QUERCETIN-3-GLUCOSIDE MEDIATES CYTOPROTECTION IN SH-SY5Y CELLS AGAINST H_2O_2 INDUCED OXIDATIVE STRESS BY INDUCING CHOLESTEROL BIOSYNTHESIS

by

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A few useful quotations

"Science may set limits to knowledge, but should not set limits to imagination."

Bertrand Russell (1872 - 1970)

"The difference between a successful person and others is not a lack of strength, not a lack of knowledge, but rather in a lack of will."

Vince Lombardi (1913 - 1970)

"There are no great limits to growth because there are no limits of human intelligence, imagination, and wonder."

Ronald Reagan (1911-2004)

"A man should look for what is, and not for what he thinks should be."

Albert Einstein (1879-1955)

Dedication

I dedicate this thesis to my paternal grandfather Dr. Francis Beverly Wishart (1908-1968), Graduate of Dalhousie Medical school class of 1936 and colleague of Sir Frederick Banting at the University of Toronto. Although we never met our lives intertwined with our love of science, medicine, sports and vacationing at Skiff Lake.

F. B. Wishart

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Abstract

Quercetin-3-glucoside (Q3G) is a natural product with strong antioxidant activity. In human neuroblastoma SH-SY5Y cells, H_2O_2 mediated oxidative stress leads to decreased cell viability, increased intracellular reactive oxygen species and cell death. Pretreatment with Q3G reverses these effects of oxidative stress. Although Q3G and related compounds are well documented cytoprotectants, the mechanism responsible for this beneficial effect is unclear. Gene expression profiling using cDNA microarrays was performed to document transcriptional changes resulting from exposure to either just Q3G or the effects of pretreatment with Q3G on the genomic response to oxidative stress. Only in cells pretreated with Q3G and then exposed to oxidative stress was there a large increase in the number of genes associated with cholesterol biosynthesis as well as an elevation of cholesterol levels. Mevastatin, an inhibitor of cholesterol biosynthesis, reversed both the elevation of cholesterol and cytoprotection produced by oxidative stress in cells pretreated with Q3G. These findings suggest that pre-incubation with Q3G increased resistance to the injurious effects of H_2O_2 by enabling SH-SY5Y cells to mount a protective response to oxidative stress by elevating cholesterol synthesis.

List of Abbreviations and Symbols Used

% - percentage

8-oxo-dG – 8-oxo-7,8-dihydrodeoxyguanosine

8-oxo-dGTP - 8-oxo-7,8-dihydrodeoxyguanosine triphosphate

ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

ATP – adenosine triphosphate

$CaCl_2$ – calcium chloride

cdk5 – cyclin dependant kinase 5

cDNA – complimentary deoxyribonucleic acid

CFCs – chlorofluorocarbons

CNS – central nervous system

CO_2 – carbon dioxide

Cy – cyanine fluorescent dye

DCFH-DA - dichlorofluorescin diacetate

DEPC – diethylpyrocarbonate

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - dimethylsulfoxide

DNA - deoxyribonucleic acid

dNTP – deoxynucleotides

DTT – dithiothreitol

EAE – experimental autoimmune encephalomyelitis

ELISA – enzyme-linked immunosorbent assay

EPO – erythropoietin

ER – endoplasmic reticulum

FBS – fetal bovine serum

FDA – food and drug administration

FDR – false discovery rate

g – gram

GSH - glutathione

H_2O_2 – hydrogen peroxide

HCl - hydrogen chloride

HDL – high density lipoprotein

HMG-CoA - 3-hydroxy-3-methylglutaryl CoA

INT - 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride

JNKK - mitogen-activated protein kinase kinase

LDH – lactate dehydrogenase

LDL – low density lipoprotein

MAP - mitogen-activating-protein

mg – milligram 10^{-3} g

mg/mL - milligram 10^{-3} g per millilitre 10^{-3} L

$MgCl_2$ – magnesium chloride

mM - millimolar 10^{-3} moles / litre

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N - normality

NaCl – sodium chloride

NaOH – sodium hydroxide

nm – nanometers 10^{-9} M

nNOS – neuronal nitrous oxide synthesase

NO – nitrous oxide

°C – degree centigrade

PBS – phosphate buffered saline

PC – positive control

pg – picogram 10^{-12} g

pH – negative log of hydrogen concentration

Q3G – quercetin-3-glucoside

RNA - ribonucleic acid

ROS – reactive oxygen species

SAM – significance analysis of microarrays

SCAP - SREBP cleavage-activating protein

SDS –sodium dodecyl sulfate

SEK1 – mitogen-activated protein kinase kinase mouse homolog

SEM – standard error of mean

SRE – sterol regulatory element

SREBP – sterol regulatory element binding protein

SSC – saline sodium citrate

tBHQ – tetra-butyl hydroquinone

U/mL – units / millilitre 10^{-3} L

UV - ultraviolet

x g – times gravity

μg/mL - microgram 10^{-6} g / millilitre 10^{-3} L

μL – microlitre 10^{-6} L

μM - micromolar 10^{-6} moles / litre

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To my parents and teachers I say thank you, after all I would not be writing my thesis if you had not taken the time to help me learn to read and write let alone build my interest in science.

And to Miss Maria Currie, I say thank you for your love and devotion through the writing of this thesis, although you're a scientist at heart, you know just when to tell me "That's enough about your experiments let's talk about something else!"

Chapter I: Introduction

Brief History Of Free Radical Research

Free radicals are a naturally occurring chemical species that are highly reactive oxidizing agents capable of causing wide range biochemical damage. These chemical species are formed by the hemolytic cleavage of a chemical bond between two unlike atoms producing an extremely volatile molecule or atom with an unpaired electron (Lehnert *et al.*, 2001). Significant amounts of energy, kilojoules / mole, are required to produce the hemolytic cleavage necessary to form a free radical (Lehnert *et al.*, 2001). Although free radical generation is a common biological phenomenon, the fleeting existence of free radicals has made them difficult to identify. In 1900, Moses Gomberg became the first chemist to correctly identify an organic free radical, triphenylmethyl radical (Tomioka *et al.*, 2001). Most types of free radicals are extremely volatile, but a few are remarkably stable. These species are stable due to π conjugation by phenolic rings (stable radical) and steric hindrance around the radical centre preventing reaction with other chemicals (persistent radical) (Nishinaga and Komatsu, 2005). Chemically, free radicals are well documented to cause polymerization by propagating chemical reactions that produce more free radicals with the addition of monomers into a growing polymeric chain (Motyakin *et al.*, 2006). Free radicals produced from chlorofluorocarbons (CFCs) by solar ultraviolet radiation destroy ozone in the atmosphere that leads to the depletion of the ozone layer (Maugh, 1984).

Biochemically, free radicals are important in combustion and intracellular signaling. Within the cell, free radicals are produced during mitochondrial respiration, where oxygen's double bond is enzymatically broken producing intermediate oxygen free radicals that ultimately form metabolic water as part of the Krebs cycle (Adam-Vizi, 2005). Nitric oxide, an important second messenger involved in intracellular signaling, is also a free radical though its half life is short lived (Engelhardt, 1999). Although free radicals play an important role within the cell, excessive concentrations result in a state known as oxidative stress that can compromise cellular integrity by oxidizing lipids (Festjens *et al.,* 2006). With oxidative stress, the acyl chains of phospholipids become more unsaturated thus introducing kinks into the chain that may lead to pore formation within the plasma membrane thus disrupting cellular integrity (Borunov *et al.,* 1986).

Pathophysiology Of Oxidative Stress

Oxidative stress is a term used to describe the physiological state that occurs when pro-oxidants such as free radicals and reactive oxygen species exceed the ability of antioxidant chemicals and proteins to neutralize them (Figure 1). Oxidative stress plays a role in a number of pathological states such as ischemic-reperfusion injury (Halestrap *et al.*, 1998) several neurodegenerative diseases (Beal, 1996) and sepsis (Gutteridge and Mitchell, 1999). Moreover, the generation of reactive oxygen species (ROS) may contribute to cellular changes associated with ageing (Lenaz, 1998). This is because our ability to scavenge free radicals decreases with age, causing oxidative stress that leads to tissue degeneration implicated in several chronic diseases such as

cataracts (Pendergrass *et al.*, 2006), diabetes and various types of neurological and cardiovascular disorders (Rice-Evans, 2004). Mitochondria represent the major source of intracellular ROS production. Even under resting conditions between 2 and 5% of molecular oxygen consumed by mitochondria is partially reduced by the electron transport chain to form superoxide and subsequently hydrogen peroxide (Powers *et al.*, 2004). Furthermore, mitochondrial ROS may inhibit one or more of the components of the respiratory chain, further accelerating the rate of superoxide formation (Turrens and Boveris, 1980). Pathological events triggered by mitochondrially-derived ROS are thought to include lipid peroxidation (Montine *et al.*, 2004), ion channel modification, DNA damage and protein oxidation as demonstrated in models where the effects of exogenous oxidizing agents have been studied (Gutteridge and Halliwell, 2000).

Mammalian cells, such as neurons, are highly dependent on aerobic metabolism to generate ATP and neurons are particularly susceptible to oxidative stress because of the high concentrations of oxygen required to supply the brain. While the brain constitutes only 2-3% of the body's total mass it consumes 20% of all the oxygen in the circulatory system (Halliwell, 2006). The high levels of oxygen present in the brain coupled with the presence of catalysts that generate free radicals such as free metals and excitotoxic amino acids can give rise to increased levels of ROS that may severely damage the brain (Halliwell, 2006). Moreover, the central nervous system (CNS) contains relatively low levels of antioxidant proteins and chemicals (Gilgun-Sherki *et al.*, 2004) by comparison to most other organs in the body. The high concentration of

mitochondria within the CNS further contributes to oxidative stress in the CNS as 2-5% of the total oxygen used by the mitochondria forms ROS (Powers *et al.*, 2004).

Oxidative Stress In Animal Models Of Neurodegenerative Disorders

Studies performed using genetically modified mice support a role for oxidative stress in neuronal cell death associated with neurodegenerative disorders such as stroke, Alzheimer's disease, multiple sclerosis and Parkinson's disease. For example, transgenic mice that overexpress the antioxidant protein superoxide dismutase display smaller brain infarcts than control mice after cerebral ischemia (Kamii *et al.*, 1996; Ying *et al.*, 2000). Additionally, it has been shown that superoxide dismutase knockout mice have increased brain infarct volumes compared to control mice after cerebral ischemia (Murakami *et al.*, 1998). Similarly genetic inactivation of the antioxidant proteins hemeoxygenase-2 and metallothionein in mice elevated neurotoxicity following transient focal neocortical ischemia resulting in greater brain damage in knockout mice compared to wild-type littermate controls (Namiranian *et al.*, 2005). Tg2576 mice, a murine model for Alzheimer's disease, have decreased levels of metallothionein 3, an immunomodulatory antioxidant protein as well as increased nNOS activity, an enzyme that generates neuronal nitric oxide (NO) (Martin *et al.*, 2006). Excessive production of NO has been linked to brain injury associated with a breakdown of the blood-brain barrier. Furthermore, it has been shown in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis that antioxidant proteins such as heme oxygenase-1 and metallothioneins are increased and play a role in the eventual resolution of EAE by limiting free radical damage (Chakrabarty *et al.*,

2003). These results not only implicate oxidative stress in neurodegenerative disorders, but also suggest that elevating concentrations of antioxidant proteins may be neuroprotective. The CNS has several free radical scavenging chemicals such as vitamin C (ascorbic acid), vitamin E (tocopherols & tototrienols), and coenzyme Q (Gilgun-Sherki *et al.*, 2004). Mice lacking the sodium-vitamin C cotransporter-2 die shortly after birth from brain hemorrhage as a result of an inability to control the increase in free radicals associated with birth related hyperoxia (Sotiriou *et al.*, 2002). Coenzyme Q is able to prevent the loss of substantia nigra neurons triggered by administration of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Ebadi *et al.*, 1996; Shults, 2003). Furthermore, high doses of the anti-oxidant α-tocopherol increase the cognitive ability of patients with Alzheimer's disease although the mechanism is not yet clear (Sano *et al.*, 1997; Grundman, 2000). Thus, altering concentrations of either antioxidant proteins or chemicals within the body leads to corresponding changes in antioxidant defense that render an organism either less or more resistant to the injurious effects of oxidative stress.

Cellular Defense To An Oxidative Insult

Cells have evolved a sophisticated defense system to cope with the oxidative stress caused by free radicals (Linford *et al.*, 2006). This defense system relies on maintaining modest levels of antioxidant proteins and chemicals, but also on the use of sets of repair enzymes to fix damaged proteins, DNA and lipids.

Reactive oxygen species (ROS) can oxidize DNA causing damage at both the base and sugar moiety. Damage to the base commonly leads to addition or loss of a hydrogen atom. ROS produce 8-oxo-7,8-dihydrodeoxyguanosine triphosphate (8-oxo-dGTP) by oxidizing guanine to 8-oxo-7,8-dihydro-2`-deoxyguanosine (8-oxo-dG) potentially resulting in an A·G mismatch (Sestili *et al.*, 1998). When ROS react with the sugar moiety it produces abasic sites, and both single and double strand breaks may occur. DNA repair endonucleases are responsible for replacing / repairing DNA bases at abasic sites after oxidative stress (Powell *et al.*, 2005). If the base is damaged, it is repaired; however, if more severe damage occurs the base is replaced. At the abasic site, endonucleases remove the backbone and ligate the strand back together (Blainey *et al.*, 2006). Antioxidant compounds are able to effectively protect DNA from oxidative damage and breakage by directly absorbing free radicals (Ahearne *et al.*, 1999; Ahearne *et al.*, 2000).

Oxidative injury to proteins usually results in damage to the amino acid methionine because it is the most sensitive amino acid to oxidative stress (Ding *et al.*, 2006). Oxidative stress alters the redox chemistry around the sulfur atom of methionine giving rise to methionine sulfoxide (Imlay, 2003). The repair enzyme methionine sulfoxide reductase is able to convert methionine sulfoxide back to methionine (Grimauld *et al*, 2001). Subcellular localization studies of methionine sulfoxide reductase indicate that this enzyme is associated with mitochondria, where the majority of cellular ROS are produced (Hansel *et al.*, 2002). Large oxidative insults may produce irreversible damage resulting in the oxidized protein being targeted for

elimination by proteasomal degradation (Davies, 2005). The 20 S proteasome is able to identify and degrade oxidized proteins (Shringarpure *et al.*, 2001), even in the absence of ubiquitin conjugation (Shringarpure *et al.*, 2003) while the 26 S proteasome is ineffective in degrading oxidized proteins, even in the presence of a functional ubiquitination system and ATP (Davies, 2001).

Because of their high degree of unsaturation and their stoichemetric abundance in membranes, lipids are at the highest risk for damage as a result of oxidative stress. Unlike DNA or protein damage, lipid peroxidation is a self-propagating event that will continue until the intermediate oxidative species is terminated (Davies, 2000). Lipid peroxidation leads to structural damage of membranes and produces oxidized products that may react with other membrane components resulting in further damage (Balestrieri *et al.*, 2003). Damaged lipids are cleaved from the membrane by phospholipase A_2 and destroyed (Halliwell, 2006). Although no lipid-specific cellular defense system has been identified, numerous studies have shown that antioxidants, such as flavonols (Rodrigeuz *et al.*, 2001; Gavino *et al.*, 1981) and strictinin (Zhou *et al.*, 2004) are able to prevent lipid peroxidation and oxidation of low density lipoprotein (Hou *et al.*, 2004; Jeong *et al.*, 2005) by directly scavenging free radicals.

