *in vitro* studies offers significant advantages over the use of the CDA based precursors, due to its presence in the blood of humans after a CDA rich meal and its *in vitro* stability compared to dietary CDAs. Thus 3,4-DHBA could potentially be used instead of CDAs for *in vitro* experiments.

![Cyanidin and 3,4-Dihydroxybenzoic acid](image)

**Figure: 29: The breakdown products of CDAs.**

CDAs breakdown into both 3,4-DHBA and 2,4-DHBA *in vitro* and *in vivo*. (Seeram et al. 2001; Azzini et al. 2010)

The work presented in this chapter examines whether the bioavailable CDA metabolites 2,4-DHBA and 3,4-DHBA can increase the activity of several endogenous antioxidant enzymes, including catalase, SOD and TrxR, which are involved in processing biologically relevant oxidants such as $\text{O}_2^-$ and $\text{H}_2\text{O}_2$. The ability of 2,4- and 3,4-DHBA to upregulate antioxidant enzyme expression is assessed.
in several different cell types to determine whether any cell-type-specific effects occur. The data presented here show that the CDA metabolite 3,4-DHBA upregulates catalase activity, SOD activity, TrxR activity and GSH concentration in three of the four cell lines tested, whereas treatment with 2,4-DHBA does not have any significant effect on these measures.

The cell lines tested in this chapter are derived from 4 different types of tissue, blood T-lymphocytes (Jurkat), neuronal cells (SH-SY5Y), intestinal cells (HT-29) and kidney cells (HEK-293). Although all are highly transformed, they still possess properties that are unique to the tissues they originated from and their examination may potentially uncover cell-type-specific effects.

6.3 Specific materials and methods

All methods used are as described in the methods section.
Cell culture was conducted as described in section 2.1.
GSH measurement was conducted as described in section 2.10.
Catalase activity measurement was conducted as described in section 2.5.
SOD measurement was conducted as described in section 2.9.
TrxR measurement was conducted as described in section 2.8.

6.4 Results

No significant effects on cell growth rates or viability were detected (Figure 30), implying that bioavailable CDA metabolites had no significant toxic effects on cells cultured under optimal conditions. Although no changes in cell viability or growth were observed (as described in section 2.1), it is possible that bioavailable CDA metabolites affect cultured cells at the biochemical level without having an overt effect on viability or growth. Therefore, we examined the effect of 2,4- or 3,4-DHBA on cellular antioxidant capacity in vitro, measured through the ability to process H$_2$O$_2$(catalase activity) (as described in section 2.5). The oxidative stress generated in cells by exogenous H$_2$O$_2$ is complex, nevertheless the ability of cell lysates to process
H$_2$O$_2$ is could be a useful measure of cellular antioxidant capacity *in vitro*. Treatment of all cell types with 100 µM 3,4-DHBA for 6 or 24 h was shown to significantly increase catalase activity, with increases also observed after 24 h in SH-SY5Y, HEK-293 and HT-29 cells when 10 µM 3,4-DHBA was used (Table 5). No significant effects were seen in any cell line at either 6 or 24 h when cells were treated with 2,4-DHBA.

Treatment of cells with 3,4-DHBA increased the SOD activity of SH-SY5Y, HEK-293 and HT-29 cells in a concentration-dependent fashion as described in section. 3,4-DHBA treatment did not have a significant effect on Jurkat cells, although an increasing trend was noted with this cell line (Table 5). Significant increases in SOD activity were seen after both 6 h and 24 h treatment with 100 µM 3,4-DHBA in SH-SY5Y, HEK and HT-29 cell lines, with a significant increase also being observed after 24 h with 10 µM treatment in HT-29 cells. There were no significant changes in SOD levels after 2,4-DHBA treatment in any of the cell lines tested (Table 5).

GSH levels were measured after treatment with 2,4- and 3,4-DHBA for 6 and 24 h (as described in section 2.10). Significant increases in GSH were seen in both the SH-SY5Y and HT-29 cell lines after both 6 and 24 h when 100 µM treatment with 3,4-DHBA was used, and after 24 h of treatment with 10 µM 3,4-DHBA in the HT-29 cell line (Table 5). There was no significant increase in GSH levels in either Jurkat or HEK-293 cells. Treatment with 2,4-DHBA did not have any significant effect on GSH levels in any cell line after either 6 or 24 h.

TrxR activities were measured 6 and 24 h after treatment with either 2,4- or 3,4-DHBA (as described in section 2.8). Significant concentration- and time-dependent increases in TrxR activity were observed in both the SH-SY5Y and HT-29 cell lines treated with 3,4-DHBA (Table 5). No significant increases in TrxR were observed in the HEK-293 cells, and only 100 µM 3,4-DHBA at the 24 h time point increased TrxR activity in Jurkat cells. Almost all the regeneration of GSH in the TrxR assay was due to TrxR activity, with very little remaining after treatment of cell lysates with the TrxR specific inhibitor Auranofin (representative data shown in Figure 31).
Figure 30: Growth rates of cell lines used for endogenous antioxidant when treated with 2,4 and 3,4-DHBA.

Cell lines used were grown to confluence in either the presence or absence of 100 µM 2,4- or 3,4-DHBA. No significant differences were seen in growth rates between treated and un-treated cells. (a) HEK-293 (b) HT-29 (c) SH-SY5Y (d) Jurkat. Data shown are means of 3 cultures ± S.E.M.
Table 5: Regulation of endogenous antioxidant enzymes in four cell lines by 2,4 and 3,4-DHBA.

Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. (Bold numbers and * indicate significant difference from control = P<0.05, a = indicates a significant difference from 10 µM treatment P<0.05). All data are expressed as % of non-treated control at the specific time point.

<table>
<thead>
<tr>
<th></th>
<th>Catalase 6h</th>
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<th>Glutathione 6h</th>
<th>TrxR 6h</th>
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Figure 31: Inhibition of TrxR activity by Auranofin treatment.
Measurement of TrxR activity by inhibition of GSH regeneration by specific TrxR inhibitor Auranofin (as described in section 2.8). Results of 3 separate experiments with 2 replicate samples expressed as means ± S.E.M. Data is presented as % of untreated control.

6.5. Discussion

6.5.1 The effects of bioavailable CDA metabolites in vitro

The main hypothesis behind these studies was that investigation of the bioavailable CDA metabolites in vitro may improve our understanding of the in vivo effects and help explain the health benefits associated with dietary CDA consumption. A secondary hypothesis was that CDA metabolites may affect the biochemistry of cultured cells in a subtle manner that is not evident at the level of cell viability and proliferation, which have often been used as experimental endpoints in previous work. It was demonstrated that 3,4-DHBA but not 2,4-DHBA significantly increased the activity of at least one endogenous antioxidant in all four cell lines without affecting cellular proliferation or viability.
6.5.2 Regulation of antioxidant enzymes by 2,4- and 3,4-DHBA

Oxidative stress has been implicated as a cause of many different disease states and may play a critical role in both the development of chronic diseases such as atherosclerosis, as well as acutely affect symptoms of chronic diseases such as Alzheimer’s disease (Madamanchi et al. 2005; Sultana & Butterfield 2009). The ability of dietary-derived compounds to positively affect oxidative stress, and hence modulate both progression of disease states and the manifestation of their symptoms, offers a unique non-medical avenue to help prevent and manage some diseases states. Here it is shown that the CDA metabolite 3,4-DHBA has endogenous antioxidant modulating capacity in multiple cell lines, increasing catalase, SOD and GSH levels or activity over time, and further that another CDA metabolite, 2,4-DHBA does not affect these measures.

Catalase activity, or the ability of the cell to process the oxidant $H_2O_2$, is a vital function required to maintain both redox balance and also to modulate potential redox-regulated signalling pathways (Rhee 1999). Mice overexpressing mitochondrial catalase exhibit extended lifespans (Schriner & Linford 2006). Further, aged naked mole rats, the longest-lived rodent, have significantly higher catalase activity than aged CB6F1 mice (Andziak et al. 2005). This research has shown that a 24 h treatment of cells in vitro with 3,4-DHBA increased catalase activity by as much as 80% in the cell lines tested (Table 5). These results are in agreement with the work by Yeh et al 2006 where the DHBA gentisic acid was shown to increase catalase activity in both intestinal cells and liver cells (Yeh & Yen 2006a). They further support the concept that various dietary phytochemicals can increase endogenous antioxidant activity, as highlighted by Na et al 2008, who showed that the major tea polyphenol EGCG increases catalase activity. EGCG treatment was further shown to regulate SOD activity (Na et al. 2008).

The antioxidant enzyme responsible for processing superoxide radicals, SOD, was originally termed the enzyme of life, with SOD activity being correlated to lifespan in primates (Ono & Okada 1984). SOD deficiency has been shown to decrease lifespan.
and increase liver carcinogenesis in a transgenic mouse model (Elchuri et al. 2005). Further, induction of increased SOD activity has been shown to protect from various cytotoxic insults in vitro (Warner et al. 1993; Brockhaus & Brune 1999). SOD activity has been positively correlated with longevity and negatively correlated with disease states such as inflammatory bowel disease and chronic lung disease (Lih-Brody et al. 1996; Juul et al. 2006). Furthermore, SOD and catalase interact in the removal of oxidants from cells since SOD activity generates $\text{H}_2\text{O}_2$, which is processed by catalase. Interestingly, synthetic SOD mimetics, when combined with catalase mimetics, have been shown to prolong survival and reduce oxidative stress in a mouse model of the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS) (Jung et al. 2001). SOD levels of cells treated with 3,4-DHBA for 24 h significantly increased in three of the four cell lines tested (Table 5). Increases in SOD activity by phytochemical treatment has been demonstrated by Yeh et al. when they showed ferulic acid could increase SOD activity in intestinal and liver cells (Yeh & Yen 2006a).

The regulation of SOD activity in conjunction with catalase activity allows for not only the detoxification of $\text{O}_2^\bullet$ into $\text{H}_2\text{O}_2$ but also processing of the resulting $\text{H}_2\text{O}_2$ into water and molecular oxygen. The combination of increased catalase and SOD activity is likely to reduce the oxidative state within the cell or body and may lead to increased levels of oxidative state-dependent proteins such as GSH. Potentially, a reduction in oxidative stress within the cell could slow the onset or reduce the symptoms of oxidative stress-associated disease.

GSH is a tripeptide that functions as a cellular antioxidant and acts to protect the cell from oxidant-induced damage. GSH is depleted in many disease states, such as HIV infection, and is also reduced during extreme exercise (Sastre et al. 1992; Herzenberg et al. 1997). Further, intravenous injections of GSH increase overall GSH levels and improve exercise-related biomarkers (Sen 1999). In this study 3,4-DHBA treatment of cells was shown to increase GSH levels in both the neuronal cell line SH-SY5Y and also the intestinal cell line HT-29, but not in the kidney or blood-derived cell lines tested (Table 5). It has previously been shown that increases in SOD and catalase levels can be induced by phenolic compounds without an increase in GSH levels, as
seen in the Jurkat and HEK cell lines, and as such the results presented here are in agreement with these findings (Yeh & Yen 2006a).

TrxR is a key cellular redox regulator, with its regulation being linked to multiple types of oxidative-stress-mediated states. TrxR regenerates many other antioxidant enzymes and compounds including GSH. Increases in TrxR activity represent an increase in the general redox stress resistance of the cell (Arner & Holmgren 2000). Increases in TrxR have previously been shown to be associated with cellular resistance to hydroperoxides (Bjornstedt et al. 1995), and can be induced by the phytochemical sulforaphane (Hintze et al. 2003). Additionally, in Saccharomyces cerevisiae, oxidative stress induced by H₂O₂ treatment also induces the expression of TrxR (Hintze et al. 2003), suggesting an oxidative stress-mediated activation of TrxR. However, several other mechanisms have been shown to exist for the activation of antioxidant enzyme expression that are not directly mediated by oxidative stress, including signalling via the estrogen receptor (Montano et al. 2004).

The upregulation of multiple antioxidant enzymes, as was seen in SHSY-5Y and HT-29 cells when treated with 3,4-DHBA, implies a generalised antioxidant enzyme response. Such responses can be mediated by activation of the ARE transcription factor binding site (Prestera et al. 1995). Further, activation of the ARE has previously been shown to be induced by phytochemical treatment of cells (Martin et al. 2004). Sulforaphane induces ARE activation by directly acting on the binding of Keap1 to Nrf2, allowing Nrf2 to localise in the nucleus and activate the ARE (Hong et al. 2005). Gallic acid has also been shown to induce the disassociation of Keap1 from Nrf2 and the subsequent activation of the ARE in a MAP kinase-dependent manner (Yeh & Yen 2006b). Interestingly, the green tea phytochemical EGCG has been shown to induce activation of cellular stress-linked MAP kinase, with this activation being inhibited by catalase co-treatment (Wu et al. 2006). This suggests a H₂O₂-mediated mechanism for EGCG-induced activation of MAP kinases, and hence may be a potential mechanism by which other phytochemicals, such as 3,4-DHBA, can induce an adaptive response.
6.5.3 Effectiveness of 3,4-DHBA over 2,4-DHBA

Potentially the formation of quinones and semi-quinones of the dihydroxy moiety of the DHBAs may be responsible for the up-regulation of antioxidant function seen in this study. Compounds that possess a catechol-type moiety, such as 3,4-DHBA, can undergo the formation of quinones and semi-quinones in cellular environments (Liehr & Roy 1990). These quinones can subsequently undergo redox cycling, generating intracellular oxidative stress (Liehr & Roy 1990) (Figure 32) that has previously been shown to induce antioxidant enzyme expression (Dinkova-Kostova & Wang). Given 3,4-DHBA treatment of SH-SY5Y cells was shown to induce a small but significant increase in intracellular oxidative stress, to increase the activity of endogenous antioxidant enzymes and is almost structurally identical to compounds that have been shown to undergo redox cycling (Kuhn and Arthur 1998), it is likely that 3,4-DHBA undergoes redox cycling via the formation of semi quinones and quinones on its dihydroxy moiety, although this was not specifically examined in this thesis. In addition to the presence of the dihydroxy moiety (as was discussed in the previous chapter), it appears likely that the orientation of the hydroxyl groups within this moiety in DHBAs may also affect the upregulation of antioxidant enzymes. It has recently been reported that orientation of the hydroxyl groups is vitally important for both the activation of the ARE and expression of antioxidant enzymes that ARE induces (Wang et al. 2010). In the ARE-reporter cell line, ARE32, it was shown that exposure to various ortho-, but not meta-, hydroquinones induced ARE-dependent transcription. We have shown that the ortho-hydroquinone, 3,4-DHBA, induced significant increases in antioxidant enzyme function, whereas the meta-hydroquinone, 2,4-DHBA, did not. The results presented here agree with the previous findings mentioned above regarding ortho- and meta-hydroquinone induction of endogenous antioxidants. Chemically, the presence of the 3,4-dihydroxy (catechol) moiety allows for the formation of a fairly stable ortho-semi-quinone radical. Specifically, a 3,4-catechol base semi-quinone has a half life of approximately 250ms (Bouheroum et al 1989), where as a 2,4 semi-quinone is so unstable that it has not currently been detected without the addition of stabilising side chains (Storm et al. 2003). Since a 2,4- quinone has previously been detected, albeit in a gas phase, a 2,4- semi-quinone must have been generated, so do in theory exist but are so unstable that they cannot be
detected (Roithová et al. 2005). The greater stability of catechol-type semi-quinones, as opposed to the unstable 2,4- (resorcinol) type semi-quinones, would potentially allow for their interaction with the sulfhydryl groups on Keap1, resulting in ARE activation. Additionally, ortho-quinones are known substrates for ARE-induced NQO1, an enzyme which is directly responsible for catalysing their two-electron reduction that facilitates redox cycling and sulfhydryl depletion-mediated activation of the ARE. Potentially an increase in substrate level would result in an upregulation of NQO1. Given NQO1 is often used as a marker for ARE activation and that a 3,4-DHBA quinone is almost certainly a substrate, the activation of the ARE by 3,4-DHBA but not 2,4-DHBA would not be an entirely unexpected result. Additionally, a recent study has determined that NAD(P)H quinone oxidoreductase 2 (NQO2) is a specific catechol quinone reductase (Fu et al. 2008), so could also potentially facilitate the redox cycling of 3,4-DHBA but not 2,4-DHBA.

Further work needs to be conducted to determine the exact mechanism of action by which 3,4-DHBA activates the upregulation of endogenous antioxidant enzyme expression and this may reveal why the structurally similar 2,4-DHBA does not.

Figure: 32: Potential formation of semi-quinones and quinones of 3,4-DHBA.

3,4-DHBA can potentially form semi-quinones and quinones that results in the generation of oxidative stress. The formation of semi-quinones and quinones can be mediated by oxygen and transition metals or by several endogenous enzymes. Additionally, quinones can by reduced back to their hydroquinone form by the actions of NADPH quinone reductase, which can result in futile redox cycling and the propagation of intracellular oxidative stress.
6.5.4 Concentration and bioavailability of 3,4-DHBA

*In vitro* experiments are usually the first step in investigating the activity of pharmaceuticals and other bioactive substances. They offer a compromise between ease of use/cost and relevance to *in vivo* activity, and provide information on biological effects and potential health benefits of phytochemicals. However, caution is needed so as not over interpret *in vitro* results, as issues of bioavailability and concentration at site of action need to be considered. It is important to note that the concentrations of 3,4-DHBA demonstrated to have effects in this work are, as are often the case when phytochemicals are tested *in vitro* (Vitaglione et al. 2007), non-physiological. Blood levels of 3,4-DHBA only reach 1 µM after the consumption of a CDA-rich meal (Vitaglione et al. 2007) and although this concentration was tested in this study, no significant changes were detected in any of the measures (data not shown). Interestingly, 3,4-DHBA itself is highly bioavailable when orally consumed in rats and concentrations as high as 20 µM have been recorded after an oral dose. Further, this dose did not appear to provide the maximum concentration in the serum and higher blood concentrations may be possible if a larger oral dose is given (Han et al. 2007). Although no oral bioavailability data exists for 3,4-DHBA in humans, the phenolic acids tend to be among the most bioavailable of the phytochemical antioxidants (Caccetta et al. 2000; Azzini et al. 2010). It therefore seems likely that 3,4-DHBA is bioavailable when fed to humans and as such, the results of this work may be more applicable to the direct dietary consumption of 3,4 DHBA rather than as a breakdown product of anthocyanins. 3,4-DHBA is the primary phenolic acid in cocoa-based products and the selection of varieties of bean with higher levels may lead to a product for consumption with potentially enhanced beneficial health effects (Ortega et al. 2008).

6.5.5 Cell line-specific effects

Antioxidant enzyme function was tested in four different cell lines: the neuronal-like cell line SH-SY5Y, the intestinal-derived HT-29, the kidney-derived HEK-293 and the blood T lymphocyte line Jurkat. These cell lines were selected based on the tissue
type that they were initially derived from and used to test for any overt tissue typespecific regulation of antioxidant enzyme function. As these are non-primary cell lines potential effects of both transformation and differentiation state may affect their responses. Previously, it has been suggested that an ARE-mediated upregulation of endogenous antioxidant function may protect multiple organs from oxidative damage. Nrf2- (an ARE inducer) knockout mice displayed greatly reduced expression or inducibility of antioxidant enzymes in the lung, intestine, liver and brain (Chan & Kan 1999; Enomoto et al. 2001; McWalter et al. 2004; Shih et al. 2005). Recently, phytochemical-induced changes in endogenous antioxidant levels have been reported to be cell line-specific (Hu et al. 2009). Hu et al (2009) reported that the phytochemicals sulforaphane and EGCG can induce an increase in levels of several endogenous antioxidant proteins (Hu et al. 2009) Additionally, they showed that this effect was inducible in the breast cancer cell lines, MDA231 cells and Michigan cancer foundation-7 (MCF7) cells, but not lung cancer-derived A549 cells. Although these investigators did not measure effects on enzyme activity, the results reported here provide evidence that cell type-specific enzyme regulation can occur and agree with their findings which show that a regulator of antioxidant enzyme expression is differently induced in different cell lines. Additionally, it was suggested, by Hu et al (2009), that the lack of antioxidant regulation in A549 cells may have been due to higher levels of basal ARE activation. Given the cell lines used, both here and in the work of Hu et al (2009), were grown in non-physiological oxygen levels, it is possible that basal levels of ARE activation are both elevated and normalised in these cell lines. Potentially, culturing cells in a more physiological low oxygen environment may reduce basal ARE levels and allow for either greater induction of endogenous antioxidants by phytochemicals, or the induction of endogenous antioxidants in cell lines that show no induction in high-oxygen environments.

6.6 Conclusion

These data support the hypothesis that dietary consumption of phytochemicals can reduce oxidative stress by increasing endogenous antioxidant capacity. The results presented here show that 3,4-DHBA, but not 2,4-DHBA, treatment can increase endogenous antioxidant function in several cell lines, and imply a common
mechanism of antioxidant enzyme regulation. Additional experiments examining the exact mechanisms behind the regulation of endogenous antioxidant enzymes *in vitro* and confirmation of these effects *in vivo* would greatly improve our understanding of how CDA-rich foods act to benefit human health.
Chapter Seven

Regulation of antioxidant enzymes in Sprague-Dawley rats by the phytochemical metabolite 3,4-DHBA

7.1 Summary

The previous two chapters examined the effect of the phytochemical blood metabolite 3,4-DHBA on regulation of endogenous antioxidant enzymes in vitro. These chapters show that 3,4-DHBA can upregulate endogenous antioxidant enzymes in multiple cell lines and that this upregulation is responsible for the protection from H$_2$O$_2$ induced cell death conferred by 3,4-DHBA treatment of the neuronal cell line SH-SY5Y. The theory underlying the use of phytochemical blood metabolites, such as 3,4-DHBA, in the in vitro cytoprotection assays, is that results gained from in vitro screening are more likely to be replicated in an in vivo animal trial. Given 3,4-DHBA was shown to regulate antioxidant enzyme activity in the previous chapter and is a phytochemical blood metabolite of CDAs as well as being present in foods as 3,4-DHBA itself, its oral feeding may be used to determine potential effects of both dietary CDAs and 3,4-DHBA. This chapter describes the effects of an oral gavage of 3,4-DHBA to healthy Sprague Dawley rats on their endogenous chemical and enzymatic antioxidant systems. These data suggest that while chemical blood antioxidant levels are decreased, endogenous antioxidant enzyme activities are increased in several tissues when rats are fed 3,4-DHBA at a dose of 100 mg per kg body weight. Further, no changes in body weight or feeding behaviour resulted from 3,4-DHBA treatment and a physiological dose of 2.5 mg per kg body weight of 3,4-DHBA did not induce any changes in the measures examined.
7.2 Introduction

Diets high in fruits and vegetables are associated with reduced risk of developing chronic disease such as cancer and CVD and to potentially slow the progression of neurological diseases such as Alzheimer’s disease (Liu et al. 2000; Dai et al. 2006). Dietary anthocyanins, the pigment compounds found in many fruits, are often associated with mediating the putative health benefits of consuming a diet high in fruits and vegetables. Although both anthocyanins and the fruits they come from are high in chemical antioxidant capacity and offer protection from oxidative stress in vitro (Choi et al. 2007), there is debate over the role of chemical antioxidants in the prevention of diseases in vivo. Chemical antioxidant action, such as direct radical scavenging and transition metal chelating, may not be an important protective factor in in vivo diseases, [reviewed in] (Stevenson & Hurst 2007). Indirect in vivo actions of anthocyanins, such as the regulation of anti-inflammatory pathways and changing of endogenous enzyme activity, may potentially mediate the health benefits linked to anthocyanin consumption (Finne Nielsen et al. 2005; Jensen et al. 2008). The aim of this study was to assess the capacity of an orally bioavailable CDA metabolite, 3,4-DHBA, to regulate both endogenous antioxidant enzyme activity and plasma chemical antioxidant levels in a healthy animal.