Antioxidant Chemicals & Nutritional Antioxidants

Antioxidant chemicals ranging in complexity from small ions to structurally large carbon-containing compounds can be found in various foods and beverages. Since antioxidants are found in many plant species, they are consumed within the human diet. The major role of antioxidant chemicals is to directly scavenge free

radicals before oxidative damage is done to the cell. Vitamins A, C and E are all well known water soluble antioxidants found in a variety of foods. Vitamin cofactors such as coenzyme Q10 and selenium have been shown to prevent oxidative damage produced by elemental zinc and manganese (Virmani *et al.*, 2005). Increasing levels of free radical scavenging compounds within cells has been shown to be protective against oxidative damage and may be a useful therapeutic strategy (Joshi *et al.*, 2004). Among the most potent free radical scavengers identified to date are a class of large polyphenolic compounds known as flavonoids that are found in wine, berries and teas (Rice-Evans and Spencer, 2004).

Flavonoids As Cytoprotective Agents

Flavonoids are naturally occurring phytochemicals produced as secondary metabolites by many plant species that provide the plant with pigmentation and protect against microbes and insects (Walgren *et al.*, 1998). The polyphenolic backbone characteristic of this chemical family consists of 15 carbon atoms arranged into 3 phenolic rings (Figure 2). The degree of unsaturation and substitution of various side groups of the backbone gives rise to the subfamilies and the biological activities of these compounds. The major dietary sources of flavonoids in the Western diet are fruits, vegetables and black tea; yielding about 1 gram (g) per day of flavonoids (Spencer *et al.*, 2004). Quercetin is the most abundantly consumed flavonoid in the diet reaching levels of 30-40 mg per day (Spencer *et al.*, 2004). The therapeutic properties of flavonoids are extensive with cardioprotective, neuroprotective, anti-inflammatory and chemoprotective properties having been attributed to various types of flavonoids. One theory to explain the diverse biological activities of flavonoids is that during their

synthesis they have developed a chemical structure that interacts with a specific fold or shape found on many proteins (Breinbauer *et al.*, 2002). This is analogous to a lock and key: where a specific protein fold in the biosynthetic enzyme (lock) forms the flavonoid chemical structure (key) and then the flavonoid (key) is able to interact with proteins that have this same fold (similar lock) post synthesis. This ability of flavonoids to bind many different types of proteins in a precise manner suggests that natural products may have evolved to bind to proteins (Breinbauer *et al*, 2002).

The free radical scavenging abilities of flavonoids are well documented, with a catechol group on the B ring increasing the antioxidant potency (Mandel and Youdim, 2004). On a mole for mole basis, flavonoids are more potent free radical scavengers than ascorbic acid (vitamin C) and coenzyme Q (Mandel and Youdim, 2004). Flavonoids also have the ability to inhibit a variety of kinases including cyclin dependent kinases (cdks) (Zapata-Torres *et al.*, 2004), protein kinase B, protein tyrosine kinases, MAP kinase kinase and SEK1 (JNKK) (Williams *et al.*, 2004). Flavopiridol shares the same chemical skeleton as flavonoids and is a potent cdk5 inhibitor in phase III clinical trials for the treatment of human neoplasms (Senderowicz, 2003).

Epidemiologically, studies have linked consumption of a single glass of red wine daily with physiological changes thought to be associated with a reduced risk of cardiovascular disease such as increased circulating high density lipoproteins (HDL), reduced cholesterol (Racek *et al.*, 2004), elevated antioxidant glutathione containing proteins and a decrease in oxidation protein products (Racek *et al.*, 2004). The French

paradox supports these findings as France shows a decreased risk of ischemic and cardiovascular diseases compared to industrialized nations in the West (Constant, 1997; Renaud, 1994). A large study that followed 34, 000 middle aged men in France found that consumption of 48 g of alcohol per day, mainly wine, decreases mortality from cardiovascular diseases by 30% establishing a paradox that consumption of wine may actually be good for you (Klatsky, 2002). Some reports suggest that this phenomenon results from elevated levels of resveratrol, a phytochemical found in red wine that is able to activate the estrogen response element, an event that has been linked to the cardioprotective effects of estrogen (Kopp, 1998).

Research Design

SH-SY5Y cells, a human neuroblastoma cell line, have been used in many *in vitro* models of neurodegenerative disorders such as Parkinson's disease (Sheehan *et al.,* 1997), Alzheimer's disease (Li *et al.,* 1996) and stroke (McCarthy *et al.,* 2004). SH-SY5Y cells are a commonly used *in vitro* model for studying the effects of oxidative stress on neuronal survival (Macleod *et al.,* 2001). These cells have low levels of antioxidants and are highly sensitive to oxidative stress (Andoh *et al.,* 2002). SH-SY5Y cells can be differentiated into neurons by addition of retinoic acid (Lopez-Carballo *et al.,* 2002) and differentiated into astrocytes by growing them in co-culture (Yu and Zuo, 1997). As SH-SY5Y cells contain biochemical and morphological characteristics of both astrocytes and neurons they can be used to model defense responses present in both cell types (O'Neill *et al.,* 1994).

Hydrogen peroxide (H_2O_2) will be used in the present study as a model pro-oxidant to induce oxidative stress in the SH-SY5Y cells (Cullen *et al.*, 1997) as this model offers an advantage over iron induced oxidative stress because it removes the possibility of chelating agents preventing the induction of oxidative stress (Zaman *et al.*, 1999). Oxidative stress harms crucial biomolecules due to excess generation of reactive oxygen species and has been shown to lead to cell death (Tsugunobu *et al.*, 2002). The H_2O_2 induced oxidative stress model is well characterized in SH-SY5Y cells (Tsugunobu *et al.*, 2002) and data from our laboratory show a 500 μM insult for 15 minutes produces a kill suitable for studying cytoprotective effects of various putitaive neuroprotectants.

Although several research groups have reported that the bioactive compound quercetin is cytoprotective against H_2O_2 induced oxidative stress, the exact mechanisms by which it prevents loss of cell viability are not well understood. Here it is demonstrated that a glycosylated derivative of quercetin, Q3G is cytoprotective against H_2O_2 induced oxidative stress by examining the effect of this compound on loss of cellular viability, cell death and generation of intracellular reactive oxygen species in SH-SY5Y cells. Comparison to a several compounds that exert a protective effect will be done to gauge the protective capabilities of Q3G. Tetra-butyl hydroquinone (tBHQ) is a potent inducer of the transcription factor Nrf2 and leads to an induction of cellular antioxidant proteins (Hara *et al.*, 2003). Vitamin C is a well known antioxidant and free radical scavenger (Lovat *et al.*, 2003) and erythropoietin is a potent neuroprotectant with strong antiapoptotic effects on SH-SY5Y cells (Um and Lodish, 2006). After

confirming the cytoprotective effect of Q3G a human 14K cDNA microarray chip was used to elucidate the protective actions of Q3G by examining the changes in gene expression that occur after either pretreatment with Q3G or Q3G pretreatment followed by exposure to H_2O_2 induced oxidative stress. These studies suggested pretreatment with Q3G primes SH-SH5Y cells in such a way that they were able to mount a protective response to oxidative stress characterized by elevated cholesterol synthesis. This finding was confirmed by measuring cholesterol levels and using an inhibitor of cholesterol synthesis, mevastatin (Bi *et al.,* 2004), to establish the role of this membrane component (cholesterol) in mediating the cytoprotective effects of Q3G.

Chapter II: Materials and Methods

Cells And General Reagents

Cell Culture

SH-SY5Y cells, a *Homo sapien* neuroblastoma cell line, that is a subline of the parental neuroblastoma cell line SK-N-SH, were obtained from the American Tissue Culture Collection (ATCC). Cells were maintained at 37°C in 95% humidity with 5% CO_2 and grown in Dulbecos Modified Eagle's Medium (DMEM, Hyclone) supplemented with 10% FBS (Hyclone), 1 mM sodium pyruvate, 2 mM L-glutamine and the antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were seeded at a density of 5×10^5 cells/ml in T-75 flasks (Corning) containing 20 mL and passaged when 80% confluency was reached. All cells were within 15 passages and in the logarithmic growth phase. The doubling time for these cells is approximately 48 hours (ATCC).

General reagents

All chemicals and reagents were obtained from Sigma-Aldrich and was of molecular biology grade unless otherwise indicated. All plasticware was obtained from Corning and was of tissue culture grade unless otherwise indicated.

Determination Of The Cytoprotective Activity Of Q3G:

Identification Of Protective Concentration Of Q3G Using The MTT Cell Viability Assay

Cell viability was measured using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay for cell viability. Cells were plated in 96 well plates at a density of 1 X 10^4 cells per well (100 µL). After 24 hours, 1 µM, 10 µM or 100 µM Q3G (dissolved in 0.05% DMSO) was added to the cells for 24 hours. The media was changed the following day and the cells were washed with PBS prior to being subjected to a 500 µM H_2O_2 insult for 15 minutes. After the conditioned culture media was removed, MTT at a final concentration of 0.5 mg/mL was added to the cells and incubated at 37°C for 4 hours. Solubilizing buffer (0.1 N HCl and 10% SDS) was added to all the wells and incubated at 37°C for 1 hour to dissolve the formazan particles that were quantified using an $ELx800_{uv}$ microplate reader (Bio-tek instrument Inc.) at 562 nM. Blank wells containing just media alone were subtracted from the readings and the results presented as a percentage of control cell viability (those receiving vehicle treatment [0.05% DMSO] and no H_2O_2 insult).

Identification Of Protective Compounds Using Cell Death ELISA

Cell death ELISA kit (Roche) measures the generation of nucleosomes produced by the cleavage of nuclear DNA between adjacent nucleosomes (DNA and histone proteins) that are associated with cell death. On day one, SH-SY5Y cells were seeded in a 24 well plate at a density of 5 X 10^4 cells per well (500 µL). On day two, Q3G 10 µM, erythropoietin (EPO) 10 pg, or tetra-butyl hydroquinone (tBHQ) 5µM

was added to different cell cultures and incubated overnight. On day three, the cells were washed with PBS to remove the compounds prior to the H_2O_2 insult (500 μM for 15 minutes). Next, the cells were washed with PBS and allowed to recover in normal media for 24 hours. The cells were then counted and normalized before being lysed in 500 μl incubation buffer (Roche) for 30 minutes at room temperature (25°C). ELISA plates were prepared by the addition of 100 μl of 1X coating solution (containing monoclonal anti-histone antibody) to each well and incubated for 1 hour at room temperature. Cell lysates were centrifuged at 20,000 g at 4°C for 10 minutes. Then 500 μl of hypertonic solution (10 M Tris, pH 7.4, 5 mM $CaCl_2$, 400 mM NaCl and 10 mM $MgCl_2$) that produces massive DNA fragmentation and nucleosome generation was added to SH-SY5Y cells and these lysates were used as a positive control, while lysates from cells receiving no pretreatment and no insult were used as a negative control. Next 100 μl of cell lysates, approximately 10^4 cell equivalents per ml, were added to each well and incubated for at room temperature 1.5 hours. After washing the wells three times with wash buffer, 100 μl of 1:10 peroxidase conjugated monoclonal anti-DNA antibody was added to the wells and incubated at room temperature for 1.5 hours. Following the antibody incubation the wells were washed and 100 μl (1 mg/ml) of detection reagent, 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate$_{(6)}$] (ABTS), was added for 15 minutes at room temperature. Absorbance was measured at 405 nm using an $ELx800_{uv}$ microplate reader (Bio-tek instrument Inc.) with the substrate solution (ABTS) as a blank. Data were expressed as enrichment factor defined as the ratio of absorbance of the dead / dying cells to that of the control non-insulted cells.

Determination Of Intracellular Reactive Oxygen Species (ROS)

Generation of intracellular ROS was measured using a substrate, 5-(and-6)-carboxy-2,7'-dichlorodihydrofluorescein diacetate, DCFH-DA (10 μM, Molecular Probes) that is oxidized to a fluorescent product in the presence of ROS. SH-SY5Y cells were seeded at a density of 5×10^5 cells per well in a 24 well plate (500 μL) in DMEM phenol red free media supplemented with 10% FBS, 1 mM sodium pyruvate and 2 mM L-glutamine. Cells were incubated with quercetin-3-glucoside (Q3G), tetra-butyl hydroquinone (tBHQ), Vitamin C (ascorbic acid) at 5 μM and 10 μM or corresponding vehicles for 24 hours (0.05% DMSO for Q3G, 0.005% DMSO for tBHQ and vitamin C). The cells were washed with PBS to completely remove compounds. Cells then were exposed to (500 μM) H_2O_2 and 0.11 mg/ml horseradish peroxidase (dissolved in PBS) for 15 minutes. The cells were then washed and maintained in standard growth medium. DCFH-DA, (10 μM, dissolved in 0.005% DMSO) was added immediately to the cells for 15 minutes at 37°C. Cells were then washed twice with PBS to completely remove DCFH-DA. Next, the cells were lysed by addition of 10 mM Tris-HCl (pH 7.2) buffer supplemented with 0.5% Tween 20. Lysates were centrifuged at 10,000 x g for 10 minutes and placed in an opaque 96 well plate (Costar). Fluorescence was measured using a microplate fluorescence reader, Flx 800 (Bio-tek instruments Inc.) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm using culture medium as the blank. ROS was expressed as fold induction compared to non-treated cells that did not receive an oxidative insult.

Determination Of The Putative Mechanism(S) Responsible For The Protective Effects Of Q3G:

cDNA Microarrays And Analysis

Isolation Of Total RNA.

This study compared alterations in gene expression between (A) SH-SY5Y cells pretreated with either 0.05% DMSO (solvent used to dissolve Q3G) or 10 μM Q3G for 6 hours and (B) SH-SY5Y cells pretreated with either vehicle or 10 μM Q3G for 6 hours then washed to remove the vehicle or Q3G followed by exposure to a 500 μM H_2O_2 insult for 15 minutes. After washing to remove H_2O_2, cells were allowed to recover in normal media for 6 hours. In both experiments A and B, SH-SY5Y cells were plated at a density of 5 X 10^5 cells/mL in a 6 well plate (3 mL) and maintained in an incubator at 37°C with 5% CO_2. At the conclusion of experiments A and B, total RNA was extracted using RNeasy mini kit (Qiagen) (Figure 3). mRNA (> 200 bases) was enriched for by using a silica membrane in the spin cup. Cells were trypsinized and centrifuged at 300 x g for 5 minutes. The supernatant was removed and 350 μL of lysis buffer RLT (containing guanidine thiocyanate and 1% β-mercaptoethanol) was added, the cells were then mixed by vortexing and homogenized. Next 350 μL of ethanol was added to the lysate, then mixed and placed in a mini column with a collection tube. The mixture was centrifuged at 8,000 x g for 15 seconds. Then 700 μL of wash buffer, RW1, was added to each column and centrifuged at 8,000 x g. After that 500 μL of purification buffer, RPE, was added to each column and centrifuged at 8,000 x g, this was done twice. RNA was eluted by adding 50 μL of RNase free H_2O onto the silica membrane and centrifuged at 8,000 x g for 1 minute. The quantity of

total RNA was determined by UV spectrophotometer and quality was assessed by the A260/A280 ratio, with all samples used for microarray analysis having ratio values in excess of 1.9. To ensure RNA integrity, RNA samples were run on a 1% formaldehyde agarose gel and DNAaseI (Qiagen) was added to the samples to degrade genomic DNA prior to microarray experiments (Gilbert *et al.*, 2003). This RNA was then exported to the University of Calgary for subsequent cDNA synthesis & purification, chip hybridization and scanning.

cDNA Synthesis & Purification

The remaining cDNA microarray experiments were carried out by the Southern Alberta Microarray Facility (SAMF, http://microarray.myweb.med.ucalgary.ca/SAMF_Home.html) at the University of Calgary. H14K 70mer oligo genomic DNA arrays (spotted cDNA) were generated in house by the Southern Alberta Microarray Facility. A fairplay microarray labeling kit (Stratagene) was used to synthesize and label cDNAs. Subsequently, 1 μg of 500 ng/μl $d(T)_{12-18}$ was added to10 μg of resuspended RNA incubated at 70°C for 10 minutes and then cooled on ice. A reaction mixture containing 2 μl of 10X StrataScript reaction buffer, 1.5 μl of 0.1M DTT, 1 μl of 20X dNTP mix (containing amino allyl-dUTP) and 0.5 μl of RNase Block (40 U/μl) was made and the RNA containing the annealed primer added. cDNA synthesis was carried out by adding 1 μl of 50 U/μl of StrataScript RT and incubating for 25 minutes at 48°C. To ensure sufficient cDNA synthesis, another 1 μl of 50 U/μl of StrataScript RT was added and further incubated at 48°C for 35 minutes. After this incubation 10 μl of 1M of NaOH was added at 70°C for

10 minutes to hydrolyze the RNA. The reaction was cooled to room temperature and after a brief spin, 10 µl of 1M of HCl was added to neutralize the solution. cDNAs were purified by addition of 4 µl of 3M sodium acetate (pH 4.5), 1 µl of 20mg/ml glycogen and 100 µl ice-cold ethanol and incubating at -20°C overnight. The solution was then centrifuged at 13,000x g for 15 minutes and the supernatant removed. The pellet was washed with 0.5 ml of ice-cold ethanol and spun at 14,000x g for 15 minutes at 4°C, supernatant was removed and the pellet air dried.