Several studies have previously evaluated the capacity of CDAAs or CDA rich extracts to prevent or protect from diseases in vivo. Cyanidin-3-O-β-D-glucoside from purple corn showed anti-obesity and anti-inflammatory effects in mice treated with a high fat diet (Tsuda et al. 2003). Additionally, cyanidin-3-glucoside has been shown to suppress the growth of Lewis lung carcinoma cells in vivo (Chen et al. 2005b). Potential neuroprotective effects of CDAAs have been shown in aged rats fed blueberry extracts. Long term and chronic blueberry feeding was shown to improve cognitive function in aged rats and was linked to the reduction in age-induced increases in oxidative stress (Joseph et al. 1999). Further, cyanidin-3-O-β-d-glucopyranoside, isolated from mulberry fruit, has been shown to be neuroprotective in a mouse model of cerebral ischemia when administrated orally after the ischemic injury was induced (Kang et al. 2006). In addition to these animal studies, a limited number of studies
have evaluated the effects of anthocyanin supplementation in humans. Plasma lipid hydro-peroxide levels, a marker of oxidative damage, were decreased in smokers that consumed 250g of blueberries a day for three weeks (McAnulty et al. 2005). Additionally, it has been shown that healthy adults that consumed a mixture of apple and blueberry juice had reduced susceptibility to oxidant induced lymphocyte DNA damage ex-vivo and a greater plasma antioxidant capacity (Wilms et al. 2007). Although this human study does superficially support a chemical antioxidant effect of anthocyanin supplementation in vivo, a closer examination reveals the protective effects are more likely due to altered regulation of other endogenous factors. Even though the changes in plasma antioxidant capacities reported in this study were significant, they were unlikely to be biologically important, as only a 2.5% increase was detected (Wilms et al. 2007). Further, the ex-vivo protection from oxidative stress induced cell death was measured in cells resuspended in PBS, hence none of the plasma that showed increased chemical antioxidant capacity was present (Wilms et al. 2007). Potentially, the protection from oxidative stress seen in the ex-vivo cell death protection assay could be due to CDA induced increases in endogenous antioxidant enzymes. Although it is possible that chemical antioxidant capacity of anthocyanins has an effect on in vivo measures of health, this would seem unlikely due to several factors that limit this mechanism in vivo. Namely, most anthocyanins are either unstable at physiological pH or are substantially modified before reaching the blood (Seeram et al. 2001; Felgines et al. 2002). Additionally, blood contains significant concentrations of endogenous chemical antioxidants and changes that can be induced by phytochemical feeding are relatively small compared to these endogenous levels (Stevenson & Hurst 2007; Wilms et al. 2007). Regulation of non-chemical endogenous antioxidant enzyme activity may represent a mechanism by which phytochemicals, such as cyanidin and its derivatives, can affect oxidative state in vivo without acting as chemical antioxidants.

Phytochemicals can act to modulate the expression and activity of various endogenous enzymes in vivo, particularly endogenous antioxidant and detoxifying enzymes. Previously CDAs and CDA rich extracts have been shown to increase the expression of detoxifying enzymes in healthy animals and to prevent the decrease of endogenous antioxidant enzyme activity in animal disease models. Levels of the
phase II detoxifying enzyme GST were increased by 45% in rats fed a diet supplemented with 10% high CDA black raspberries (Reen et al. 2006). Additionally, anthocyanin rich bilberries have been shown to increase concentrations of liver GST in rats (Dulebohn et al. 2008). Disease-induced decreases in antioxidant enzyme expression have been inhibited in several rat models by anthocyanin feeding. CDA were shown to partially prevent a high fat diet induced reduction in both catalase and SOD activity in a mouse model of obesity (Lee et al. 2009). Further, CDA feeding was shown to prevent the depletion of cellular GSH levels in a diabetic mouse model (Guo et al. 2007). Although CDA have been shown to be protective in some animal models, its poor bioavailability and the differences between its absorption in humans and animals affect the relevance of these results to humans (Tsuda et al. 1999; Vitaglione et al. 2007).

Anthocyanins exhibit poor bioavailability in both humans and rats, with the phenolic acid 3,4-DHBA being the primary blood breakdown product of its consumption in both species (Tsuda et al. 1999; Vitaglione et al. 2007). After oral consumption of CDAs in humans, 3,4-DHBA is present in the blood at levels approaching 1 µM, 2000 times higher than the concentration of either cyanid or CDAs found in the blood (Vitaglione et al. 2007). Although 3,4-DHBA is also the primary blood metabolite of CDAs in rats, a significantly greater proportion (12% of the 3,4-DHBA concentration) of CDAs are seen in the blood of rats after its oral consumption (Tsuda et al. 1999). Given that there is a significantly greater amount of CDAs in the blood of rats after its oral consumption, the oral feeding of CDA to rats does not accurately represent the human situation and may not be a good model for human CDA consumption. Interestingly, 3,4-DHBA is highly orally bioavailable in rats and can potentially be used to deliver 3,4-DHBA to the blood of a rat at concentrations that represent human blood levels after oral CDA feeding (Han et al. 2007). Additionally, 3,4-DHBA itself is present in many different fruits (Herrmann 1989) and has been linked to the putative health benefits of cocoa and several Chinese herbal medicines (Ye et al. 2003; Ortega et al. 2008).

Given the good oral bioavailability of 3,4-DHBA (Han et al. 2007), the modulation of endogenous antioxidant activity by CDAs in animal disease models (Reen et al.
2006), the human data suggesting anthocyanin feeding can increase resistance to oxidative stress (Wilms et al. 2007) and the data from chapters 5 and 6 on the regulation of antioxidant enzymes by 3,4-DHBA _in vitro_, this chapter examines the capacity for the orally bioavailable CDA metabolite, 3,4-DHBA, to increase antioxidant enzymes expression in a healthy rat. Concentrations were used that represent either: 1, a blood 3,4-DHBA concentration that is detected in humans after a CDA rich meal (2.5 mg per kg body weight) or 2, a dose that could be given in a functional food format (100 mg per kg body weight) (Appendix 1 FDA calculation from Human Equivalent Dose.)

This trial was conducted to both confirm the theory that _in vitro_ assays that use phytochemical blood metabolites are a valid screening tool for predicting _in vivo_ results, and also to provide an important step in the development of a 3,4-DHBA or 3,4-DHBA precursor rich functional food.

### 7.3 Specific methods

Chemical antioxidant measures, ORAC and FRAP, were conducted as described in chapter 2.2 and 2.3 respectively using a plasma sample.

3,4-DHBA was dissolved in a solution of 0.5 % propylene glycol, 99.5% MQ-H₂O, which was pre-warmed to 37°C, to make a solution containing 20 mg per mL 3,4-DHBA. This solution was then vortexed for 120 s to ensure all of the 3,4-DHBA was dissolved and this was used for the high dose treatment. To make the low dose treatment, the solution was further diluted 1 in 40 with 0.5 % propylene glycol 99.5% MQ-H₂O to make a 0.5 mg per mL 3,4-DHBA solution. Animals were given an oral gavage of 5 mL solution per kg body weight. The solution of 20 mg per mL 3,4-DHBA equated to a treatment of 100 mg per kg body weight. The treatment with 0.5 mg per mL 3,4-DHBA equated to the treatment of 2.5 mg 3,4-DHBA per kg body weight. A vehicle only gavage of 0.5 % propylene glycol 99.5% MQ-H₂O was given to the control group.
7.3.1 Animals

The rat feeding trial was carried out in the Food Evaluation Unit, Plant & Food Research, Palmerston North, in a room maintained at a temperature of 22 ± 1°C, humidity of 60 ± 5%, air exchange of 12 times/hour, and with a 12 hour light/dark cycle (lights on from 7am to 7pm).

Weanling male rats (21 days, 45-55 g) were housed in family groups and fed a control powdered diet ad libitum until they were 6 weeks of age. At 6 weeks of age the rats were transferred to individual hanging cages and continued on the casein-based control diet (Table 6). The experiment started two weeks after individual housing started to ensure that the animals were adapted to their housing condition. The rats were weighed weekly and their live-weights recorded. At 8 weeks of age (approximately 200 to 280 g live-weight), rats were gavaged once daily for 14 days with the experimental treatments (control, 2.5 mg per kg bodyweight 3,4-DHBA, 100 mg per kg bodyweight 3,4-DHBA). Rat weight and food intake was measured daily during the 14 day experimental period. Rats were staggered onto the experimental treatments to accommodate the time required to harvest tissues at the end of the experimental period. Fresh faeces from each rat were collected on day 14 of experimental feeding. On day 15 rats were fasted overnight (16 h) to ensure feeding did not influence the endpoint measurements. Rats were euthanased via an inhalational CO₂ overdose. Blood samples were taken via cardiac puncture for plasma collection using EDTA pre-coated tubes. Plasma was collected from blood by centrifugation (300 g, 10 mins at 4°C) and immediately stored at -20°C. After taking the blood sample, the rats were transcardially perfused with cold saline to ensure all blood was removed from the tissues and organs prior to their removal, as the presence of blood may have effected measurements of endogenous antioxidant enzyme activity. The organs (liver, kidney, intestine and brain) were then removed, weighed, snap frozen in liquid nitrogen and stored for subsequent analysis.

7.3.2 Diet

Sprague Dawley rats were fed on a nutritionally complete powered diet that was limited but not deficient in vitamin E, 50 units per kg diet vs 75 units per kg for the
AIN93 standard diet. The vitamin E level in this diet was deliberately limited as it is both significantly elevated in the standard AIN93 rat diet (Reeves et al. 1993) and could potentially interfere with any adaptive response mediated induction of antioxidant enzyme activity that may be induced by a moderate treatment induced pro-oxidant effect (Eder et al. 2002). The diet was made on-site from pure ingredients (Table 6).

**Table 6: Ingredient composition (g per kg) of the control diet**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Diet (g per kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein&lt;sup&gt;2&lt;/sup&gt;</td>
<td>120</td>
</tr>
<tr>
<td>Corn oil&lt;sup&gt;3&lt;/sup&gt;</td>
<td>65</td>
</tr>
<tr>
<td>Sucrose&lt;sup&gt;4&lt;/sup&gt;</td>
<td>40</td>
</tr>
<tr>
<td>Corn starch&lt;sup&gt;5&lt;/sup&gt;</td>
<td>625</td>
</tr>
<tr>
<td>Cellulose&lt;sup&gt;6&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>Low vitamin E vitamin mix&lt;sup&gt;7&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>Salt mix&lt;sup&gt;8&lt;/sup&gt;</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>1</sup> All diet components were phenolic acid free.

<sup>2</sup> Alacid 80 mesh, New Zealand Milk Products, Wellington, New Zealand.

<sup>3</sup> Davis Trading Company, Palmerston North, New Zealand.

<sup>4</sup> Chelsea Sugar Refinery, Auckland, New Zealand.

<sup>5</sup> Wheaten corn flour, Starch Australasia, Goodman Fielder Group, Tamworth, NSW, Australia.

<sup>6</sup> Ceolus PH102, Commercial Minerals Ltd, Auckland, New Zealand.

<sup>7</sup> A low vitamin E mixture prepared at Plant & Food Research that supplies: (mg kg<sup>-1</sup> diet) retinol acetate 5.0, DL-α-tocopheryl acetate 50.0, menadione 3.0, thiamine hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg kg<sup>-1</sup> diet) ergocalciferol 25.0, cyanocobalamin 50.0.

<sup>8</sup> A mixture prepared at Crop & Food Research supplied: (g kg<sup>-1</sup> diet) Ca 6.29, Cl 7.79, Mg 1.06, P 4.86, K 5.24, Na 1.97; (mg kg<sup>-1</sup> diet) Cr 1.97, Cu 10.7, Fe 424, Mn 78.0, Zn 48.2; (μg kg<sup>-1</sup> diet) Co 29.0, I 151, Mo 152, Se 151.

### 7.3.3 Enzymatic antioxidant assays

Enzymatic antioxidant measures were conducted as described in the methods chapter, (GSH section 2.10, catalase activity section 2.5, SOD section 2.9, TrxR section 2.8) with the additional step of lysate preparation. Organs were thawed on ice prior to the
addition of lysis buffer (as described in chapter 2). Tissues were homogenised by sonication using an Omni Sonic Ruptor 400™ ultrasonic homogeniser (Omnisonic, Kennesaw USA). A setting of 30% on the Omni Sonic Ruptor 400™ was used with the microprobe attached. Individual bursts of up to 30s were used with samples being left to chill on ice between sonication runs. Liver, testis and brain samples were sonicated for 30s. Kidney samples were sonicated for 60 s in two 30 s bursts. Intestine samples were ground by mortar and pestle under liquid nitrogen prior to suspension in lysis buffer and sonication for 30 s. Sonication of testicular samples was unable to break down the tough outer skin of the testicle so testicular samples had this skin well perforated before sonication. Protein concentration was determined by Bradford protein assay. Samples were then prepared and analysed as described in chapter 2 with an additional dilution step of up to 50 fold, depending on the protein concentration of the sample and assay being conducted, occurring after the centrifugation at 16,000 g for 15 min.

7.4 Results

7.4.1 Food intake & body weight

Food intake and body weight of the rats was not significantly affected by either dose of 3,4-DHBA. Average food intake was found to be 18.6 +/- 0.42, 17.6 +/- 0.38 and 19 +/- 0.73 g per day for the control, low dose DHBA and high dose DHBA groups (Figure 33). Food intake of the rats undergoing either daily gavage of 2.5 mg per kg, 100 mg per kg or a control gavage had their daily food intake measured. Average daily food intake was approximately 18 g and did not significantly vary between treatment groups. As changes in food intake could potentially affect antioxidant enzyme activity, the lack of treatment induced changes in food intake indicated that any changes observed in endogenous antioxidant enzyme activity were most likely due to the direct effect of 3,4-DHBA treatment.

Mean body weights of the treatments groups were between 240 and 255 g at the start of the trial and between 304 and 317 g after the two week intervention (Figure 34 a and b). In addition to bodyweight and food intake, organ weights were also measured.
to identify any overtly toxic organ effects. No differences were found in the weight of the kidneys, brain or heart between either of the treatment and control groups (data not shown).

Figure 33: Effect of daily gavage of 3,4-DHBA on food intake of Sprague Dawley rats.
Food intake of Sprague Dawley rats undergoing a daily gavage of either, 2.5 mg per kg 3,4-DHBA, 100 mg per kg 3,4-DHBA or a control gavage, was measured. Food intake was determined by measurement of food removed from feeding bowl minus food that was discarded though the cage. No significant differences from the control animals were induced by either 3,4-DHBA treatment. Results shown are expressed as the mean ± S.E.M of 10 animals per group.
Figure 34: Effect of daily gavage of 3,4-DHBA on weight gain of Sprague Dawley rats.

Bodyweight of Sprague Dawley rats undergoing a daily gavage of either, 2.5 mg per kg 3,4-DHBA, 100 mg per kg 3,4-DHBA or a control gavage, was measured. (a) Daily mean body weights of rats from the control and both treatment groups during the duration of the trial. (b) Average weight gain of the rats from the different treatments groups over the length of the trial. No significant differences from the control animals were induced by either 3,4-DHBA treatment. Results shown are expressed as the mean ± SEM of 10 animals per group.
7.4.2 Plasma chemical and tissue enzymatic antioxidant capacity

Chemical antioxidant potential of plasma and enzyme antioxidant activity of the intestine, liver, kidney, brain and testis were measured. The high dose 3,4-DHBA induced a significant decrease in the chemical antioxidant capacity of plasma as measure by FRAP (P < 0.05) but not by ORAC (Figure 35 a and b). Low dose 3,4-DHBA treatment did not induce any significant changes in plasma antioxidant activity determined by either FRAP or ORAC (as described in section 2.2 and 2.3 respectively). Significant upregulation of endogenous antioxidant activity in several tissues was observed in the high 3,4-DHBA treatment group. 100 mg per kg 3,4-DHBA treatment for 14 days resulted in a 33% increase in liver catalase activity and an 81% increase in liver SOD activity (Table 7). Intestinal antioxidant enzyme activity was also increased by the high dose 3,4-DHBA treatment. Intestinal catalase, SOD, TrxR activities and GSH levels were all significantly increased by 86%, 30%, 31% and 70% respectively in rats given 100 mg per kg 3,4-DHBA. No changes were observed in enzymatic antioxidant activity in the kidney, testis or brain of rats given the high DHBA treatment or in any of the organs of rats given the low DHBA treatment.
Sprague Dawley rats were administered a daily gavage of either 2.5 mg per kg 3,4-DHBA, 100 mg per kg 3,4-DHBA or a control solution. After 14 days of daily gavage rats were starved overnight then blood was taken via cardiac puncture for assessment of plasma chemical antioxidant action via both FRAP and ORAC assays. (a) Chemical antioxidant capacity of the blood plasma determined by the FRAP assay (as described in section 2.2). (b) Chemical antioxidant capacity of the blood plasma determined by the ORAC assay (as described in section 2.3). Results shown are expressed as the mean ± S.E.M of 10 animals per group.

Figure 35: Plasma FRAP and ORAC after 3,4-DHBA treatment.

(a)

(b)
Sprague Dawley rats were administered a daily gavage of either 2.5 mg per kg 3,4-DHBA, 100 mg per kg 3,4-DHBA or a control solution. After 14 days of daily gavage, the rats were transcardially perfused with saline and harvested for organs. Endogenous antioxidant capacity of the tissues was determined by enzymatic or biochemical assays (as described in section 2.5, 2.8, 2.9 and 2.10 for catalase activity, TrxR activity, SOD activity and GSH concentration respectively). Results shown are expressed as the mean ± S.E.M of 8 animals per group. * denotes significantly different from rats treated with control gavage only (* and numbers in bold = P<0.05)

<table>
<thead>
<tr>
<th></th>
<th>Catalase (U per mg)</th>
<th>SOD (U per mg)</th>
<th>Glutathione (µM per mg)</th>
<th>TrxR (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.36 ± 3.09</td>
<td>19.21 ± 3.28</td>
<td>12.09 ± 1.92</td>
<td>100 + 11.6%</td>
</tr>
<tr>
<td>2.5 mg per kg</td>
<td>22.97± 2.42</td>
<td>19.64 ±2.93</td>
<td>11.67 ± 2.37</td>
<td>97.2 + 9.7%</td>
</tr>
<tr>
<td>100 mg per kg</td>
<td><strong>28.15 ±2.86.</strong></td>
<td><strong>34.87 ±5.09.</strong></td>
<td>14.56 ±3.08</td>
<td>106 + 12.7%</td>
</tr>
<tr>
<td><strong>Intestine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.18 ± 2.16</td>
<td>25.70 ± 2.85</td>
<td>8.14 + 2.10</td>
<td>100 + 10.2%</td>
</tr>
<tr>
<td>2.5 mg per kg</td>
<td>10.19+ 2.97</td>
<td>27.12 + 4.19</td>
<td>10.31+ 1.83</td>
<td>104 + 12.5</td>
</tr>
<tr>
<td>100 mg per kg</td>
<td><strong>17.03+3.56.</strong></td>
<td><strong>33.58+ 3.64.</strong></td>
<td><strong>13.85 + 2.47.</strong></td>
<td><strong>131 + 10.9.</strong></td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>10.30 + 1.04</td>
<td>16.07 ± 3.14</td>
<td>6.86 +1.35</td>
<td>100 + 8.6%</td>
</tr>
<tr>
<td>2.5 mg per kg</td>
<td>10.78 + 1.43</td>
<td>15.93 + 3.66</td>
<td>6.47 +1.14</td>
<td>105 + 9.7%</td>
</tr>
<tr>
<td>100 mg per kg</td>
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<td>16.56 + 2.74</td>
<td>7.09 + 1.60</td>
<td>94.0 + 10.3%</td>
</tr>
<tr>
<td><strong>Brain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.56+ 0.14</td>
<td>9.32 + 1.52</td>
<td>4.27 + 0.76</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mg per kg</td>
<td>0.49 + 0.11</td>
<td>9.59 +1.37</td>
<td>3.99 + 0.62</td>
<td>ND</td>
</tr>
<tr>
<td>100 mg per kg</td>
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<td>9.24 + 2.05</td>
<td>4.63 + 1.18</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Testes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.43 ± 0.07</td>
<td>8.83 + 0.92</td>
<td>10.43 + 1.09</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mg per kg</td>
<td>0.41±0.10</td>
<td>8.49 + 0.67</td>
<td>10.67 + 1.00</td>
<td>ND</td>
</tr>
<tr>
<td>100 mg per kg</td>
<td>0.41+0.06</td>
<td>8.65 + 1.16</td>
<td>10.40 + 0.86</td>
<td>ND</td>
</tr>
</tbody>
</table>
7.5. Discussion

Sprague Dawley rats were fed on a powered diet containing vitamin E levels that were less than the standard AIN93 diet, but sufficient (Reeves et al. 1993), for both the duration of the intervention and the two weeks immediately preceding the intervention. Vitamin E is a potent chemical antioxidant that can act to quench oxidative stress in both *in vitro* and *in vivo* systems and, when given in excess, has previously been shown to inhibit the oxidative stress-induced up regulation of catalase activity *in vivo* (Eder et al. 2002). Given 3,4-DHBA treatment was shown to have both pro-oxidant effects and to induce an upregulation of catalase activity *in vitro* (chapter 5), any 3,4-DHBA mediated pro-oxidant induced upregulation in endogenous antioxidant activity could potentially be either suppressed or reduced by excessively high dietary vitamin E. As such, vitamin E levels were reduced in this trial (Table 6).

During the course of the intervention no significant treatment induced changes in either body weight or food intake were observed (Figures 33 and 34). Changes in body weight and food intake are potentially important in animal feeding trials and are often overlooked when evaluating endogenous antioxidant activity *in vivo*. Previously, it has been shown that weight loss induced by dietary restriction can induce significant increases in catalase activity in rats. Heart catalase activities were seen to double in Sprague Dawley rats that had undergone dietary-restriction induced weight loss (Lammi-Keefe et al. 1981). Further, caloric restriction has been shown to increase catalase and SOD activity in *S. cerevisiae* (Agarwal et al. 2005). Given the importance of feeding behaviour in the measurement of endogenous antioxidant enzymes, the palatability of the test compound and hence their effect on feeding behaviour must be considered. It is well documented that phenolics, including 3,4-DHBA, are unpleasant to the palette and may adversely affect feeding behaviour if added directly to diets. For example, gallic acid, a phenolic acid with a structure similar to 3,4-DHBA, has been shown to reduce food intake and body weight of rats when added to their diet (Glick 1981). Since food intake, specifically its restriction, can induce endogenous antioxidant expression, any decrease in food intake could have affected measures of antioxidant function in this experiment. As 3,4-DHBA was given by oral gavage and no changes to feeding behaviour were detected, any changes
induced by 3,4-DHBA treatment are most likely the result of the 3,4-DHBA treatment.