Dye Coupling Reaction, Purification And Hybridization

cDNAs were evenly labeled by incubating the pellet in with 5 µl of 2X coupling buffer at 37°C for 10 minutes and then adding 5 µl of Cy 3 or Cy 5 fluorescent dyes, mixing by pipetting up and down and incubating for 1 hour at room temperature in the dark. The fluorescent Cy dyes react with amino groups present on the amino allyl-dUTP effectively and evenly labeling the cDNA. The labeled cDNAs were purified by adding a wash solution containing 90 µl of DEPC H_2O to the cDNA and 100 µl of DNA binding solution and 100 µl of 70% ethanol then pipetting forcing the cDNA into the aqueous solution and contaminants into the organic solution. The solution was then transferred to a microspin cup with receptacle tube and centrifuged at 15,000x g for 30 seconds. The cDNAs were washed twice by the method described above and a second set of washings done by adding 750 µl of 75% ethanol to a microspin cup and centrifuging at 15,000 x g for 30 seconds, this was done twice. cDNAs were eluted from the columns by addition of 50 µl of 10 mM Tris base (pH 8.5) and incubating at room temperature for 5 minutes then centrifuged 15,000 x g for 30 seconds. To ensure

the maximum amount of cDNA was recovered the flow through was placed back into filter cup incubated at room temperature for 5 minutes then centrifuged 15,000 x g for 30 seconds. Samples were then placed into a vacuum centrifuge and the volume reduced to 3 µl. Hybridization solution consisted of 90 µl of DIG Easy Hyb (Roche), 5 µl yeast tRNA (Promega) and 5 µl salmon sperm DNA (Promega) heated at 65°C for 2 minutes and then cooled to room temperature before use. Then 70 µl of total volume (64 µl of hybridization solution and 6 µl of combined labeled cDNAs) were placed on a 14K microarray slide and the slides hybridized face to face for 18 hours at 37°C in a humidified chamber and washed three times the next day with saline-sodium citrate (SSC) buffer to remove any non-specific binding.

Microarray Analysis

For statistical analysis a balanced design was used for the experiments with each biological treatment completed in duplicate (Gilbert *et al.*, 2003). A dye swap method was used for each biological set to account for Cy3/Cy5 dye labeling bias. Both experiments A and B were performed in duplicate yielding two biological samples (total RNA) that were run in duplicate (dye swap) for each biological sample. Therefore, 4 determinations were used for calculating the changes in gene expression for each treatment condition (i.e. 2 microarrays/replicate (dye swap) × 2 replicates/group, N = 4).

A ScanArray 500 microarray scanner (Perkin-Elmer) was used to scan the microarray slides at 550 nm (Cy3) and 649 (Cy5). QuantArray 3.0 software (Perkin-Elmer) was used to capture and analyze images, saved as TIFF image files and then the

resultant data (QuantArray files and images) transferred to GeneTraffic Duo 2.5 microarray analysis/database package (http://Iobion.com). This data was then sent back to our laboratory for analysis and annotation. GeneTraffic was used for spot flagging, filtering, annotation and normalization of the data. After determining valid spots, Statistical Analysis of Microarrays, SAM, (http://www-stat.stanford.edu/tibs/SAM/index.html) was performed on elements using the following criteria: one class analysis, median centre arrays and 100 permutations. Multiple T-tests are performed by SAM to identify genes whose expression was significantly altered. In addition to the multiple T-tests, SAM calculates a false discovery rate (FDR) that is indicative of type I error allowing for differing degrees of confidence in the data obtained. In our procedure, a FDR < 8% was used to ensure a high degree of confidence that the genes we chose to further explore were actually altered before determining the genetic basis for the protective effects of Q3G.

Gene ontology and potential linkages between genes with significantly altered expression were examined using PathwayArchitect (Stratagene) (http://www.stratagene.com/products/displayProduct.aspx?pid=733), a software package that assists interpreting experimental results in the context of signal transduction pathways, gene regulation networks and protein interaction maps. Significantly upregulated and downregulated genes were transferred into PathwayStudio and interaction networks generated.

Confirmation Of Array Results Measuring Gene And Protein Expression: Cholesterol Assay

cDNA microarray experiments indicated alterations in pathways associated with cholesterol biosynthesis. These results were confirmed by measuring changes in cholesterol levels. Cholesterol levels were measured using a cholesterol assay kit (Biovision). SH-SY5Y cells were plated at a density of 5 X 10^5 cells per well in a 6 well plate (3 mL). Then 24 hours later 10 μM Q3G and vehicle were added to the media for 6 hours. Compounds were then removed and the cells washed with PBS before being subjected to a 500 μM of H_2O_2 for 15 minutes. The cells were allowed to recover for 6 hours and then removed by scraping the cells and 8.5 X 10^5 cells counted. Lipids were extracted by adding a 2:1 chloroform-methanol mixture to the cells and centrifuging at 10,000 x g for 10 minutes and dried overnight at room temperature (25°C). Dried lipids were then re-dissolved in 20 μl of 2-propanol containing 10% Triton X-100. Next 2 μl of sampxle was added to 48 μl of cholesterol reaction buffer and 50 μl of reaction mix (44 μl cholesterol reaction buffer, 2 μl cholesterol probe, 2 μl enzyme mix and 2 μl cholesterol esterase) were added to the sample. Samples were incubated at 37°C for 60 minutes protected from light. Absorbance was measured at 562 nm using an $ELx800_{uv}$ microplate reader (Bio-tek instrument Inc) using a cholesterol reaction buffer as a blank. Sample absorbance levels were compared to those generated by a standard curve and cholesterol levels determined by comparison to the standard curve.

Cholesterol Inhibition And Cell Viability

Cell viability was measured using an MTT assay for cell viability. Cells were plated in 96 well plates at a density of 1 X 10^4 cells per well. After 24 hours, 10 μM Q3G was added to the cells for 6 hours, the media was then changed and the cells were subjected to a 500 μM H_2O_2 insult for 15 minutes. After the H_2O_2 insult, 1 μM mevastatin (LKT laboratories) was added to the cells for 18 hours. After the conditioned culture media was removed, MTT at a final concentration of 0.5 mg/mL was added to the cells and incubated at 37°C for 4 hours. Solubilizing buffer (0.1N HCl and 10% SDS) was added to the cells at 37°C for 1 hour to dissolve the formazan particles that were quantified using an $ELx800_{uv}$ microplate reader (Bio-tek instrument Inc.) at 562 nM. Blank wells containing just media alone were subtracted from the readings and the results presented as a percentage of control cell viability (those receiving vehicle treatment and no H_2O_2 insult).

Membrane Integrity

Membrane integrity was assessed by measuring lactate dehydrogenase (LDH) release from the cell. Cells were plated in 96 well plates at a density of 1 X 10^4 cells per well in phenol red free DMEM supplemented with 5% FBS, 1 mM sodium pyruvate and 2 mM L-glutamine. After 24 hours, 10 μM Q3G was added to the cells for 6 hours, the media was then changed and the cells were subjected to a 500 μM H_2O_2 insult for 15 minutes. After the H_2O_2 insult 1 μM mevastatin was added to the cells for 18 hours. On day 2, 10X lysis buffer (formulation under patent) was added to the cells for 45 minutes to rupture the cell membrane (positive control). The plates were then

centrifuged at 250 x g for 4 minutes at room temperature and 50 µl of media was removed and placed into empty 96 well plates. Then 50 µl of substrate solution containing 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) salts and diphorase was added to the media, mixed, and incubated for 30 minutes at room temperature and protected from light. After incubation 50 µl of stop solution (formulation under patent) was added to the wells to halt further reaction. Absorbance was measured at 490 nm using an $ELx800_{uv}$ microplate reader (Bio-tek instrument Inc.). Background values (media only and non insulted cells) were subtracted from the readings and the results presented as a percentage of positive control LDH release (those receiving 10X lysis buffer for 45 minutes).

Statistical Analysis

Data are expressed as mean ± SEM from at least three independent experiments performed in quadruplicate (3 independent experiments * 4 determinations per experiment = 12 determinations). Statistical analysis was performed using GraphPad Prism software by one-way ANOVAs. If significant, group comparisons were performed using Tukey's test. Probability values were considered significant at $P < 0.05$.

Chapter III: Results

Q3G Pretreatment Reduces Loss Of Cell Viability After An Oxidative Insult

To determine the effects of increasing concentrations of Q3G on the viability of SH-SY5Y cells exposed to an oxidative insult, cells were incubated for 24 hr with 1 μM, 10 μM and 100 μM of Q3G. Cell viability was measured after the H_2O_2 insult using MTT, a compound that is broken down by the enzyme succinate dehydrogenase in viable mitochondrial to purple formazan particles. Exposure to H_2O_2 (500 μM, 15 minutes) resulted in decreases in viability ranging from 40%-60% with the mean being 50% relative to vehicle-treated cells. Pretreatment with 1 or 10 μM Q3G before H_2O_2 exposure increased viability by approximately 40% (1 μM) and 55% (10 μM) relative to cells treated with H_2O_2 alone (Figure 4). Treatment with 10 μM Q3G alone did not significantly alter cell viability relative to control cells that did not receive flavonoid and the oxidative insult. These results indicate that of 10 μM Q3G provided maximal protection from the loss of viability produced by the H_2O_2 insult and that Q3G was not cytotoxic nor did it alter the rate cell division.

Q3G Pretreatment Reduces Cell Death

To determine the cytoprotective effects of Q3G, a cell death ELISA was performed. Various reports have shown that flavonoids, most specifically quercetin have the ability to prevent loss of viability produced by an oxidative insult such as H_2O_2. Although the ability to prevent loss of viability is important, the cell is not necessarily dead and many repair mechanisms may be under way to halt cell death. Measuring nucleosomes that have been cleaved by proteases from cellular DNA are a

more definite measure of cell death. Q3G, tBHQ and EPO were added to SH-SY5Y cells for 24 hours, the next day conditioned culture media was removed and the cells subjected to 500 μM H_2O_2 for 15 minutes. Cells were lysed and the ELISA performed as described in the Methods. Pretreating SH-SY5Y cells with Q3G (10 μM) caused a significant reduction in cell death compared to the hypertonic treated (positive control, PC) and non-pretreated H_2O_2 insulted cells as measured by cell death ELISA (Figure 5). The level of cytoprotection from cell death achieved by Q3G is comparable to that of the well known neuroprotectant EPO.

Q3G Pretreatment Reduces Intracellular ROS

Quercetin is well documented to be a free radical scavenger, however, little is known about the free radical scavenging abilities of the glycosylated form of quercetin, Q3G. Q3G (5 μM and 10 μM), tBHQ (5 μM and 10 μM), a compound documented to induce antioxidant genes, and vitamin C (5 μM and 10 μM), another well documented free radical scavenger were added to SH-SY5Y cells for 24 hours. The next day the cells were washed and ROS production induced by the addition of H_2O_2 (500 μM) and horseradish peroxidase (0.11 mg/ml) for 15 minutes. Cells were lysed and intracellular ROS levels measured by DCFH-DA. Pretreatment with Q3G (5 μM and 10 μM) significantly reduced the induction of free radicals as did tBHQ (5 μM) while vitamin C (5 μM and 10 μM) had no effect (Figure 6). Pretreatment with 5μM of Q3G almost returned intracellular ROS levels to those of cells not exposed to the oxidative stress. Vehicle treated samples reduced intracellular ROS levels, as DMSO is able to chemically react with free radicals, though the reduction was not statistically significant. Since Q3G produced a greater decrease in intracellular ROS levels than did

vitamin C and tBHQ, we reasoned that other factors are responsible for the ability of Q3G to reduce hydrogen peroxide-induced cell death. To obtain some insight into the processes that might mediate Q3G-induced cytoprotection, we conducted microarray experiments to examine changes in gene expression produced by Q3G alone or pretreatment with Q3G followed by exposure to oxidative stress.

Q3G Treatment Alone Does Not Alter Gene Expression

Microarray experiments were conducted by exposing SH-SY5Y cells to 10 μM of Q3G or equivalent vehicle (0.05% DMSO) for 6 hours. The 6 hour time point was chosen because we hypothesized that any changes in gene expression resulting in the protective nature of this compound would take place soon after uptake of the compound, estimated to be 6 hours after addition to the media. RNA was extracted using the RNeasy mini kit and mRNAs reverse transcribed to cDNAs labeled using fluorescent Cy 5 and Cy 3 dyes. Treatment with Q3G alone did not alter gene expression in SH-SH5Y cells compared to those treated with vehicle (Figure 7). Only the expression of one gene, histone 2B was significantly altered with a standard error of 20.6%.

Q3G Pretreatment Alters The Genomic Response To Oxidative Stress

Another microarray experiment was conducted to compliment the Q3G or vehicle alone array by adding an oxidative insult (500 μM H_2O_2, 15 minutes) to cells pretreated with Q3G or vehicle. This microarray experiment yielded 28 candidate genes with altered gene expression, 25 genes were upregulated and 3 down regulated (Figure 8). Many of the genes with elevated expression (Table 1) were functionally

categorized (Figure 9) as belonging to a family that serves a critical role in cholesterol biosynthesis. Putative functional linkages between the significantly altered genes were identified using a program called Pathway Architect (Figure 10). Since 16 out of the 25 (62%) significantly upregulated genes are involved in cholesterol metabolism or lipid metabolism, we hypothesized that the protective effects of Q3G may be mediated by cholesterol biosynthesis thus preventing or repairing damage due to lipid peroxidation.

Q3G Pretreatment Results In Elevated Cholesterol Biosynthesis After Oxidative Stress

Since many of the upregulated genes are involved in cholesterol biosynthesis, a cholesterol assay was performed. SH-SY5Y cells were treated with 10 μM Q3G, vehicle (0.05% DMSO) or control (no treatment) for 6 hours, then exposed to 500 μM H_2O_2 for 15 minutes. After the oxidative insult the cells were allowed to recover for 6 hours and lysed and the assay performed as described in the Methods. Absorbance values were compared to those of a standard curve and cellular cholesterol levels determined (Figure 11). Cells treated with vehicle and then exposed to oxidative stress did not show an increase in cholesterol biosynthesis over control cells exposed to oxidative stress (Figure 12). By contrast, cells pretreated with Q3G and then exposed oxidative stress showed a significant increase of cholesterol levels, approximately 35%, over vehicle treated cells exposed to the oxidative insult (Figure 12). These findings were consistent with the microarray results indicating elevated cholesterol biosynthetic capacity in cells pretreated with Q3G and subjected to oxidative stress.