### 7.5.1 Regulation of endogenous antioxidant enzymes by 3,4-DHBA in vivo.

The 100 mg per kg dose of 3,4-DHBA was shown to upregulate several antioxidant enzymes in both the intestine and liver but not other more peripheral tissues of healthy rats (Table 7). This upregulation of endogenous antioxidant enzymes might explain part of the mechanisms behind the observed health benefits conferred by diets high in CDAs.

Endogenous antioxidant enzyme activities are related to the pathogenesis and progression of multiple disease states and as such, their regulation by exogenous compounds offers a potential mechanism to prevent or slow the progression of these diseases (Mates et al. 1999). Perhaps the best examples of compounds that are protective against disease via the regulation of endogenous antioxidant enzyme activity are the catalase and SOD mimetics. Catalase and SOD mimetics are synthetic compounds that mimic the enzymatic effects of catalase and SOD and effectively increase the overall catalase or SOD activity of a system. Catalase mimetics have been shown to protect cells from oxidative stress mediated death and damage in vitro (Day et al. 1995). In vivo, mimetics have been shown to delay death in a prion mouse disease model, suppress oxidative stress mediated damage by global cerebral ischemia in a Mongolian gerbil model and protect from oxidative stress induced complications in a mouse model of Alzheimer’s disease (Melov et al. 2005; Sharma & Gupta 2007; Brazier et al. 2008). Potentially, increasing the activity of endogenous catalase and SOD with 3,4-DHBA treatment could have similar beneficial effects in vivo.

Several studies have examined the effect of dietary phytochemicals on measures of endogenous antioxidant enzymes in vivo. P-coumaric acid, a phenolic acid found in a wide variety of plants, has been shown to induce significant increases in both catalase and SOD activity in the intestine and liver of rats when given by oral gavage (Yeh & Yen 2006a). 100 mg/kg body weight p-coumaric acid resulted in a doubling of liver...
and intestine catalase activity and a 90% and 50% increase in increase in liver and intestine SOD activity respectively (Yeh & Yen 2006a). The results presented in this chapter agree with these results and support the theory that non-physiological dosages of phenolic acids can induce increased endogenous antioxidant activity in rats. Dietary p-coumaric acid is, however, normally found as an ester, and as such, its bioavailability relies on gut esterase activity that may affect its pharmacokinetics in humans (Andreasen et al. 2001). Hence results for an intervention in rats with non-esterified p-coumaric acid are potentially less likely to be relevant to humans than results from an intervention in rats using 3,4-DHBA.

The effect of both 3,4-DHBA and a CDA rich extract on antioxidant enzyme expression during disease states has previously been measured in rodents. An anthocyanin rich blueberry extract was shown to reduce diabetes induced decreases in catalase, SOD and GSH levels (Nizamutdinova et al. 2009). Additionally, direct feeding of 3,4-DHBA in another diabetic mouse model showed similar effects (Lin et al. 2009). During this 8 week intervention, mouse diets were supplemented with between 1-4% 3,4-DHBA. The 4% 3,4-DHBA diet group showed a 50% and 30% resistance to diabetes-induced suppression of catalase activity in the heart and kidney respectively. However, there was no non-supplemented non-diabetic control and the food intake of the treatment groups was not recorded, making it difficult to determine if catalase activity of the tissues would have increased in a non-diseased animal or if the increase in catalase activity was, in part, affected by suppression of food intake. Although the capacity for both CDAs and 3,4-DHBA to increase antioxidant enzymes expression supports the findings presented here, there exist two important distinctions between the results presented here and these previous findings. Firstly, this intervention examined the effects of oral 3,4-DHBA in a healthy animal as opposed to a disease model and secondly, no changes in either body weight or food intake were found in this study. (Lin et al. 2009). Further, as the basal levels of antioxidant enzymes were reduced by the disease models used, the relative increase seen after CDA or 3,4-DHBA treatment were potentially due to the prevention of the disease-induced decrease and not to a specific upregulation of their activity, as is seen in the study reported here.
In this study, increased antioxidant enzyme activity was observed in the liver and intestine of rats given the high dose of 100 mg per kg 3,4-DHBA, with no significant changes in activity detected in the kidney, brain or testis in any of the treatment groups (Table 7). Given that the liver and intestine are the two most peripheral organs they would likely be exposed to significantly higher concentrations of 3,4-DHBA than the other organs. It is therefore unsurprising that they were shown to have the greatest response to oral 3,4-DHBA treatment. As the kidneys would also be likely to be exposed to high levels of 3,4-DHBA, one might also have expected the kidneys to undergo similar regulation of antioxidant enzymes; however, no changes in enzymatic antioxidant function of the kidneys were detected. Given the kidneys are a site for the concentration and excretion of dietary derived phenolic compounds present in the blood, they are regularly exposed to significant levels of phenolic compounds similar to 3,4-DHBA (Rechner et al. 2002). The chronic exposure of the kidneys to concentrated levels of phenolics may have either 1) already maximised their expression of antioxidant enzymes or 2) resulted in the kidneys being resistant to upregulation of antioxidant activity by additional phenolic exposure.

No significant change in endogenous antioxidant enzyme activity was seen in either the brains or testis of rats treated with 3,4-DHBA. This lack of regulation is potentially due to the restrictive nature of the capillaries that both supply blood and dictate the transfer of nutrients to these tissues. The transfer of compounds from the blood to the brain and the blood to the testis is regulated by the blood brain barrier (BBB) and blood testis barrier (BTB) respectively. The BBB’s function is to protect the brain from changes in blood homeostasis, such as fluxes in the levels of ions, amino acids, peptides and other potentially bioactive compounds such as 3,4-DHBA (Bradbury 1984). It is located at the brain’s blood capillaries and acts to prevent water-soluble substances from crossing into the brain by preventing their diffusion between cells (Bradbury 1984). Although fat soluble compounds can diffuse though the cells plasma membrane, they are often actively pumped back into the blood by efflux pumps in the capillary wall (Cordon-Cardo et al. 1989). Given 3,4-DHBA is highly water-soluble and has a structure similar to compounds that are known substrates for the BBB efflux pumps, it is very unlikely to have access into the brain proper after oral feeding. Hence the lack of 3,4-DHBA induced antioxidant enzyme
upregulation in the brain is not unexpected. The BTB has a structure similar to the BBB, in that tight junctions between adjacent Sertoli cells prevent diffusion of hydrophilic compounds across the barrier (Pelletier & Byers 1992). Given the similar function of the BTB to the BBB, it is again unsurprising that no changes in endogenous antioxidant enzyme activity were detected in the testis.

Although up regulation of antioxidant enzymes is generally regarded as beneficial, it should be noted that increases in endogenous antioxidant enzymes can also be induced by potentially damaging compounds. Exposure to cigarette smoke has been shown to increase catalase and SOD activity in the testis of dogs after short term exposure (Ozyurt et al. 2006). Since long term tobacco smoking in humans is linked to increased chance of testicular cancer (Kuper et al. 2002), it could be deduced that catalase and SOD activity upregulation are potentially an adaptive response to smoke induced damage and could in theory be intermediate markers of testicular cancer. Given this example, one could argue that high dose 3,4-DHBA may be inducing upregulation of antioxidant enzymes as a precursor to disease. Although upregulation of endogenous antioxidant enzymes may in some cases be a marker of future disease, since no increase in disease rates have been associated with high phytochemical intake in healthy humans, it would seem unlikely that 3,4-DHBA induced endogenous antioxidant upregulation would indicate future negative health outcomes. Additionally 3,4-DHBA, when given to rats for 52 week at a higher concentration than was used in this study, did not induce any toxic effects (Hirose et al. 1992).

Previously the effect of oral anthocyanin feeding on phase II enzyme expression in healthy rats has been examined (Reen et al. 2006; Dulebohn et al. 2008). Phase II enzymes, which relate to antioxidant enzymes activity through common transcriptional regulation (Zhu et al. 2005), act to process toxins and drugs as they are absorbed into the body. The effect of various types of blueberry phytochemicals on several measures of phase II enzyme expression where examined by Dulebohn et al (2008) in young Sprague-Dawley rats. They showed increases in hepatic GST in rats fed either 10% blueberries, a concentrate of blueberry polyphenols or 1% concentrated blueberry flavonoids. A different study by Reen et al showed significantly greater increases in hepatic GST activity in rats fed 10% black
raspberries (Reen et al. 2006). The greater increase in GST seen after black raspberry feeding could potentially be due to the different anthocyanin profiles in blueberries and black raspberries. Blueberries used by Dulebohn et al (2008) were high in peonidin, petunidin and CDAs, where as CDAs were the primary anthocyanins present in the black raspberries used by Keen et al. Although GST activity was not measured in this study, significant increases in related hepatic antioxidant enzymes are shown. The intervention required to induce hepatic antioxidant enzymes expression in this study, 100 mg per kg, equates to approximately 0.5% of the total weight of the diet. This is the same concentration of phytochemicals used to induce significant increases in GST activity by black raspberry anthocyanins, and as such, their results agree with the finding that CDAs or their orally bioavailable metabolite, 3,4-DHBA, can upregulate hepatic enzymes at dietary levels of 0.5%.

7.5.2 Relationship between chemical and enzyme antioxidant function

3,4-DHBA was shown to both increase endogenous antioxidant enzyme activity and decrease plasma FRAP levels following two weeks of oral gavage (Figure 35 and Table 7). Previously, changes in both plasma chemical antioxidant potential and endogenous antioxidant enzyme activity by phytochemical treatments have been reported. EGCG, the primary and generally considered most active green tea catechin, has been shown to have no effect on plasma FRAP levels after short term consumption (Van Amelsvoort et al. 2001) and to decrease plasma FRAP after long term consumption (Kimura et al. 2002). Additionally, several green tea catechins, including but not exclusively EGCG, have been shown to upregulate endogenous antioxidant enzyme expression in vitro (Murakami et al. 2002; Bahia et al. 2008). Although this interesting dichotomy of inverse changes in chemical and enzymatic antioxidant actions may seem counter intuitive, it is seen in some diseases states. For example, during the progression of nephrotic syndrome, plasma FRAP levels are seen to increase despite decreases in both the endogenous antioxidant activity of the organs and plasma thiol concentrations (Karthikeyan et al. 2008). Given kidney damage in nephrotic syndrome is mediated by oxidative stress, it appears that high plasma FRAP does not offer protection, at least in the case of nephrotic syndrome, from oxidative
damage to organs, and further, that the endogenous antioxidant activities of tissues are not positively linked to plasma FRAP values. Mechanistically, this is difficult to explain and there are great inconsistencies in the reported links between plasma FRAP and endogenous antioxidant enzyme activity of tissues. It is possible that increases in plasma FRAP are due to a response to greater levels of oxidative stress being generated by the tissues (due to lower levels of antioxidant enzymes) or conversely, decreased FRAP levels are resultant from decreased levels of oxidative stress being generated by the tissues (due to higher levels of antioxidant enzymes). Recently it has been proposed that high plasma chemical antioxidant levels may in fact inhibit the adaptive response to exercise by removing the oxidative stress that is required to induce increases in endogenous antioxidant enzymes (Gomez-Cabrera et al. 2008). Potentially therefore, elevated FRAP levels in the blood may prevent the upregulation of antioxidant enzymes induced by phytochemical compounds.