The Effects Of Cholesterol Biosynthesis On Cell Viability

Having demonstrated that cholesterol biosynthesis was increased in cells pretreated with Q3G and exposed to oxidative stress, next the role of elevated cholesterol biosynthesis on the ability of Q3G to reduce cell loss produced by oxidative stress was determined. Cells were treated with Q3G (10 μM) for 6 hours, exposed to H_2O_2 (500 μM) for 15 minutes and then a 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitor mevastatin (1 μM) added as described in the Methods. Pretreatment with Q3G followed by exposure to H_2O_2 decreased the loss of cell viability produced by H_2O_2 by about 25%, (Figure 13). This protective effect of Q3G was reversed by addition of mevastatin after the H_2O_2 insult (Figure 13). Mevastatin at the concentration used (1 μM) was did not alter cell viability after the H_2O_2 insult, nor did it alter the viability of cells when added on its own (Figure 13).

Q3G Pretreatment Prevents Loss Of Membrane Integrity Due To H_2O_2 Insult

After confirming that cholesterol biosynthesis was essential for the ability of Q3G to prevent the loss of cell viability triggered by oxidative stress, we sought to determine the effects of this process on membrane integrity as assessed by release of the intracellular enzyme LDH. Since cholesterol is a major constituent of the plasma membrane and contributes to membrane integrity, elevated cholesterol biosynthesis post insult may protect against oxidative stress-induced cell death. Cells were treated with Q3G (10 μM), exposed to H_2O_2 (500 μM) for 15 minutes and an HMG-CoA reductase inhibitor mevastatin (1 μM) added as described in the Methods. Pretreatment with Q3G reduced the increase in LDH release produced by H_2O_2, by approximately

66% relative to those cells exposed to H_2O_2 alone (Figure 14). Addition of mevastatin partially reversed the protective effects of Q3G pretreatment. Mevastatin at the concentration of 1 μM after H_2O_2 insult resulted in a substantial loss of membrane integrity (35%) compared to the positive control (lysis buffer), while mevastatin alone did not result in a substantial loss of membrane integrity (Figure 14).

Figure 1: A schematic diagram showing the causes and effects of free radicals. Various sources such as inflammation, the electron transport chain, toxicity, ischemia, X-rays and air pollutants can generate free radicals that alter the redox balance within a cell resulting in oxidative stress. Oxidative stress leads to undesirable events such as lipid peroxidation, protein modification, and DNA damage that can ultimately trigger cell death.

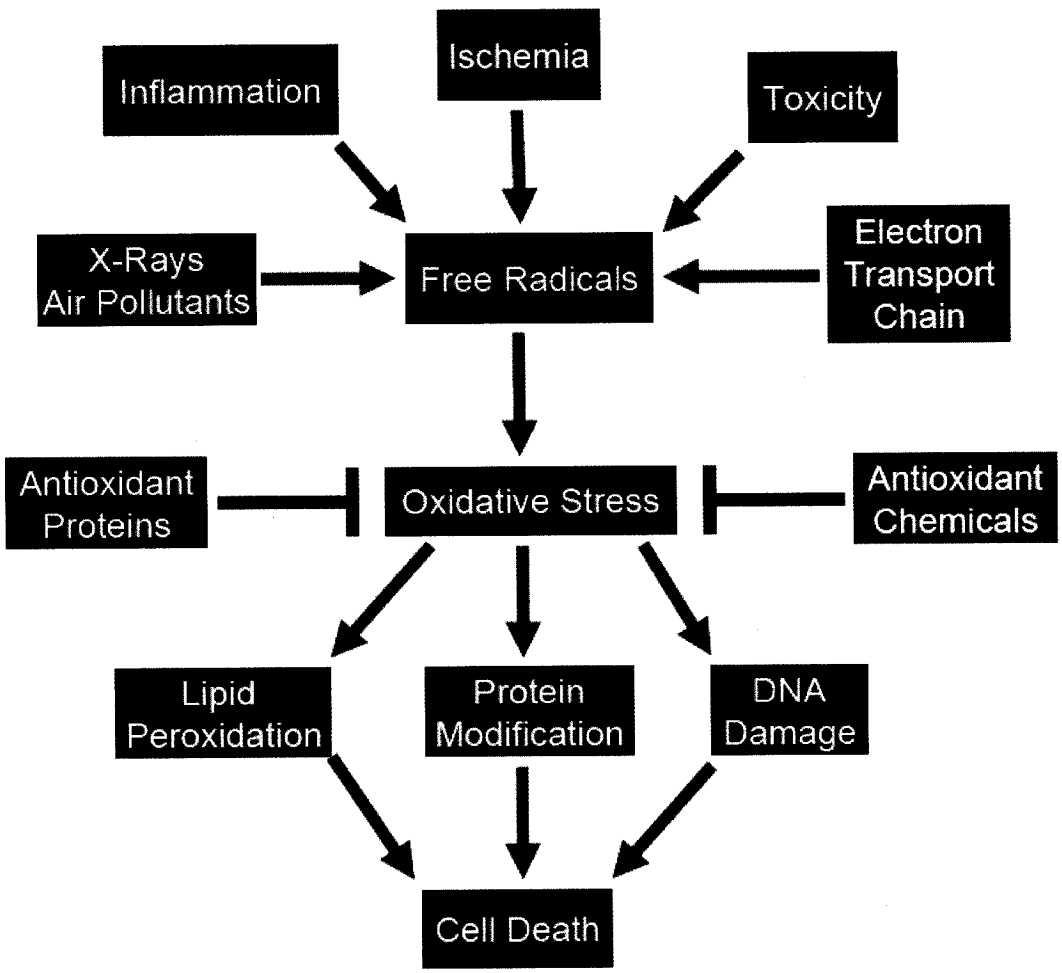
Inflammation
Ischemia
Toxicity
X-Rays
Air Pollutants
Free Radicals
Electron
Transport
Chain
Antioxidant
Proteins
Oxidative Stress
Antioxidant
Chemicals
Lipid
Peroxidation
Protein
Modification
DNA
Damage
Cell Death

Figure 1

Figure 2: The 15 carbon polyphenolic backbone characteristic of the flavonoid family is composed of 3 rings: A, B, and C. Alterations in the degree of saturation of the C ring and side group substitution of these rings gives rise to the various subfamilies and determines the biological activity of these compounds. Quercetin-3-glucoside: R6, R8, R3' = H, R7, R4', R5' = OH, R3 = glucose ($C_6H_{12}O_6$)

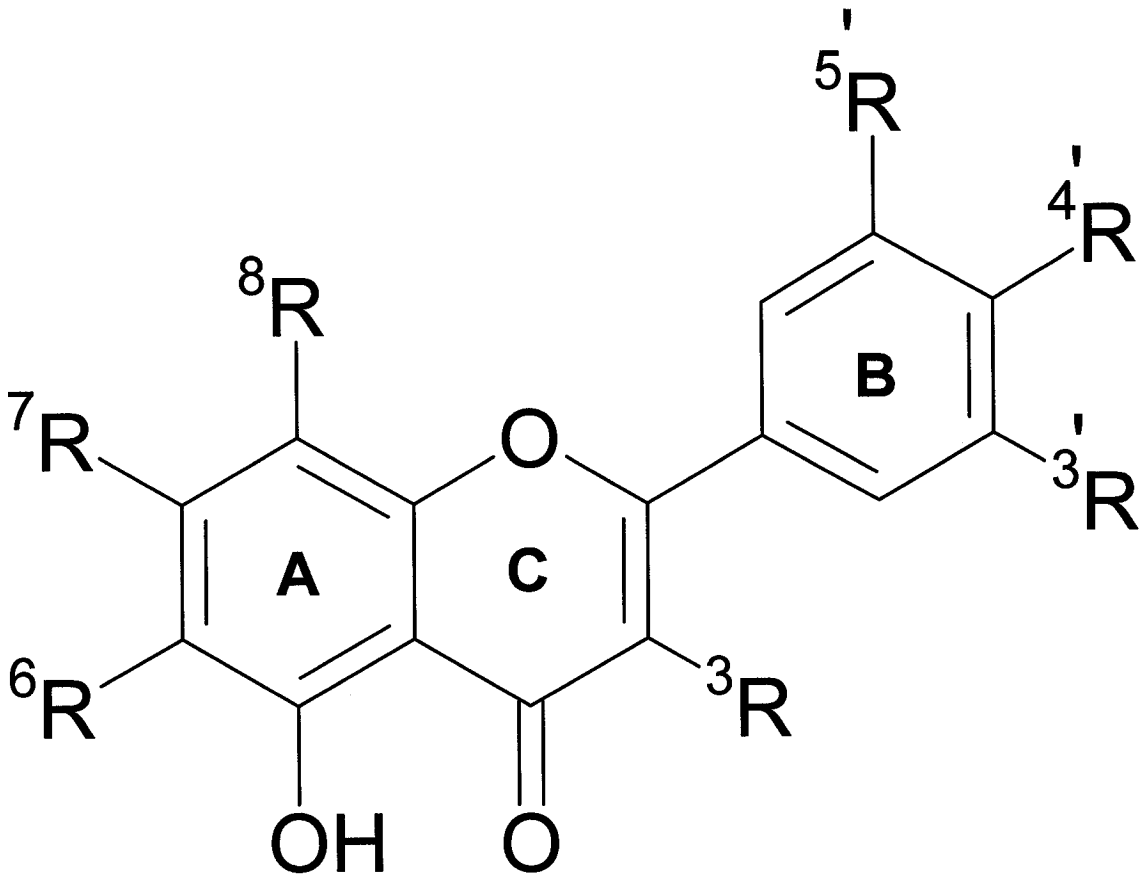

Figure 2

Figure 3: A schematic representation of the microarray protocol. A) SH-SY5Y cells were plated at a density of 5 X 10^5 cells / mL in a 6 well plate and 10 μM of Q3G or an equivalent vehicle (0.05% DMSO) for 6 hours. B) Compounds were removed and the cells were washed with PBS and total RNA extracted using an RNeasy mini kit. C) Fluorescent Cy labeled cDNAs were then synthesized using reverse transcriptase and Cy 3 and Cy 5 dyes coupled to amino allyl-dUTPs. To control for effects related to dye labeling bias, dye swapping was employed. D) 14K human microarray chips contained 13,972 spots of cDNA respectively synthesized by the University of Calgary Microarray Facility. E) Equal amounts of cDNAs derived from total RNA from each treatment group were hybridized to the chips for 18 hours and then the chips were washed. F) Chips were scanned using a ScanArray 500 microarray scanner. G) Data analysis was performed using QuantArray 3.0 and Gene Traffic Duo 2.5 software.

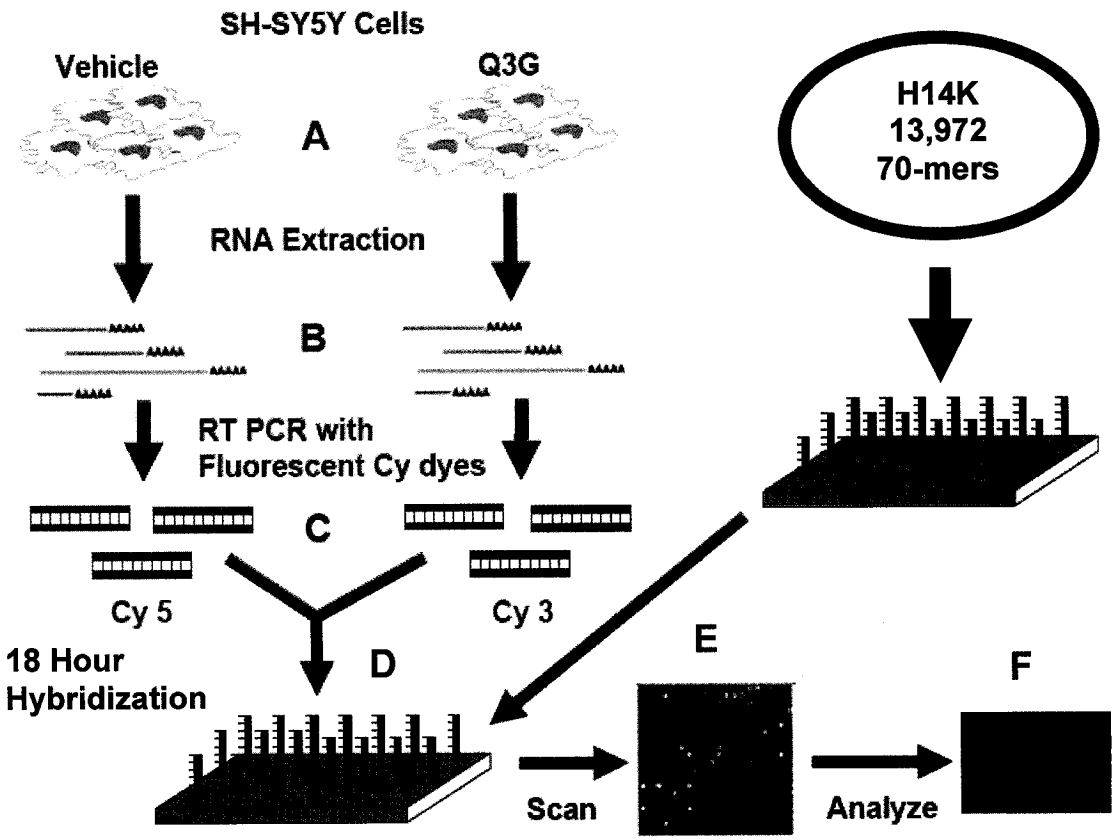
SH-SY5Y Cells
Vehicle
Q3G
A
H14K
13,972
70-mers
RNA Extraction
B
RT PCR with
Fluorescent Cy dyes
C
Cy 5
Cy 3
18 Hour
Hybridization
D
E
F
Scan
Analyze

Figure 3

Figure 4: A MTT assay measuring cell viability after H_2O_2 insult in SH-SY5Y cells. SH-SY5Y cells were pretreated with vehicle (control), 1 μM, 10 μM, or 100 μM of Q3G for 24 hours. After the incubation the conditioned culture media was removed and replaced with fresh media. The cells were then exposed to 500 μM H_2O_2 for 15 minutes. The next day cell viability was assessed by a MTT assay. Data are expressed as % viability compared to control cells receiving no Q3G and no insult. Each bar is representative of the mean ± S.E.M. of 20 determinations performed over 3 independent experiments. P*<0.001(1 way ANOVA, Tukey post hoc test) versus H_2O_2 treated cells. Cells receiving just 10 μM Q3G showed no significant changes in viability, thus 10 μM Q3G for 24 hours is not cytotoxic, nor did it alter cell proliferation.