Speculatively, short term increases in the chemical antioxidant capacity of the plasma may positively affect organ antioxidant activity via a chemical antioxidant sparing effect that protects the endogenous antioxidants from oxidative stress mediated inactivation (Sastre et al. 1992). Whereas longer term interventions may have an inverse relationship between FRAP and antioxidant enzyme activity for the reasons discussed above (Kimura et al. 2002), no definitive or consistent relationship appears to exist between plasma FRAP and tissue enzymatic antioxidant activity. Perhaps the more transient nature of plasma FRAP levels, and particularly the acute response plasma FRAP has to feeding, makes determining an exact relationship elusive (Kimura et al. 2002). Regardless of the specific relationship between plasma FRAP and organ antioxidant activity, the results presented here, showing decreased FRAP and increased organ antioxidant activity, are in agreement with at least some of the published literature.

**7.5.3 Physiological relevance**

When conducting animal trials it is important to consider both the physiological relevance of the intervention being used, as well as potential toxicity of the compound if given to humans. In animal experiments using phytochemical compounds, issues of
bioavailability, peak blood concentrations and potential differences in toxicity of the compound between the animal used and humans must be addressed. Data on both the blood concentrations of 3,4-DHBA in humans after a CDA rich meal and also the bioavailability of 3,4-DHBA itself in rats has previously been reported. Blood concentrations of 3,4-DHBA in humans after a CDA rich meal have been shown to be as high as 1 µM and account for 40% of the orally consumed CDA (Vitaglione et al. 2007). 3,4-DHBA is highly orally bioavailable in rats, with blood levels reaching the 10’s of µM after a large oral dose of 3,4-DHBA (Han et al. 2007). The doses used during this animal trial were chosen to represent both a low, physiological dose (2.5 mg per kg body weight) and a high, functional food type dose (100 mg per kg body weight). The low dose of 2.5 mg per kg was chosen as it would result in similar blood 3,4-DHBA concentrations as are observed in humans after a CDA rich meal. The high dose of 100 mg per kg was chosen as: 1, concentrations of 3,4-DHBA greater than this have previously been shown to be safe in rats and 2, it would result in a human equivalent dose (HED) of 1g, when calculated using the American food and drug administration (FDA) guidelines (see appendix 1 for rational and calculation), which is achievable in a functional food format (FDA 2005). Given the bioavailability of both 3,4-DHBA in rats and similar phenolic acids in humans is high, and no additional factors that infer increased toxicity of 3,4-DHBA in humans are known, a dose of 1g 3,4-DHBA would likely be safe and well tolerated in humans.

Although no significant changes were seen in any measures at the low dose, there was a trend towards increased levels of antioxidant enzymes activity. Although significant results observed in the high dose treatment group may be applicable to humans if given a high 3,4-DHBA functional food, they are not directly relevant to the effects of normal dietary exposure to 3,4-DHBA or CDAs in humans. A future clinical trial assessing the effects of high dose 3,4-DHBA on endogenous antioxidant enzyme expression in humans may further validate a potential use for 3,4-DHBA or 3,4-DHBA rich extracts in the functional food space.
7.6 Conclusion

This chapter shows for the first time that 3,4-DHBA induces a slight but significant increase in endogenous antioxidant enzymes activity in specific organs of healthy adult rats when given orally at a non-physiological dose of 100 mg per kg body weight. Further it examines the effect of physiological concentrations of 3,4-DHBA and shows no significant changes in the same measures. Potentially, a functional food could be formulated for human use, which contained 1 g of 3,4-DHBA, and tested for endogenous antioxidant enzyme regulating capacity in humans. Further, the regulation of endogenous antioxidant enzymes by 3,4-DHBA may in part explain the health benefits of its dietary precursor CDAs in humans and may offer protection from the development of chronic diseases.
Chapter 8 Final discussion

8.1 Introduction

The results presented in this thesis describe the effects of several phytochemical blood metabolites, primarily the CDA metabolite 3,4-DHBA, on measures of oxidative stress both in vitro and in vivo. In this chapter the relevance of results presented and potential future directions are discussed.

This thesis proposed the following questions:

1. Do phytochemical metabolites offer protection from oxidative stress in vitro and can the combination of phytochemical metabolites give synergistic protection from oxidative stress induced cell death in vitro?
2. If so, what mechanism(s) underlie the protection phytochemical metabolites have in vitro?
3. Are these potential protective effects conserved across multiple cell types?
4. Can in vitro screening using phytochemical metabolites be used to better predict in vivo efficacy?

This chapter will address how successfully these questions were answered in addition to other questions that have been highlighted by the work presented here.

8.2. What is the role of cell culture density in assays involving oxidative stress?

The role of cell culture condition in assays that evaluate oxidative stress is an often overlooked parameter in in vitro assays. Potentially, specific cell culture conditions may adapt cells to stress and result in resistance to subsequent insult. The results presented in chapter 3 show that cell culture conditions, both at the time of exposure
to the oxidative stress and during the growth of cells prior to their exposure, can affect the sensitivity of Jurkat cells to H$_2$O$_2$-induced cell death. Associated with this change in sensitivity to H$_2$O$_2$ were changes in endogenous catalase activity, ATP concentration and alterations in the basal level of the cellular redox state.

These results highlight a possible redox stress mediated mechanisms for cell culture condition derived changes in sensitivity to H$_2$O$_2$ toxicity, and suggest that the consistent maintenance and reporting of culture conditions is important for studies that evaluate oxidative stress in vitro. Very few reports have examined the role of cell culture density in catalase activity, ROS generation and ATP production, and had shown they are regulated by changes in cell culture density, in certain cell types, in isolation of each other. However, no work had been conducted that examine all of these measures concurrently or the transient effect that long term culture conditions might have on assays evaluating oxidative stress. Given the data presented here, it would appear that changes in cell culture density, both acutely and chronically, can dramatically affect cellular responses to oxidant exposure and should be carefully considered when conducting experiments that involve oxidative stressors in vitro.

The long term culturing of Jurkat cells at low densities resulted in a relative increase in the level of cellular oxidative stress. This is potentially important for various assays that examine factors related to redox state. Given it has previously been shown that ATP is required to maintain catalase activity and that the generation of ATP causes a cellular oxidative load (Cross & Ruis 1978; Garcia-Ruiz et al. 1995), one might consider that the relatively greater nutrient levels available when cells are grown at low density may allow for greater production of ATP and hence higher catalase activity. However, caution must be taken not assume that a similar profile of redox state/cell culture density applies to all cell types or lines. Indeed, a previous study examining the relationship between cell culture density and basal levels of redox stress in HeLa cells showed increases in redox state as culture density increased (Bello et al. 2004). It is unclear why this study illustrates a positive relationship between cell density and redox state, as opposed to the inverse relationship that is presented here. It is possible that the adherent nature of HeLa cells allows for cell to cell commutation that affects cellular redox state as cell culture density increases, as
opposed to the free-floating Jurkat cells. Regardless of the exacting mechanisms underlying cell culture density regulation of basal redox levels, it is apparent that there is no consistent relationship between cell culture density and cellular redox state between different cell types. Hence, until a more conclusive examination of the mechanisms underlying these effects is conducted, the extension of the results presented here beyond Jurkat cells is unwarranted.

Perhaps of greater importance than the protection from H$_2$O$_2$-induced cell death conferred by culturing cells at lower densities, is the change in the mode of cell death that was observed. The treatment of Jurkat cells with H$_2$O$_2$ has previously been shown to induce both necrosis and apoptosis, with greater concentrations inducing necrosis and lower concentration inducing apoptosis (Hampton & Orrenius 1997). The culturing of cells at very high densities appeared to both sensitise the Jurkat cells to H$_2$O$_2$-induced cell death and resulted in greater levels of necrosis. The capacity of compounds to protect against cell death can potentially relate to the mode of cell death being induced (Vantieghem et al. 1998; Champagne et al. 1999). Due to this capacity, the shift in mode of death from apoptosis to necrosis may potentiate the cytoprotective effects of compounds that protect from necrosis, while reducing the apparent potency of compounds that mediate cytoprotection via interactions with apoptotic mechanisms. Additionally, increasing the sensitivity of cells to a cytotoxic agent may result in an insult that is too great to protect from with any given compound. It is therefore important to consider both the potential mechanism of action by which a putative cytoprotective agent may function as well as the effects the cell culture conditions may have on this mechanism, when designing and evaluating this type of experiment.

The results described in chapter 3 of this thesis highlight the potential importance of cell density and cell culture conditions in assays that evaluate responses to oxidative stress. Based on the results presented, it may be prudent to both provide more details of cell culturing conditions in scientific publications and to evaluate cells for potential changes in redox state before conducting such research.
8.3. How effective & representative are phytochemical metabolites vs dietary phytochemicals in vitro assays?

Previous work has clearly demonstrated the effectiveness of phytochemicals in the prevention of a wide array of cellular damage in vitro. Recently, work alluding to the mechanisms of digestion and absorption of these dietary phytochemicals has shown that many phytochemicals are substantially modified into specific blood metabolites during the absorption process. The use of phytochemical blood metabolites, as opposed to their dietary forms, may therefore improve the relevance of in vitro assays to the in vivo reality. The results from chapter 4 show that, of the DHBA and HBA phytochemical metabolites tested, only 3,4-DHBA conveyed significant cytoprotective effects in the model system used. Further, the cytoprotective activity of 3,4-DHBA was significantly less than that of the dietary phytochemical controls of (+) catechin and quercetin, and no synergies between 3,4-DHBA and either the DHBAs, HBA or positive controls was detected. Although the blood metabolites examined had limited bioactivity as compared to the positive control compounds, of (+) catechin and quercetin that are not bioavailable, they could be included alongside or instead of dietary phytochemicals to aid in improving the relevance of in vitro phytochemical screening.

The DHBAs and HBAs tested represent bioavailable metabolites of multiple dietary phytochemicals (Pedersen & FitzGerald 1984; Koskela et al. 2007), and as such, are excellent examples of phytochemical metabolites that can be used for in vitro assays examining the putative health benefits of their parent compounds. However, determining what the most appropriate form of other phytochemical metabolites are, particularly ones that are conjugated with sulphates or glucuronidated during absorption, to test in in vitro assays is more complicated. For example, when the phytochemical resveratrol is absorbed into the body it is modified by the actions of enteric hydrolysis and then phase 1 and 2 enzyme activity of the intestine and liver, and then circulates as a sulphated form (Walle et al. 2004). Logic would dictate that this sulphated resveratrol would be the compound of choice to use in in vitro assays that attempt to replicate the in vivo situation.
However, some target cells within the body express both sulphotransferase and sulphatase enzymes (Munroe & Chang 1987; Dooley et al. 2000), which can remove or add sulphate conjugates respectively, and these activities need to be considered. In vivo there are several sulphotransferases enzymes that can sulphate phytochemicals, including P-form phenol sulphotransferase and M-form phenol sulphotransferase that target phytochemicals (Galijatovic et al. 1999), as well as the steroid sulphotransferases of estrogen sulphotransferase and dehydroepiandrosterone (DHEA) sulphotransferase that also have specificity for a broad array of sulphated phytochemical metabolites (Miksits et al. 2010). The steroid sulphotransferases, in conjunction with sulphatases, act to regulate the activity of circulating steroid hormones. Steroid hormones circulate in the blood in an inactive sulphated form. When they reach a target tissue they are desulphated by the relevant sulphatase and become active (Reed et al. 2005). Potentially, as mentioned above, this can also occur to phytochemicals that are sulphated during absorption and would result in the phytochemical being present within the cell in its aglycone form. It has also been shown that endogenous sulphotransferases and sulphatases have some specificity for resveratrol and resveratrol sulphates respectively (Miksits et al. 2010). Hence it is possible that the circulating resveratrol sulphate may be desulphated in certain tissues in vivo, indicating that the addition of un-conjugated resveratrol to in vitro assays may more closely represent what occurs in vivo. Equally, it is possible that resveratrol may be sulphated by cells that express sulphotransferase activity in vitro and then, the addition of sulphated resveratrol may more closely represent the in vivo situation. Additionally, the expression of these sulphatase and sulphotransferase enzymes may be different in the transformed cell lines used in vitro as compared to their in vivo equivalent cell types. Furthermore, genetic polymorphism in at least two of the sulphotransferases enzymes which have specificity for phytochemicals, has been reported in humans leading to potentially significant inter-personal differences in phytochemical metabolism and hence, bioactivity (Basler et al. 1992; Brookes et al. 2008). Until further work examining the expression and regulation of sulphatases and sulphotransferases on phytochemicals is conducted, it is not possible to determine whether the use of a sulphated form would increase the relevance of in vitro work.
The most potent of the positive controls tested in chapter 4, quercetin, is present in the circulation of humans as various glucuronidates following oral consumption (Manach et al. 1998). As is the case with sulphated forms of phytochemicals, there is the potential for these glucuronide groups to be removed by the actions of endogenous enzymes. Glucuronidases, enzymes that remove the glucuronide conjugate, have been shown to be expressed during inflammation and as such, circulating glucuronidated phytochemicals may be processed back to their dietary forms. Shimoi et al. (2005) examined the effect that systemic inflammation, induced by injection of lipopolysaccharide (LPS) to rats, had on beta-glucuronidase activity. They showed that systemic inflammation caused both increased activity of beta-glucuronidase in rat plasma and increased the plasma concentration of free luteolin, relative to the concentration of luteolin monoglucuronide, after oral feeding. Further, they examined this same effect in vitro, by evaluating the activity of beta-glucuronidase in four different cell types and determining the effect of immune stimulation on its activity. It was shown that all four cell types, neutrophils, human umbilical vein endothelial cells, IMR-90 and Caco-2 cells, possessed beta-glucuronidase activity and that this activity increased the most in neutrophil cells when they were activated by cytochalasin B and ionomycin (Shimoi & Nakayama 2005). This combined with the in vivo rat data indicates that phytochemical glucuronidates may be converted back to their aglycones both in vitro and in vivo. Although the results of this study may argue that the use of dietary forms of phytochemicals in in vitro assay as being physiologically relevant, there is probably still too little data on glucuronidase activity with relation to phytochemicals, to draw such a conclusion. Further, as previous in vitro assays showed that quercetin has different bioactivities to its glucuronidated metabolites (Day et al. 2000), it is likely that in vivo beta-glucuronidase activity is either not sufficient or not rapid enough to remove all the conjugates before they elicit biological effects.

3,4-DHBA as used in the studies presented here, has not been detected in the blood of humans in either a sulfated or glucuronidated form, is therefore unlikely to be affected by these mechanisms and represents a sound target for investigation. Given the current state of knowledge regarding phytochemical breakdown, absorption, conjugation, bioavailability and biotransformation by cells, the use of non-conjugated
metabolites of phytochemicals, such as the DHBAs and HBAs, in in vitro assays is likely preferable to the use of conjugated phytochemicals, which is again preferable to the use of their dietary forms.

Given many phytochemical compounds are conjugated during absorption into the body, comparing the effects of glucuronidated and sulfated phytochemical metabolites to the activity of the DHBAs and HBA is perhaps a more correct way of determining which dietary phytochemicals are likely to have more potent in vivo effects. Although conjugated phytochemicals were not examined in this thesis, due to issues of purity and availability, several reports have previously examined the relative effects of conjugated and non-conjugated forms of dietary phytochemicals. Stevenson et al. (2008) examined the cytoprotective potential of phytochemicals conjugated with glucuronidates against H$_2$O$_2$-induced cell death. It was shown that phytochemical glucuronidates were less potent cytoprotectants than their dietary forms. Quercetin, one of the non-bioavailable control phytochemicals used in this thesis, was a weaker cytoprotectant when glucuronidated than when not (Stevenson et al. 2008). Further, several studies have shown that sulfation of resveratrol greatly alters its biological effects (Day et al. 2000; Hoshino et al. 2010). Generally, reduction of activity of the metabolites tested here, relative to the positive controls, agrees with previous finding that show glucuronidated and sulfated phytochemicals metabolites have greatly reduced antioxidant capacity and often have reduced effectiveness in in vitro assays. Although it would have been preferable to examine the dietary precursor of 3,4-DHBAs in the in vivo experiments conducted in this thesis, issues previously discussed concerning the stability and the complex nature of their breakdown process made this non-feasible.

8.4. What are the roles of phytochemical metabolites in the protection from oxidative stress? Endogenous antioxidant enzyme effects vs chemical antioxidant action.

Conventionally, phytochemicals were believed to mediate potential health benefits by acting as chemical antioxidants that directly quench free radicals before they can
induce cellular damage. Currently, some debate exists as to whether chemical antioxidant action or the regulation of endogenous cellular systems is more relevant to the in vivo effects of phytochemical consumption. Chapter 5 examines the mechanisms underlying the cytoprotection seen after DHBA treatment, starting with the conventional hypothesis that this protection is mediated by chemical antioxidant action. The data presented in chapter 5 indicates that the protection against H$_2$O$_2$-induced cell death offered by phytochemical pre-treatments, at least in the case of 3,4-DHBA, is likely mediated by upregulation of endogenous antioxidant enzymes and is not directly related to the chemical antioxidant action of these compounds.

Although the data presented in this thesis would indicate that chemical antioxidant capacity is not the mechanism of action by which 3,4-DHBA mediates its cytoprotection, this does not preclude other phytochemical compounds from having significant antioxidant effects via chemical antioxidant action, particularly if they are either highly bioavailable or not orally administered. Certainly, significant physiological effects of phytochemicals have been proposed to be mediated by chemical antioxidant actions (Heitzer et al. 1996). Perhaps the best example of these can be seen during treatments with either vitamin C or E. Vitamin C and E are potent chemical antioxidants, are orally bioavailable, can have their blood concentrations significantly elevated by oral supplementation and are transported into cells (Burton & Traber 1990; Levine et al. 1996; Liang et al. 2001; Stocker 2004). One could surmise that they are in essence the ideal examples of phytochemicals that could mediate effects via a direct chemical antioxidant mechanism.

In vitro, vitamin C and E exhibit potent antioxidant effects, including cytoprotection, protection from oxidant-induced DNA damage and reduction in oxidative stress mediated lipid peroxidation (Wagner et al. 1996; Noroozi et al. 1998). In vivo, vitamin C and E can acutely decreases elevated levels of oxidative stress or damage in a variety of situations, including exercise induced increases in lipid peroxidation and diabetes-induced oxidative damage (Dillard et al. 1978; Sharma et al. 2000). The combination of this in vitro and in vivo data suggests that vitamin C and E can have significant biological effects via chemical antioxidant actions in vivo, hence suggesting that other phytochemical compounds may also have such effects in vivo.
However, long term health benefits, such as improvements in cardiovascular health, protection from cancer and neurological diseases are not observed in the majority of studies using interventions with either vitamin C or E (Sharma et al. 2000; Gray & Whitney 2003; Boothby & Doering 2005; Lee et al. 2005; Miller et al. 2005; Cordero et al. 2010). In fact, vitamin C supplementation has been shown to inhibit exercise-induced improvements in cardiovascular health (Gomez-Cabrera et al. 2008), despite acutely protecting from oxidative stress in vivo (Thompson et al. 2001). Some might argue that the regulation of vitamins, particularly vitamin C, by endogenous systems of the body is stringent and that excess consumption leads to greater levels of excretion; hence lack of long term efficacy is not unexpected. While this is partially true for large doses of vitamin C, supplementation with moderate amounts does result in long term increases in plasma vitamin C concentrations and an associated increase in plasma antioxidant capacity (Levine et al. 1996; Hamilton et al. 2000). Although it is perhaps somewhat simplistic to attribute all the actions of vitamin C and E to their antioxidant effects, given their other cellular roles, examining their effects does highlight that an overt increase in blood antioxidant capacity does not necessarily correlate with long term positive health outcomes and further, indicates that chemical antioxidant action may not be important for long term health benefits in vivo.

Previous reports have indicated that the capacity of phytochemicals to regulate biological markers is directly associated with their chemical antioxidant capacity. These studies are in disagreement with the results presented in this thesis, which found no correlation between antioxidant capacity and protection from H\textsubscript{2}O\textsubscript{2}-induced cell death. Yeh et al (2003) showed that the chemical antioxidant capacity of phenolic acids was directly related to their ability to induce the activity of P-form sulfotransferase P. Further Burns et al (2000) correlated the antioxidant capacity of various red wines to their capacity to induce vasodilatation of thoracic aortas ex-vivo. Although these reports indicate that chemical antioxidant capacity is important in predicting biological effects, there are several important considerations that potentially conflict with this indication. Firstly, although Yeh et al (2003) showed a correlation between chemical antioxidant action and biological effects in vitro, when
they measured a similar effects in vivo they found the less potent chemical antioxidant compounds were in some cases more potent inducers of biological effects (Yeh & Yen 2006a). Secondly, a detailed examination into the relative chemical antioxidant potential of polyphenols using various in vitro assays, found there was no correlation between the results of the FRAP, ORAC and a lipid peroxidation assays used. Further, they found that the results of only one of the three assays tested correlated with the results from a cellular cytoprotection assay (Zhang et al. 2006). These studies suggest that while it may be possible to class a compound as an antioxidant with various chemical antioxidant assays, it is difficult to predict exact antioxidant capacity of compounds, due to the variation between assays. The source of this variation and lack of relation to cellular events is likely due to 2 reasons. i) That different chemical antioxidant assays measure different aspects of chemical antioxidant behaviour and ii) that the events that occur during a chemical antioxidant assay do not necessarily correlate with what occurs in a cellular environment.

The results from chapter 5 and 6 show that 3,4-DHBA was both a more active chemical antioxidant and a more potent inducer of endogenous antioxidant enzymes than 2,4-DHBA, and hence, at least for the compounds tested, appears to support a correlation between chemical antioxidant capacity and biological effects. However, the inconsistent nature of measures of chemical antioxidant capacity brings such a conclusion into doubt. Previous reports on the relative chemical antioxidant capacity of 2,4 and 3,4-DHBA show inconsistent results, with neither 2,4 nor 3,4-DHBA being consistently the more potent chemical antioxidant. Makris et al (2001), showed that 2,4-DHBA was more potent in 3 measures of antioxidant capacity that 3,4-DHBA (Makris & Rossiter 2001), whereas the results presented in this thesis show that 3,4-DHBA is more potent. These results again support the finding that a consistent relationship between chemical antioxidant action and biological effects does not necessarily exist.

The results presented in this thesis, combined with many previous scientific studies indicate that chemical antioxidant action is not a likely mechanism behind the long term health benefits attributed to the consumption of fruits and vegetables. The upregulation of endogenous antioxidant enzymes however, potentially represents a
mechanism by which phytochemical metabolites may mediate long term health benefits. The data presented in chapters 5, 6 and 7 shows that 3,4-DHBA can induce a wide variety of antioxidant enzyme activities, in a variety of cell types and that this activity is associated with the in vitro protection from H$_2$O$_2$-induced cell death. The importance of these endogenous antioxidant enzymes in the progression of chronic disease has been highlighted in several key studies.