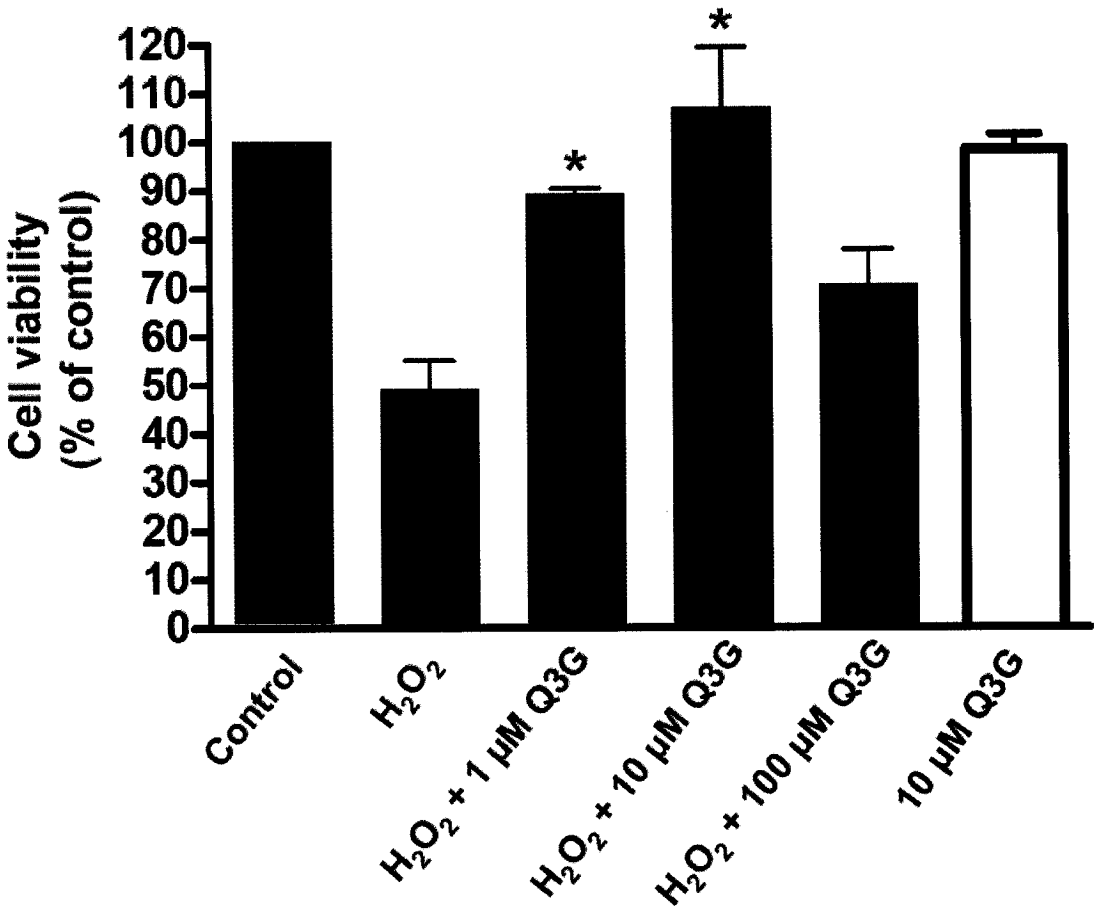
Cell viability
(% of control)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
*
Control
H_2O_2
H_2O_2 + 1 µM Q3G
H_2O_2 + 10 µM Q3G
H_2O_2 + 100 µM Q3G
10 µM Q3G

Figure 4

Figure 5: A cell death ELISA measuring DNA fragmentation in SH-SY5Y cells. SH-SY5Y cells were pretreated with either 10 μM Q3G, or 5 μM tBHQ, or 10 pg EPO for 24 hours. After the incubation the cells were washed with PBS and exposed to 500 μM H_2O_2 for 15 minutes. After 24 hours, cells were lysed and the ELISA performed as described in the Methods. A hypertonic solution was used as a positive control (PC) and all are data represented as fold increase over control cells receiving no pretreatment with compounds and not subjected to oxidative stress. The data shown are the mean ± S.E.M. from 12 samples in 3 independent experiments, $P^*<0.05$ (1 way ANOVA, Tukey post hoc test), versus H_2O_2 non-treated cells.

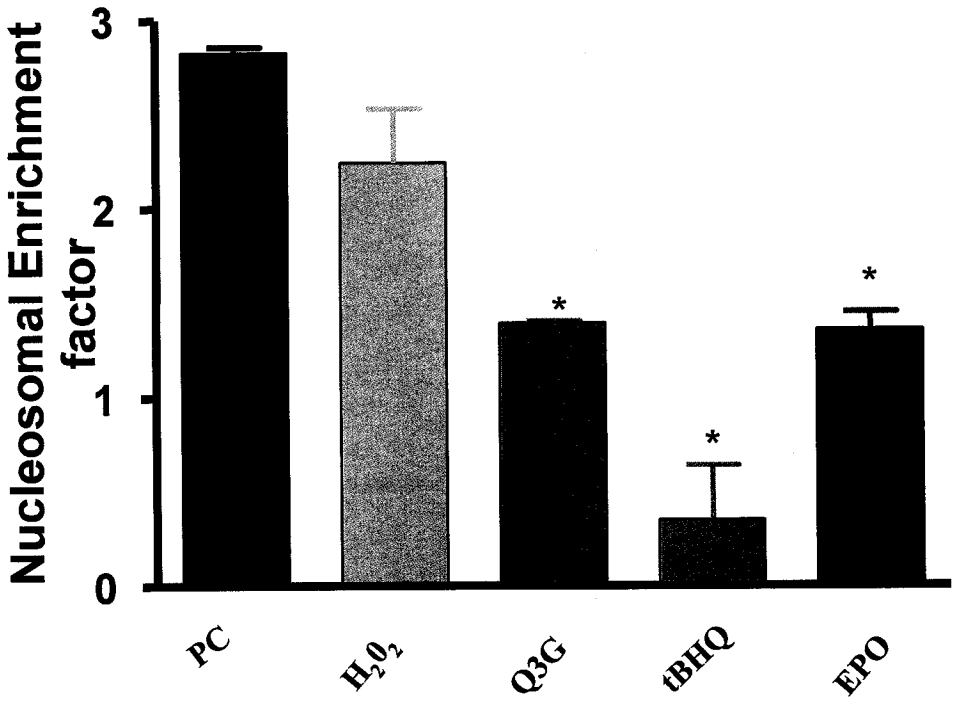

Figure 5

Figure 6: Reactive oxygen species (ROS) produced in SH-SY5Y cells immediately after exposure to 500 μM H_2O_2 and 0.11 mg/ml horseradish peroxidase for 15 minutes. SH-SY5Y cells were pretreated with quercetin-3-glucoside (Q3G), tert-butylhydroquinone (tBHQ) and Vitamin C (ascorbic acid) or vehicles for 24 hours. ROS levels were measured immediately after insult using 5-(and-6)-carboxy-2,7'-dichlorodihydrofluorescein diacetate, DCFH-DA. Each bar is representative of the mean ± S.E.M. from 12 samples in 4 independent experiments, P*<0.05, P**<0.01 (1 way ANOVA, Tukey post hoc test) versus H_2O_2 treated cells. ROS production is expressed as the fold elevation compared to SH-SY5Y cells that were not exposed to oxidative stress.

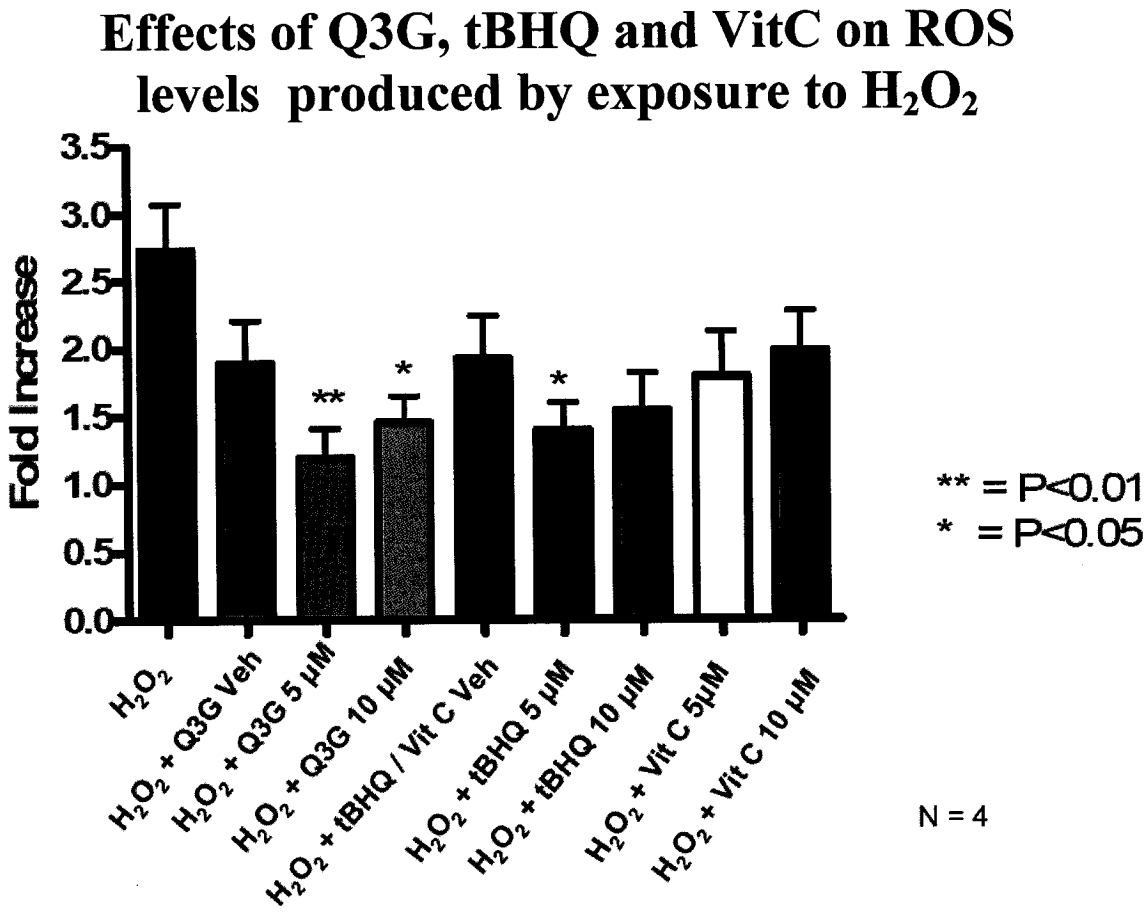
Effects of Q3G, tBHQ and VitC on ROS levels produced by exposure to H_2O_2
Fold Increase
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
H_2O_2
H_2O_2 + Q3G Veh
H_2O_2 + Q3G 5 µM
H_2O_2 + Q3G 10 µM
H_2O_2 + tBHQ / Vit C Veh
H_2O_2 + tBHQ 5 µM
H_2O_2 + tBHQ 10 µM
H_2O_2 + Vit C 5µM
H_2O_2 + Vit C 10 µM
** = P<0.01
* = P<0.05
N = 4

Figure 6

Figure 7: Significance Analysis of Microarray (SAM) plot of all the genes examined after treatment of SH-SY5Y cells with quercetin-3-glusoside (10 μM, 6 hrs) versus vehicle treatment (0.05% DMSO, 6 hrs). With a standard error value equal to 20.6% and a false discovery rate equal to 0%. Only 1 gene, histone 2B, displayed significantly altered expression (upregulated, red dot).

Effects of Q3G and vehicle on gene expression

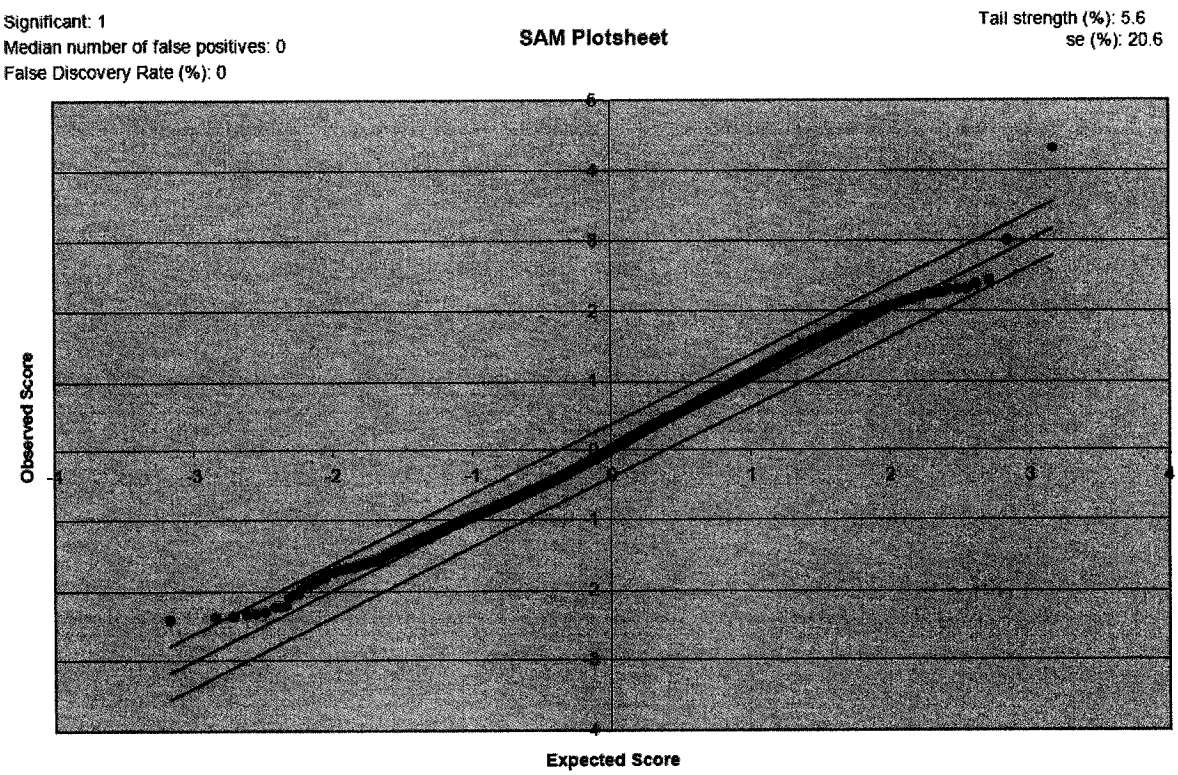

Figure 7

Figure 8: Significance Analysis of Microarray (SAM) plot of all the genes examined after treatment of SH-SY5Y cells with quercetin-3-glusoside (10 μM, 6 hrs) plus 500 μM H_2O_2 insult (15 minutes, 6 hr recovery time) versus vehicle treatment (0.05% DMSO, 6 hrs) plus 500 μM H_2O_2 insult (15 minutes, 6 hr recovery time). With a standard error value equal to 23.5% and a false discovery rate equal to 7.31%. It was determined that 28 genes had significantly altered expression, with 2 false positives. Significantly upregulated genes (25) are indicated as red dots and significantly downregulated genes (3) are indicated as green dots.

Effect of Q3G and vehicle on gene expression in cells exposed to oxidative stress

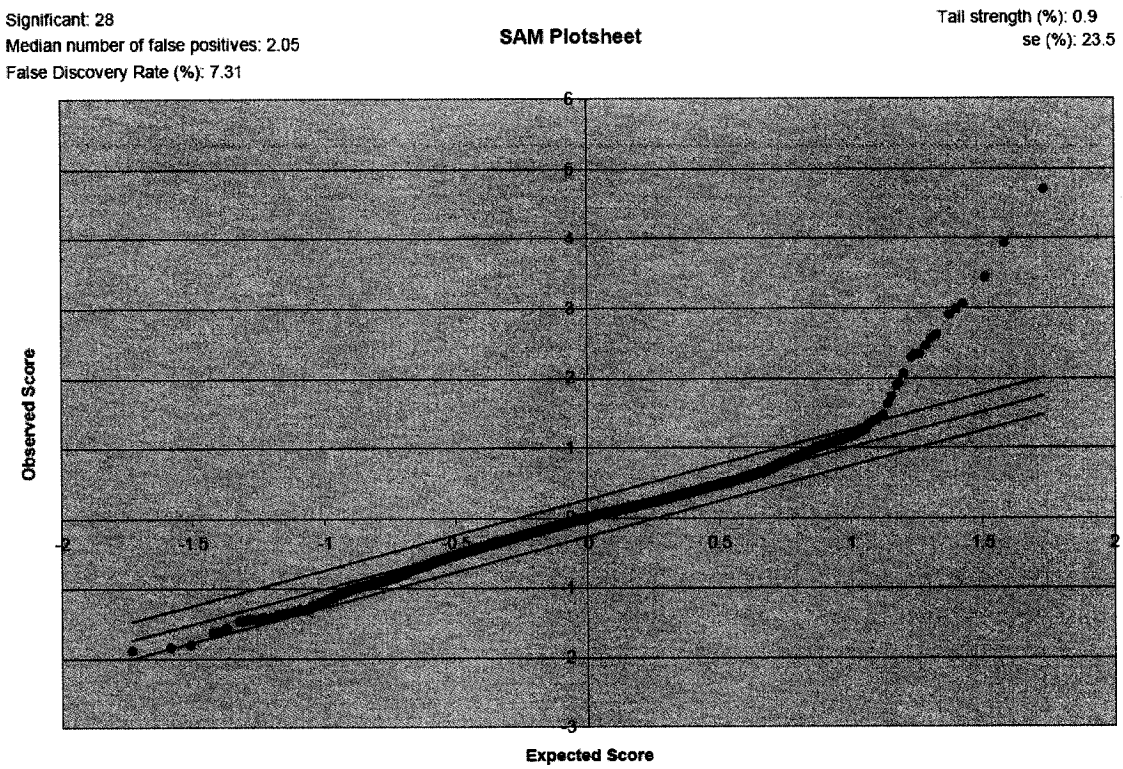

Figure 8

Table 1: Genes whose expression was significantly altered as a result of exposing SH-SY5Y cells to quercetin-3-glusoside (10 μM, 6 hrs) plus 500 μM H_2O_2 insult (15 minutes, 6 hr recovery time) compared to cells treated with vehicle (0.05% DMSO) and 500 μM H_2O_2 insult (15 minutes, 6 hr recovery time). Data are expressed as fold increase / decrease over mRNA levels of SH-SY5Y cells exposed to vehicle treatment (10 μM equivalent, 6 hrs) plus 500 μM H_2O_2 insult (15 minutes, 6 hr recovery time).

Table 1

Gene name	Gene Symbol	Accession No	Function	Fold change
Upregulated				
Stearoyl-CoA desaturase	SCD	AB032261	Fatty acid biosynthesis	2.81
Farnesyl-diphosphate farnesyltransferase 1	FDFT1	X69141	Cholesterol biosynthesis	2.76
Cytochrome P450 family 51, subfamily A, polypeptide 1	CYP51A1	U23942	Cholesterol biosynthesis	2.63
Sterol-C4-methyl oxidase-like	SC4MOL	U60205	Sterol biosynthesis	2.62
Squalene epoxidase	SQLE	D78130	Sterol biosynthesis	2.42
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	HMGCS1	X66435	Cholesterol biosynthesis Lipid metabolism	2.42
Isopentenyl-diphosphate delta isomerase 1	IDI1	X17025	Cholesterol and Isoprenoid biosynthesis	2.38
Unknown		L00352		2.24
Fatty acid desaturase 2	FADS2	AF126799	Fatty acid biosynthesis and desaturation	2.1
7-dehydrocholesterol reductase	DHCR7	AF034544	Cholesterol biosynthesis	1.94
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	HMGCR	NM_000859	Cholesterol biosynthesis	1.88
Unknown		AB016247		1.71
RAB3A interacting protein	RAB3IL1	NM_013401		1.669
Glutamate receptor, ionotropic, N-methyl D-aspartate 1	GRIN1	L13266	Regulation of synaptic plasticity	1.651
Unknown		U96876		1.651
Isocitrate dehydrogenase 1	IDH1	AF020038	Carbohydrate metabolism	1.61
Transmembrane protein 97	TMEM97	L19183	Regulation of cell growth	1.589
Fatty acid synthase	FASN	U29344	Fatty acid biosynthesis	1.57
Farnesyl diphosphate synthase	FDPS	D14697	Cholesterol and Isoprenoid biosynthesis	1.5
Zinc finger protein 364	ZNF364	AL079314		1.464
Jun D proto-oncogene	JUND	X56681	Transcription	1.43
Unknown		D85606		1.36
Potassium voltage-gated channel, subfamily H	KCNH7	AF035290	Signal transduction and Potassium ion transport	1.358
Unknown		AF077754		1.28
Downregulated				
Sestrin 1	SESN1	AF033122	Negative regulation of cell cycle	-1.61
Methyltransferase like 7A	METTL7A	AL050159	Transferase activity	-1.42
Ectodermal-neural cortex	ENC1	AF059611	Development and Neurogenesis	-1.51

Figure 9: Pie chart showing the functional distribution of the 25 genes significantly up regulated in response to 6 hours of 10 μM Q3G treatment plus 500 μM H_2O_2 insult of 15 minutes, with a 6 hr recovery time compared to cells treated only with vehicle and 500 μM H_2O_2 insult. Significantly altered gene expression was identified by microarray analysis using Gene Traffic software and SAM. **Note:** The summation of the categories in each graph does not equal the number of significant genes as some genes fall into more than one category.

Functional categories of genes whose expression was upregulated by Q3G and H_2O_2 exposure compared to treatment with vehicle and H_2O_2

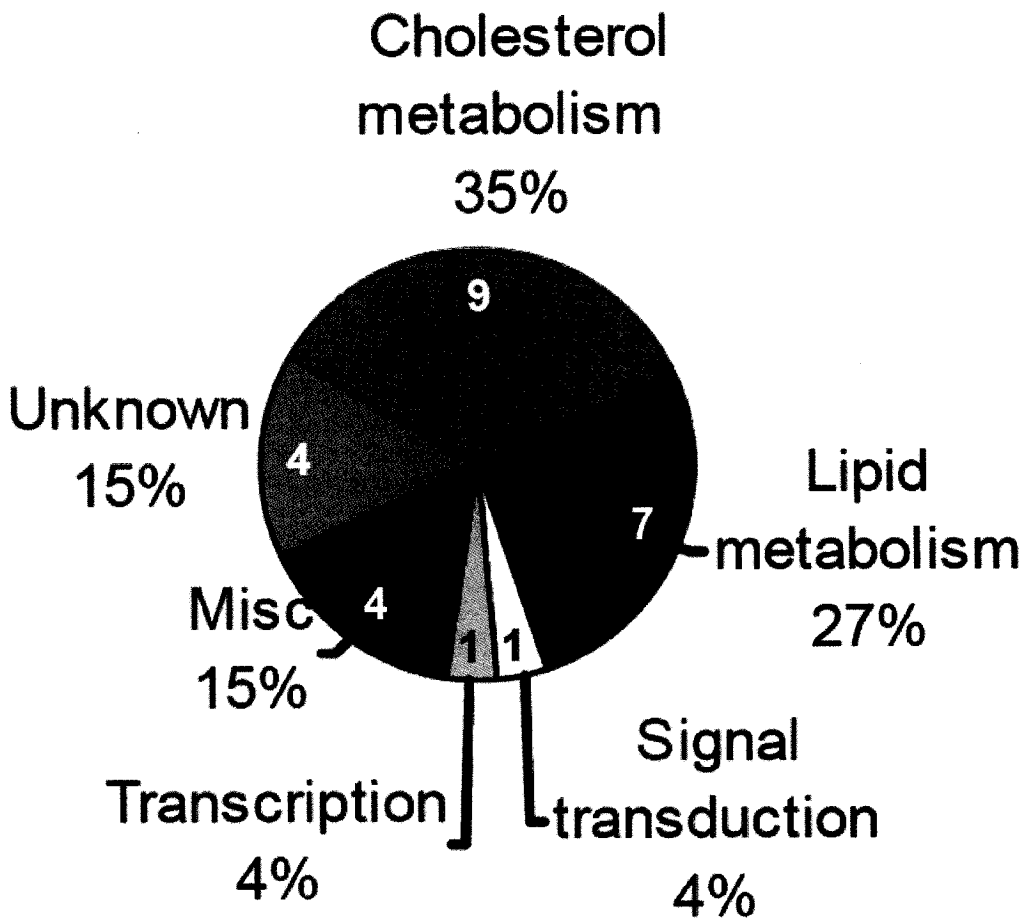

Figure 9

Figure 10: A pathway assist diagram showing potential functional linkages and signal transduction pathways between genes upregulated after treatment with quercetin-3-glucoside (10μM, 6 Hrs) plus 500 μM H_2O_2 insult (15 minutes, 6 hr recovery time). The red circles in the diagram are a set of proteins involved in cholesterol biosynthesis whose transcription is increased by Q3G pretreatment and H_2O_2 insult. The blue circles are proteins not examined in the array that connect to the increased genes. Green circles are small molecule intermediates (enzyme substrates, enzyme products) that link the proteins. Green squares on arrows indicate regulation, blue squares indicate binding, and purple diamonds indicate metabolism. For abbreviations in red circles consult Table 1.

Blue circles: NOVA1 = neuro-oncological ventral antigen 1, ITGAM = integrin αM, MAX = Myc associated factor X, ONECUT1 = one cut domain family member 1, E2F4 = E2F transcription factor 4, TAF1 = TATA box associated factor 1 RNA polymerase II, HNF4A = hepatocyte nuclear factor 4.

Green circles: A = 2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17-hexadecahydro-1H-cyclopenta[a]phenanthren-3-ol, B = [2-(6-aminopurin-9-yl)-5-[[[[5-(5-carbamoyl-1-pyridyl)-3,4-dihydroxy-tetrahydrofuran-2-yl]methoxy-hydroxy-phosphoryl]oxy-hydroxy-phosphoryl]oxymethyl]-4-hydroxy-tetrahydrofuran-3-yl]oxyphosphonic acid, C = 17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol, D = cGMP, E = 2-amino-5-[1-(carboxymethylcarbamoyl)-2-mercapto-ethyl]amino-5-oxo-pentanoic acid.

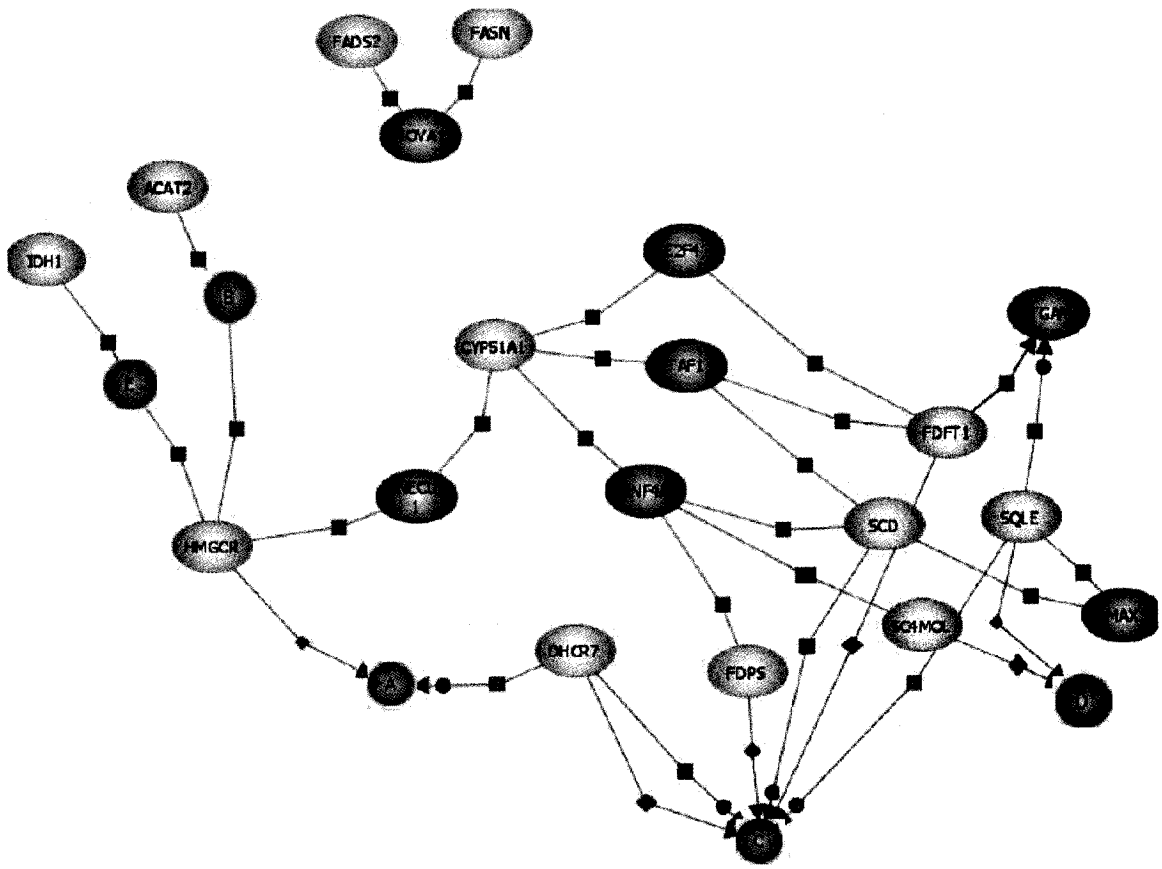
FADS2
FASN
ACAT2
IDH1
CYP51A1
SCD
FDFT1
SQLE
HMGCR
LSS
DHCR7
FDPS

Figure 10

Figure 11: A standard curve of cholesterol concentration versus absorbance at 562 nm for the cholesterol assay. Cellular cholesterol levels were determined by fitting sample absorbance to the line and using the equation $Y = AX + B$ to determine the level of cholesterol.

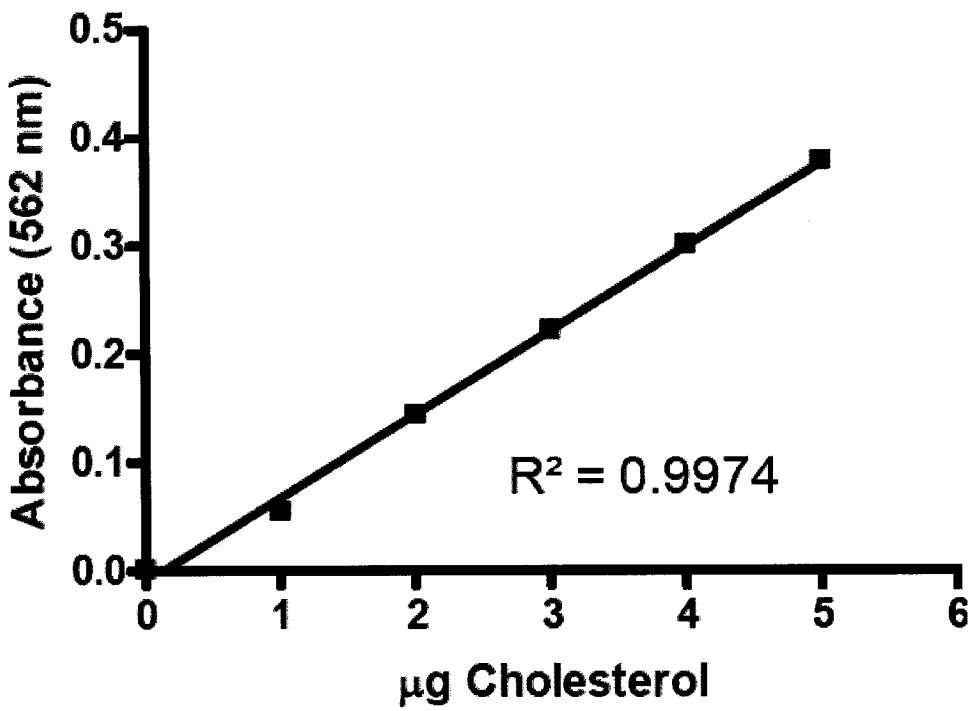
Absorbance (562 nm)
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
μg Cholesterol
R² = 0.9974

Figure 11

Figure 12: The level of cholesterol in SH-SY5Y cells was measured using a cholesterol assay kit. Q3G and vehicle were added to the cells and incubated for 6 hours, then cells were subjected to 500 μM H_2O_2 for 15 minutes and allowed to recover for 6 hours. Cells were then equalized to 8.5 X 10^5 cells and lipids extracted by addition of a chloroform-methanol solution. Following a 15 minute incubation the solution was centrifuged and the organic phase collected and evaporated. The lipids were re-dissolved in 20 μL of 2-propanol. Afterward 2 μL of sample was added to 48 μL reaction buffer and 50 μL of reaction mix was added (44 μl cholesterol reaction buffer, 2 μl cholesterol probe, 2 μl enzyme mix and 2 μl cholesterol esterase), samples were incubated at 37°C for 60 minutes. Absorbance was measured at 562 nm and the amount of cholesterol present was determined by fitting the absorbance values to the standard curve. Each bar is representative of the mean ± S.E.M. from 12 determinations in 3 independent experiments. $P^* < 0.001$ (1 way ANOVA, Tukey post hoc test) versus H_2O_2 treated cells.