The results described in this thesis shows that 3,4-DHBA can increase SOD activity both in vitro and in vivo. SOD activity and expression has been implicated in cardiovascular health and related diseases. SOD activity is decreased in the smooth muscle cells of atherosclerotic lesions and is theorised to regulate the balance between $^\bullet$O$_2$- and NO, thus affecting vasodilatation (Luoma et al. 1998). Additionally a direct effect of SOD activity has been shown in a rat model of hypertension. The injection of liposome-entrapped SOD to Sprague-Dawley rats was shown to decrease angiotensin II-induced hypertension (Laursen et al. 1997). Several human studies using the SOD rich melon extract Glisodin, which increases blood SOD activity in humans (Vouldoukis et al. 2004), have shown potential cardiovascular health benefits. A two year supplementation study with oral Glisodin reduced malondialdehyde, a marker of lipid peroxidation, by 34 % and significantly decreased carotid artery intima media thickness, a measure of atherosclerosis, in high CVD risk individuals (Cloarec et al. 2007). Acutely, daily administration of Glisodin combined with beta-glucan and Coenzyme Q10 improved time to exhaustion and blood lipid hydroperoxide concentration in soccer players undergoing pre-season fitness training (Arent et al. 2010). SOD activity of blood has also been shown to indicate future susceptibility to both cancer and overall mortality (Ito et al. 2002). Further, over expression of the mitochondrial located SOD-2 in a mouse model of Alzheimer’s disease, protects mice from Alzheimer’s disease-related learning and memory deficits and decreased $^\bullet$O$_2$- levels in the brain (Massaad et al. 2009). Although it is difficult to directly contribute the health effects of fruit and vegetable consumption to antioxidant enzyme mediated protection from oxidative stress, it is becoming increasingly clear that moderate increases in endogenous antioxidant enzymes are beneficial to human health and also, that at least some phytochemicals can induce increases in endogenous antioxidant enzyme activity.
8.5. Does the use of phytochemical metabolites in \textit{in vitro} assays improve their relevance to \textit{in vivo} trials?

Chapters 6 and 7 examined the effects of 3,4-DHBA on antioxidant enzymes expression \textit{in vitro} and \textit{in vivo} respectively. These chapters tested the key hypothesis of this thesis, that \textit{in vitro} assays using phytochemical metabolites are more relevant to \textit{in vivo} effects than assays using dietary phytochemicals. The results of chapter 6 show that 3,4-DHBA can upregulate the activity of endogenous antioxidant enzymes in several cell lines derived from multiple tissue types. Additionally they show time and dose dependent changes in these activity levels. Chapter 7 examined the effect of oral 3,4-DHBA feeding on the endogenous antioxidant enzyme expression in rats. Significant increases in the activity level of antioxidant enzymes were seen in the intestine and liver of rats given 100 mg per kg 3,4-DHBA per day. Combined, the results from chapter 6 and 7 show that in the case of 3,4-DHBA, \textit{in vitro} assays using a blood metabolites can predict \textit{in vivo} effects. However, more work with a larger number of phytochemical metabolites is required to determine whether these predictions can be more widely applied.

Phytochemical metabolite screening \textit{in vitro} may offer a more directed and representative approach to predicting \textit{in vivo} effects. However several potential issues can limit its usefulness. Some of the major issues to consider are, i) the differential bioavailability of compounds between humans and animal models used, ii) the inter-individual variation in gut efflux pumps and phase 1 and 2 enzymes that conjugate and excrete phytochemicals, and iii) the effect of changes in gut microflora have on phytochemical absorption.

As was highlighted in the work presented here, it is not always possible to feed an animal a dietary phytochemical and obtain the same blood metabolites, in the same relative concentrations, as is seen in a human. It is easy to suggest that all \textit{in vitro} phytochemical experiments should be conducted using human blood metabolites and that animal experiments should use dietary interventions, which result in a blood metabolite profile that is representative of a human. The reality is that this would
require robust and comparable bioavailability data for both humans and the animal model being used, as well as information about the in vitro behaviour of the compound being tested. Additionally, as many dietary factors potentially affect the bioavailability of a compound, such as the food matrix it is presented in and the type of conjugates that are bound (Adam et al. 2002; Ke et al. 2008), it is not unheard of to have dramatically conflicting reports concerning bioavailability of a phytochemical (Azuma et al. 2000; Gonthier et al. 2003; Lafay et al. 2006b), potentially making determining the correct metabolite to use difficult. Although there are clear advantages to using phytochemical metabolites in in vitro assays, more data on the bioavailability of phytochemicals in humans and animals is needed before such a practice can become standard.

8.6 Future Experiments

The experiments described in this thesis have shown that in vitro screening using blood metabolites can be representative of in vivo effects and that the CDA metabolite 3,4-DHBA can induce endogenous antioxidant expression both in vitro and in vivo. To further the research presented here several directions for future experiments both in vitro and in vivo are discussed below.

The data presented in this thesis has shown that 3,4-DHBA can induce increases in endogenous antioxidant enzyme expression both in human cell lines and in Sprague Dawley rats. The logical progression from these experiments is to test for 3,4-DHBA mediated regulation of antioxidant enzyme activity in humans. Although this experiment might determine if 3,4-DHBA can regulate endogenous enzymes in humans several questions need to be considered before such an experiment is carried out.

1. Would a dietary amount of CDAs be expected to induce significant effects on endogenous antioxidant activity in humans?

2. Given the limitation in tissue sampling when testing compounds in humans, could sample tissues that are likely to be affected by CDA supplementation be taken?
3. Could 3,4-DHBA be used instead of CDAs?

Data presented in chapter 7 clearly shows that oral consumption of an amount of 3,4-DHBA that equates to a dietary serving of CDAs in humans, does not induce the expression of antioxidant enzymes in rats after a 2 week intervention. It is therefore unlikely that a similarly short intervention would induce significant changes in antioxidant enzyme expression in humans that consumed CDAs. Further, as the changes observed in the rat study were in liver and intestine, it would not be feasible to take biopsy samples of these organs from humans. It is possible that a longer term intervention may result in significant increases in endogenous antioxidant enzyme activity. Also, if the intervention is long enough in duration, it may be possible to see CDA-induced upregulation of antioxidant enzymes in red blood cells, which develop from bone marrow and circulate for up to 120 days before being replaced. If a human trial feeding with CDAs was to be undertaken, perhaps the best methodology would potentially be a 120 day intervention, and then include blood and muscle sampling.

Due to the differential bioavailability of CDAs in rats and humans, the dietary intervention used in chapter 7 included the direct feeding of the CDA metabolite, 3,4-DHBA, to rats in order to emulate the metabolic profile of human CDA consumption. It may be possible to directly feed 3,4-DHBA to humans in larger doses, as was done to the rats in chapter 7, and then measure antioxidant enzyme activity as described above. However, to date no bioavailability data for 3,4-DHBA in humans exists. Although it is likely that 3,4-DHBA would be highly bioavailable, before an intervention study is conducted in humans, a short term bioavailability study would need to be carried out to determine the exact pharmacokinetic of oral 3,4-DHBA in humans.

The data presented in chapter 5 showed that 3,4-DHBA induces endogenous antioxidant enzyme expression and that this expression is responsible for protection from H$_2$O$_2$-induced cell death in SH-SY5Y cells. Further, this chapter begins to examine the mechanisms by which these antioxidant enzymes may be upregulated by 3,4-DHBA treatment. As this thesis is being written, a report examining the
mechanisms of 3,4-DHBA-induced upregulation of antioxidant enzymes in a rat immune cell line has been published (Vari et al. 2010). The authors report that 3,4-DHBA induces upregulation of antioxidant enzymes via MAPKinase mediated activation of the ARE. These results agree with the finding of the preliminary examination of MAPKinase activation conducted in this thesis and suggest that this mechanism of activation may also be present in human tissue cell lines. An examination of MAPKinase mediated ARE activation in human tissues, could potentially be conducted to further both the work presented here and this recent publication.

In addition to the role of MAPKinase in the induction of antioxidant enzyme expression by 3,4-DHBA, chapter 5 examined the possible role oxidative stress may have. The results of chapter 5 suggest that 3,4-DHBA can induce intracellular oxidative stress in SH-SY5Y cells and that this stress can be partially blocked by the actions of exogenous catalase. To further our understanding of the exact role increased intracellular oxidative stress has during 3,4-DHBA-induced upregulation of endogenous antioxidant, a more complete investigation could be completed. The results presented in this thesis indicate that the most likely mechanisms for activation of endogenous antioxidant enzymes are via either:

i) Extracellular formation of H$_2$O$_2$, via the reaction of 3,4-DHBA with the cell culture media, which then diffuses into the cells and activates either oxidative-stress sensitive MAPKinase or directly affects NRF2 binding to the ARE.

ii) The intracellular generation of oxidative stress by 3,4-DHBA undergoing redox cycling and generating both H$_2$O$_2$ and semiquinone radicals of 3,4-DHBA that can both activate the ARE via reactions with sulfhydryl groups on NRF2.

iii) A combination of these 2 factors.

To determine the relative contribution of these mechanisms, an experiment could be conducted that used cells treated with 3,4-DHBA and then co-treated with either excess catalase, to prevent extracellular formation of H$_2$O$_2$, excess pegylated catalase,
to prevent the formation of excessive intracellular H$_2$O$_2$, or GSH, to react with any semiquinone radicals of 3,4-DHBA that form from redox cycling.

8.7 Final conclusion

The data presented here has shown that the CDA metabolite 3,4-DHBA can induce endogenous antioxidant enzyme expression both \textit{in vitro} and \textit{in vivo}. It suggests that the use of phytochemical blood metabolites, in \textit{in vitro} assays, may be better able to predict \textit{in vivo} outcomes than the use of non-bioavailable dietary phytochemicals. Finally, it has been demonstrated that cell culture conditions may be an important factor in experiments that evaluate oxidative stress \textit{in vitro} and should be considered when planning, conducting and reporting results.
Appendix 1: Calculation of Human Equivalent Dose (HED)

Estimations of HED in this thesis were done using the non-draft FDA guidelines 2007. These guidelines use a ‘conversion factor’ that attempts to accurately estimate the amount of drug required to have the same effectiveness in a human as in the animal model used. The conversion factor is based primarily on the surface area to volume ratio of the animals being compared, as this can be considered to be representative of the animal’s metabolic rate/capacity to metabolise the drug(s). The conversion factor has also been tested using historical data from previous animal and human experiments and has been found to be broadly accurate. The FDA approved HED calculations used in this thesis were discussed for several years, by both the industry and academia, before being ratified by the FDA in 2007 and represent a robust and internationally acceptable systems for estimating HED for clinical trials using drugs. It should be noted that the primary compound being tested in this thesis, 3,4-DHBA, is not a drug per se and it could be argued that it may not be appropriate to use these FDA guidelines for a dietary phytochemical. However, as many drugs are in fact either derived from phytochemical or are phytochemicals themselves and the outcomes measured, concentrations used and the administration of the compound in a pure form are all ‘drug-like’. The use of the FDA guidelines for the calculation of the HED should be a scientifically sound method and potentially better than the direct conversion based on mg/kg bodyweight.

Calculations:

FDA HED conversion factor Rat to Human = 6.2
FDA reference Human body weight = 60kgs
Conversion of rat dose in mg/kg to human dose in mg/kg = Rat dose (mg/kg) / Rat to human conversion factor = 100 / 6.2 = 16.13
100 mg/kg body weigh rat = 16.13 mg/kg body weight human
Equivalent amount for a 60 kg Human = 967.7 mg
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