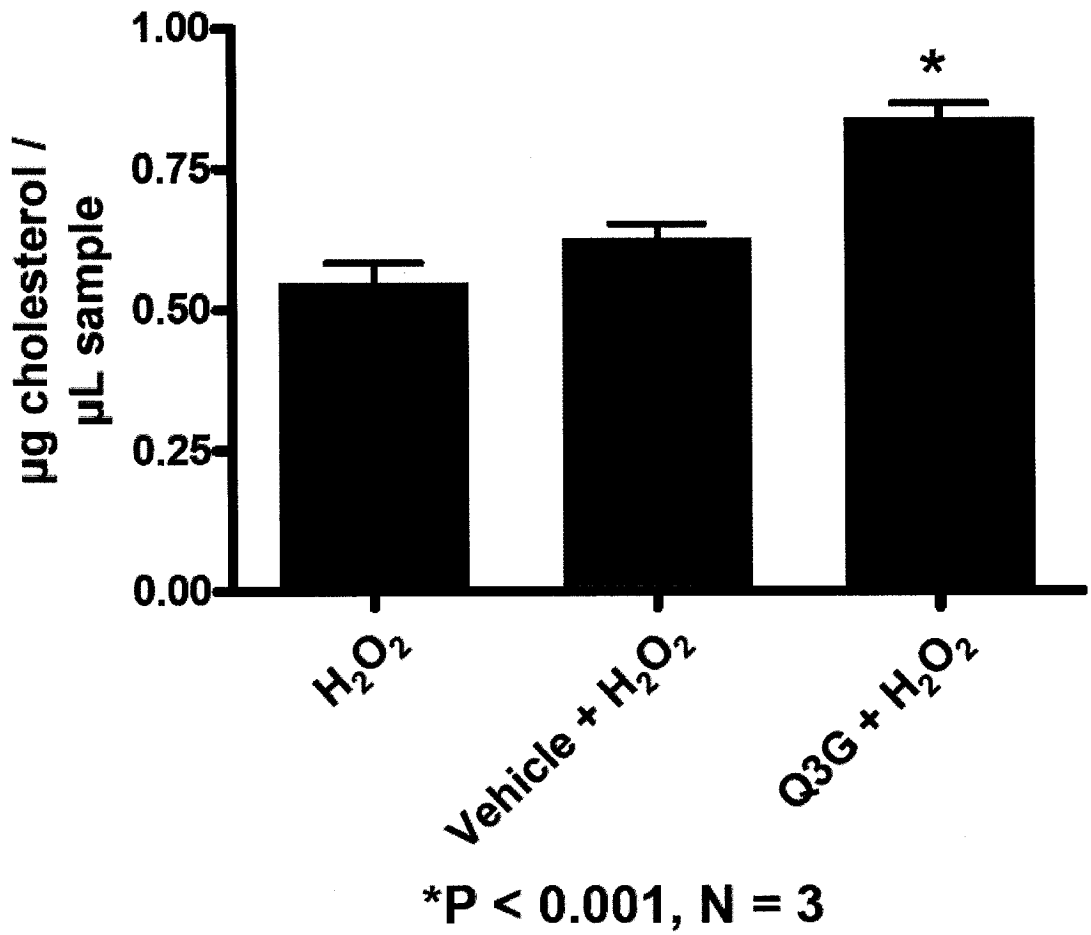
1.00
0.75
0.50
0.25
0.00
µg cholesterol / µL sample
*
H2O2
Vehicle + H2O2
Q3G + H2O2
*P < 0.001, N = 3

Figure 12

Figure 13: Cell viability after addition of mevastatin, a HMG CoA reductase inhibitor. Cells were plated in a 96 well plate at a density of 1 X 10^4 cells per well. After 24 hours quercetin-3-glucoside was added to the wells for 6 hours. The conditioned media was removed and the cells subjected to 500 μM of H_2O_2 for 15 minutes, 1 μM of mevastatin was added to the cells for 18 hours and cell viability assessed by a MTT assay. Data are expressed as % viability compared to control cells receiving no Q3G and no insult. Each bar is representative of the mean ± S.E.M. from 20 determinations in 3 independent experiments. P*<0.001(1 way ANOVA, Tukey post hoc test) versus H_2O_2 treated cells. P†<0.001(1 way ANOVA, Tukey post hoc test) versus Q3G + H_2O_2 treated cells.

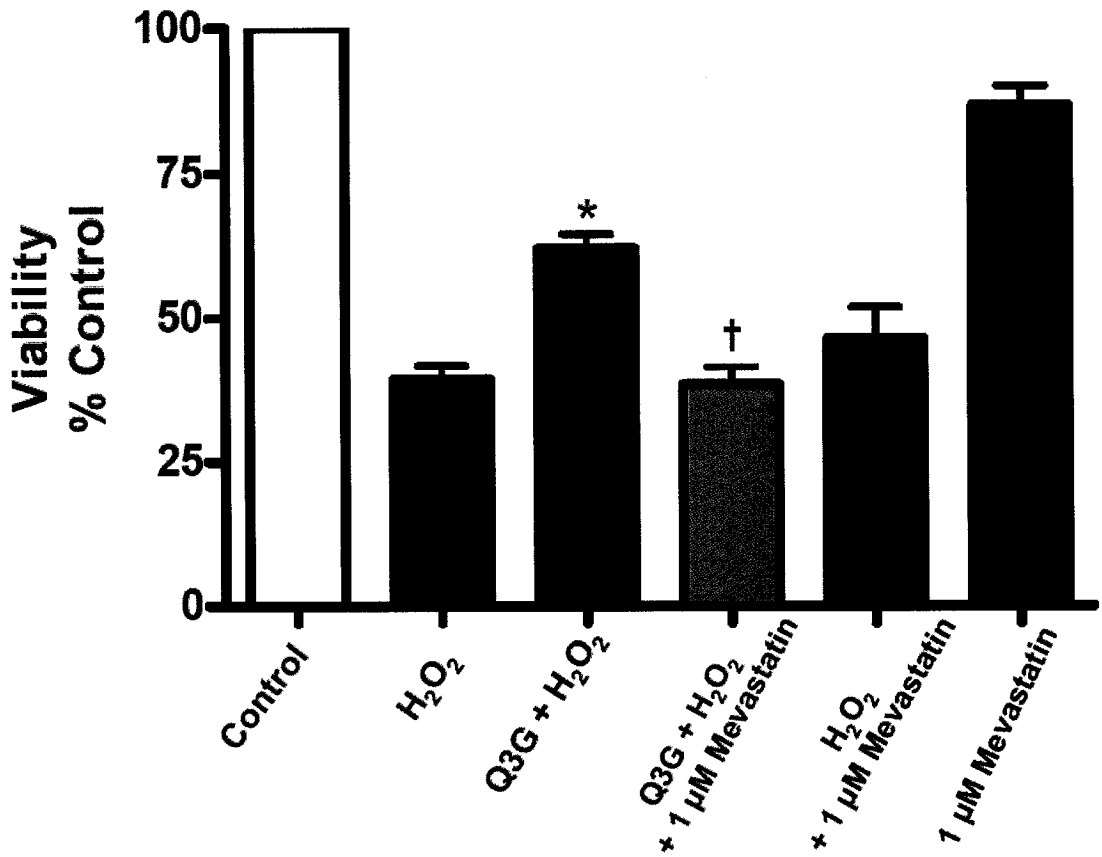
Viability
% Control
100
75
50
25
0
*
†
Control
H_2O_2
Q3G + H_2O_2
Q3G + H_2O_2 + 1 µM Mevastatin
H_2O_2 + 1 µM Mevastatin
1 µM Mevastatin

Figure 13

Figure 14: SH-SY5Y cell membrane integrity after H_2O_2 insult and addition of mevastatin, a HMG CoA reductase inhibitor. Cells were plated in a 96 well plate at a density of 1 X 10^4 cells per well. After 24 hours quercetin-3-glucoside was added to the wells for 6 hours. The conditioned media was removed and the cells subjected to 500 μM of H_2O_2 for 15 minutes, 1 μM of mevastatin was added to the cells for 24 hours and the membrane integrity was assessed by examining lactate dehydrogenase (LDH) release. Data are expressed as % LDH release compared to positive control cells receiving incubation with lysis buffer. Each bar is representative of the mean ± S.E.M. from 24 determinations in 4 independent experiments. P*<0.001(1 way ANOVA, Tukey post hoc test) versus H_2O_2 treated cells. P†<0.001(1 way ANOVA, Tukey post hoc test) versus Q3G + H_2O_2 treated cells.

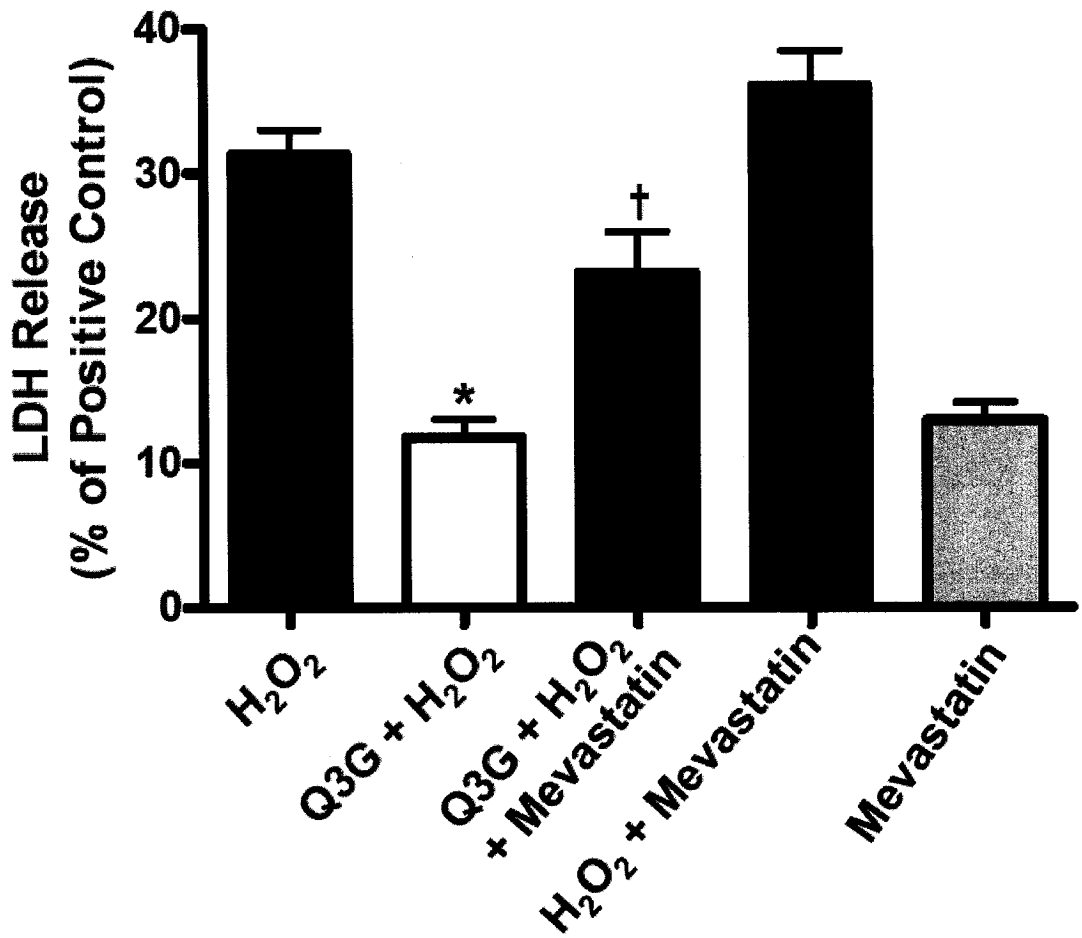
LDH Release
(% of Positive Control)
40
30
20
10
0
*
†
H_2O_2
Q3G + H_2O_2
Q3G + H_2O_2 + Mevastatin
H_2O_2 + Mevastatin
Mevastatin

Figure 14

Figure 15: A schematic of the proposed mechanisms for the cytoprotective effects of quercetin-3-glucoside. Q3G is able to enter the cell and activate SREBP after H_2O_2 stress leading to increased expression of several proteins such as sterol co-A desaturase 1, HMG-CoA synthase and HMG-CoA reductase that are involved in cholesterol biosynthesis resulting in an overall increase in cholesterol. Q3G can associate with and enter cholesterol enriched membranes protecting them from oxidative stress. These effects were reversed in cells treated with the HMG CoA reductase inhibitor, mevastatin.

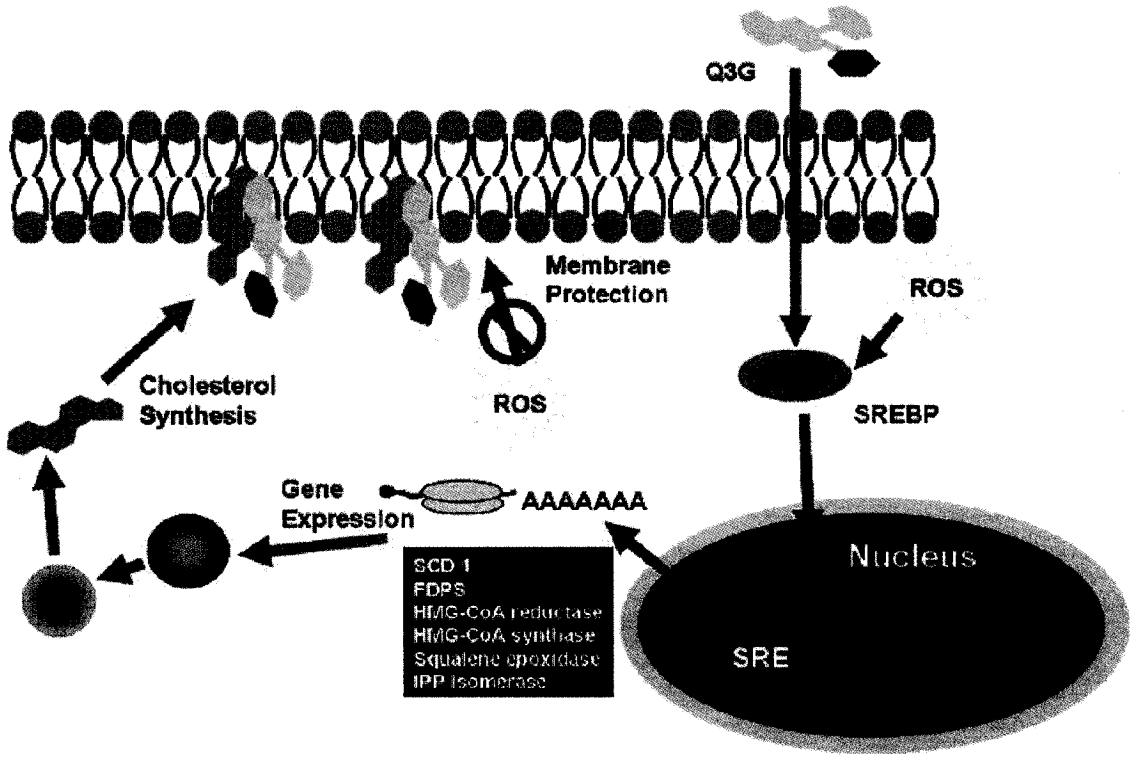
Q3G
Membrane
Protection
ROS
ROS
Cholesterol
Synthesis
SREBP
Gene
Expression
AAAAAAA
SCD 1
FDPS
HMG-CoA reductase
HMG-CoA synthase
Squalene epoxidase
IPP isomerase
Nucleus
SRE

Figure 15

Chapter IV: Discussion

General Discussion Of Data Obtained

A major finding of the present study was that the flavonoid Q3G protected SH-SY5Y cells from injurious concentrations of H_2O_2 that otherwise trigger cell death by creating excessive oxidative stress. **Moreover, a novel mechanism for the cytoprotective effects of Q3G was elucidated** (Figure 15). This mechanism involved the ability of Q3G exposure to prime the cell for a cytoprotective response that is triggered by H_2O_2 insult. Evidence was presented that this adaptive response consisted of increased expression of genes responsible for cholesterol biosynthesis that may increase plasma membrane integrity by reducing lipid peroxidation and enhancing the availability of structural components necessary for membrane repair after oxidative injury.

Initial experimentation presented in this study showed that pre-treatment of SH-SY5Y cells with Q3G followed by exposure to H_2O_2 reduced the loss of cellular viability normally seen after the H_2O_2 insult. Many other investigators have reported that quercetin is able to reduce cell death resulting from oxidative stress (Su *et al.*, 2003; Choi *et al.*, 2003; Cesquini *et al.*, 2003). Quercetin is able to protect against cell death triggered by excessive oxidative stress as demonstrated in the present study by decreased nucleosome fragmentation and LDH release (loss of membrane integrity), markers of cell death. As reported here using SH-SY5Y cells and by others using cultured chicken spermatogonial cells, quercetin, and chemically related compounds

reduces necrotic cell death by preventing lipid peroxidation thereby maintaining membrane integrity (Zhang, 2005). Quercetin may also prevent H_2O_2 induced apoptosis by decreasing caspase-3 activation in H9c2 cardiomyoblasts (Park *et al.*, 2003) and increasing levels of heme oxygenase 1, an antioxidant protein found in brain and macrophages (Chow *et al.*, 2005). Both of these mechanisms were examined in the present study and neither was found to be responsible for the cytoprotective effects of Q3G against oxidative stress in SH-SY5Y cells (data not shown).

Similar to the findings of other studies, we have linked the protective cellular effects of quercetin to its free radical scavenging ability. The ability of quercetin to directly absorb free radicals and form complexes with metal ions, that promote oxidative stress, enable the compound to protect against single stand DNA breaks, lipid peroxidation and protein damage (Jeong *et al.*, 2005; Chow *et al.*, 2005).

Quercetin can be incorporated in the plasma membrane of cells where it is intercalated between the acyl chains of phospholipids (Movileanu *et al.*, 2000). This action is mediated by the hydrophobic nature of quercetin. The degree of membrane intercalation for quercetin can be altered by changing the pH of the media (Lopez-Revuelta *et al.*, 2006). For example, at acidic pH, the hydrophobicity of quercetin is elevated enabling this compound to intercalate to a higher degree in the plasma membrane. Another study has implicated pi-pi interactions, a noncovalent interaction between organic compounds containing aromatic moieties, enabling quercetin to enter the lipid bilayer in a cholesterol-dependent fashion (Saiji *et al.*, 1995). Membranes

enriched in cholesterol achieve greater levels of quercetin incorporation, thus increasing resistance to lipid peroxidation (Saiji *et al.*, 2003). Conversely, membranes depleted of cholesterol display increased lipid peroxidation and an elevated loss of membrane integrity (Lopez-Revuelta *et al.*, 2006). Similar effects were observed in the present study where cholesterol depletion after an oxidative insult resulted in increased loss of membrane integrity and decreased cell viability.

Others have performed cDNA microarray experiments to profile changes in gene expression that occur after exposure to quercetin, however little research has been done on the effects of quercetin exposure on gene expression in SH-SY5Y cells. Using the prostate cancer cell lines PC-3 and DU-145, several cDNA microarray studies have implicated genes related to the control of cell cycle and cell growth in the chemotherapeutic effects of flavonoids (Nair *et al.*, 2004). In line with these findings, quercetin has been reported to alter the transcription of genes involved in apoptosis, cell-cycle control and xenobiotic metabolism in the colon-carcinoma cell lines CO115 (Murtaza *et al.*, 2006) and Caco-2 (van Erk *et al.*, 2005). In addition to the alterations in the expression of genes that regulate cell division, there is also some evidence that the transcription of tumor suppressor genes is upregulated by quercetin (van Erk *et al.*, 2005). However, by comparison to the present study, all of these studies used significantly higher concentrations of quercetin (50 μM -100 μM) and none of them subjected the cells to an oxidative insult or examined the ability of quercetin to influence the transcriptional response to oxidative stress. Although SH-SY5Y cells were derived from a neuroblastoma cancer cell line, in the present study changes in the

expression of genes relating to either cell division or tumor suppression following exposure to Q3G were not present. Moreover, significant alterations in gene expression were only observed in cells after an oxidative insult in cells pre-treated with Q3G.

Plausible Mechanisms For Cytoprotection

Cholesterol biosynthesis is highly regulated and entails numerous enzymatic processes. At the heart of this synthetic pathway are two transcription factors, sterol regulatory element binding protein 2 (SREBP-2) and SREBP-1 that can be alternatively spliced giving rise to SREBP-1a and SREBP-1c (Kuhn *et al.*, 2004). Both transcription factors are anchored to the endoplasmic reticulum membrane in a hairpin like structure with their amino and carboxyl termini exposed to the cytosol (Loewen and Levine, 2002). To become transcriptionally active, SREBPs must be cleaved by a SREBP cleavage-activating protein (SCAP) releasing the NH_2 terminal ends of these proteins into the cytoplasm where they can dimerize via basic helix-loop-helix leucine zipper motifs following which translocation to the nucleus occurs (Loewen and Levine, 2002). Once in the nucleus, SREBPs bind to sterol regulatory element (SRE) triggering the transcription of genes necessary for cholesterol biosynthesis. When cholesterol biosynthesis is required, an endoplasmic reticulum (ER) bound protein that monitors cholesterol levels called Insing activates SCAP resulting in the cleavage and activation of SREBPs (Loewen and Levine, 2002). To date, there are no reports in the literature demonstrating that flavonoids or polyphenolic compounds can alter the activities of SCAP or Insing. However, the ability of Q3G pre-treatment to prime SH-SY5Y cells

to increase the expression of cholesterol biosynthesis after exposure to H_2O_2 implicates SCAP and Insing in this Q3G-mediated cytoprotective response to oxidative stress.

Many of the genes found to be increased in the present cDNA microarray studies are under control of the transcription factor SREBP-2, that is continuously synthesized by the cell and degraded via ubiquitin mediated proteasome degradation. A few studies have demonstrated that treatment with polyphenolic genistin plus a H_2O_2 insult increases levels of mSREB-2, the membrane cleaved form of SREBP-2 (Mullen and Shay, 2004; Mullen *et al.*, 2004). The polyphenol epigallocatechin gallate has been reported to increase levels of SREBP-1 though this leads to a lowered cellular cholesterol concentration by promoting cholesterol efflux (Bursill and Roach, 2006).

Alternatively, ester containing polyphenols, such as Q3G, may increase the expression of many proteins involved in cholesterol biosynthesis such as SREBP-2 by inhibiting proteasome mediated protein degradation leading to increased levels of cholesterol (Mullen *et al.*, 2004). Inhibition of proteasome mediated protein degradation has already been proposed as a mechanism for establishing neuroprotection because many compounds such as morphine (Rambhia *et al.*, 2005), lactacystin (Nam *et al.*, 2001), reversatrol (a polyphenolic compound found in grapes and red wine) are able to inhibit the proteasome leading to reduced levels of amyloid-beta peptides implicated in Alzheimer's disease (Marambaud *et al.*, 2005). In cultured cerebellar granule neurons that are dependent on depolarizing concentrations of potassium (50 mM) in the media to survive, proteasome inhibitors reduce the death of these neurons

when extracellular concentrations of potassium are lowered (Canu *et al.*, 2000). These proteasome inhibiting compounds must be added at the time potassium reduction occurs to be neuroprotective because they are no longer effective if added 3 hours after exposure. Animal studies have also supported a protective action of proteasome inhibition against the injurious effects of cerebral ischemia (Phillips *et al.*, 1999; Phillips *et al.*, 2000).

An oxidation sensitive subunit exists in the 26 S proteasome identifying it as a protein sensitive to oxidative stress (Reinheckel et al., 1998). Under electrophilic induced oxidative stress, proteasome activity is decreased in SH-SY5Y cells (Shibata *et al.*, 2003). Oxidative damage to the 26 S proteasome that reduces enzymatic activity of this complex combined with subsequent inhibition of the proteasome with polyphenolic compounds would further reduce the ability of the 26 S proteasome to degrade proteins suggesting this combination may be the mechanism for cytoprotection. It is also possible that Q3G must undergo reaction with H_2O_2 and glutathione (GSH) to produce a pro-oxidant quinoid species (Pinto and Macais, 2005) that may mediate the cytoprotective effects of Q3G shown in the present study. This is supported by the fact that no alterations in gene expression occurred when Q3G was incubated with SH-SY5Y cells that were not subjected to subsequent oxidative stress.

Inhibition of the proteasome is thought to mediate the ability of the drug bortezomib to eliminate cancerous myeloma cells by inhibiting degradation of the cyclins, cyclin-dependent kinases, tumor suppressors, and nuclear factor-kB thereby

arresting cells in the M/G_2 growth phase and sensitizing them to apoptosis (Joazeiro *et al.*, 2006). Bortezomib has gained Food and Drug Administration FDA approval for the treatment of myeloma and second generation compounds such as NPI-0052 and PR-171 are undergoing clinical testing (Joazeiro *et al.*, 2006). Although inhibition of the proteasome produces apoptosis in cancer cells, this appears to be a dose dependant effect since quercetin protects cardiomyocytes from anoxia-reoxygenation at a low concentration but induces apoptosis at a higher concentration (No listed author, 2006).

Both the SREBPs must be phosphorylated at key serine and threonine residues by glycogen synthase kinase-3β (GSK3β) (Sundqvist *et al.*, 2005) to attract the ubiquitin ligase Fbw7 that ubiquitionates these proteins and targets them for proteosomal degradation (Minella *et al.*, 2005). Given the large polyphenolic structure of Q3G of approximately 400 g/mol and the fact that flavonoids are able to inhibit various kinases it is possible that the cytoprotective effects observed in the present study may be mediated by inhibition of GSK3β leading to increased levels of SREBPs that initiate elevated cholesterol synthesis.

An enzyme complex known as p300/CBP is able to associate with SREBP and acetylate the lysine residues in the transcription factor complex thereby blocking the ubiquitation process leading to a longer survival rate of p300/CBP/SREBP and higher cholesterol biosynthesis (Giandomenico *et al.*, 2003). Promotion of acetylation via stabilization of the p300/CBP/SREBP complex or increasing the acetylation ability of this complex could be potential mechanisms for the effects of Q3G on cholesterol

biosynthesis seen in this study. By contrast, curcumin, a structurally distinct polyphenolic compound that is thought to exhibit similar effects as quercetin, has been shown to inhibit the p300 acetyltransferase (Marcu *et al.*, 2006) which would yield lower levels of cholesterol biosynthesis.

Epidemiological & Pharmacokinetic Studies Supporting Future Research

Epidemiological evidence supports the cytoprotective effects of quercetin. Following ingestion, Q3G is absorbed by the gut where it is converted into free quercetin (Crespy *et al.*, 1999). Three hours after ingestion, concentrated red grape juice elevated the antioxidant capacity of the plasma, increased HDL, while reducing concentrations of oxidized LDL, apolipoprotein B-100 and LDL-cholesterol in blood of the peripheral circulatory system (Castilla *et al.*, 2006). In a randomized trial of male smokers, consumption of dealcoholized red wine significantly reduced urine levels of F2-isoprostane, a marker of lipid peroxidation (Caccetta *et al.*, 2001). Similarly in a placebo controlled study, lyophilized grape powder reduced plasma LDL, cholesterol, plasma triglycerides, apolipoproteins B and E and F2-isoprostane in pre- and post-menopausal women (Zern *et al.*, 2005).

Black tea is the major source of flavonoids in the Western world. The Rotterdam study examined the effects of black tea consumption on the incidence of myocardial infarction in 4807 subjects 55 years or older. The findings of this study suggested that the relative risk for myocardial infarction decreased in patients who consumed more than 375 ml of tea daily (Geleijnse *et al.*, 2002). Likewise,

epidemiological data from the Zutphen study that followed 552 men aged 50-69 for 15 years suggested total dietary flavonoids, mainly quercetin, consumed from black tea reduced the incidence of stroke in a manner inversely proportional to tea intake (Keli *et al.*, 1996).

By contrast, a large study of 38,445 women generated findings at odds with these studies by reporting that there was no significant positive correlation between flavonoid intake and cardiovascular disease (Sesso *et al.*, 2003). Nonetheless, this study showed that roughly 3% of women consuming greater than 946 ml of tea daily were at a reduced risk for adverse vascular events.

Pharmacokinetic studies in humans show that glycosylation of quercetin leads to a higher bioavailability of this flavonoid and that only glycosylated products, not free quercetin, could be detected in serum (Graefe *et al.*, 2001). Similar results were found in a study of pigs that were fed flavonoids as glycosylated flavonoids proved to be more abundant in serum samples than unglycosylated flavonoids (Cermak *et al.*, 2003). Furthermore, consumption of a high fat diet may increase quercetin levels in the serum following oral administration of this flavonoid (Lesser *et al.*, 2004). Other studies have shown that incubation of glycosylated quercetin products with human saliva caused the breakdown of these products into free quercetin within minutes (Walle *et al.*, 2005). However large inter-subject variability was reported in the ability to break down glycosylated quercetin into free quercetin.

Quercetin but not glycosylated forms of this flavonoid show high apical to basolateral permeability in Caco-2 cells, an *in vitro* model of intestinal absorption (Walgren *et al.*, 1998). Methylated quercetin shows 8 fold higher apical to basolateral permeability than quercetin. Methylated flavonoids also show reduced levels of hepatic metabolism making them more metabolically stable thereby increasing oral bioavailability (Wen and Walle, 2006). Rat feeding studies showed that the intestinal tract contained 94-100% unmetabolized quercetin, but intestinal tissues contained 11 different metabolites of the parent compound (Graf *et al.*, 2006). Tissue distribution studies of quercetin in rats and pigs show high levels of this flavonoid in rat lungs, pig liver and kidney but low levels in brain and spleen of both species (de Boer *et al.*, 2005). Taken together, these studies suggest that oral consumption of Q3G **may exert** superior cytoprotective effects compared to quercetin by increasing bioavailability. In summary, the results of the present study suggest that dietary supplementation with Q3G **may reduce the risk** of cardiovascular disease such as stroke by enhancing membrane repair associated with oxidative stress.

Chapter V: Conclusions

1. Quercetin-3-glucoside is cytoprotective against H_2O_2 induced oxidative stress in SH-SY5Y cells at a concentration of 1 μM and 10 μM.
2. Under H_2O_2 induced oxidative stress, quercetin-3-glucoside elevated the expression of several genes involved in cholesterol biosynthesis.
3. The increase in expression of these genes involved in cholesterol biosynthesis is essential for maintaining cell viability, and membrane integrity following oxidative stress.
4. Inhibition of cholesterol biosynthesis prevented the cytoprotective effects of Q3G.

Future Work

In order for quercetin-3-glucoside to become a therapeutic, work needs to be done in finding the exact molecular mechanism that leads to an increase in genes controlling cholesterol biosynthesis after exposure to this compound. Once the mechanism is found, enzymatic and cell based screens may be used to identify other compounds that are cytoprotective against oxidative insults in primary neuronal cell cultures before moving to *in vivo* models. Therapeutic potential in the field of neuroprotection exists for quercetin-3-glucoside because it appears to be a safe natural product that is consumed daily in various food products. Studies have shown that structurally similar flavonoid compounds such as amentoflavone (30 mg/kg) are neuroprotective in neonatal models of hypoxic-ischemic brain injury (Shin *et al.*, 2006).

Although quercetin-3-glucoside is very hydrophobic, a novel microemulsion drug delivery system containing clove oil, Tween 20 and water is able to encapsulate quercetin and thereby enable effective delivery of this drug *in vivo* (Gupta *et al.*, 2006).

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