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Small Molecular Weight Modulators of Apoptosis and Senescence

By Lee-Anne Fochesato

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
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Windsor, Ontario, Canada

2011

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Small Molecular Weight Modulators of Apoptosis and Senescence

By Lee-Anne Fochesato

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ABSTRACT

Age-related neurodegenerative diseases are a major problem in industrialized nations, and affect millions of people worldwide. The pro-apoptotic protein Bax plays a crucial part in apoptosis. We screened a library of low molecular weight compounds for a potential Bax inhibitor. One compound that we have discovered (C22) was found to block Bax association to the mitochondria and prevent mitochondrial membrane destabilization. In addition, C22 may have the ability to prevent neuronal cell death in an *in vivo* model of stroke

WS-CoQ_{10} is a potent anti-oxidant and is known to stabilize the mitochondria. The aim of this study was to evaluate the ability of WS-CoQ_{10} to the induction of stress-induced premature senescence in presenilin-1 (PS-1) mutated Alzheimer's Disease (AD) fibroblasts. It was found that WS-CoQ_{10} was able to inhibit senescence in these cells as well as decreasing the protein levels of senescence-associated proteins.

DEDICATION

I dedicate this thesis to the endless search for that million dollar sound.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Siyaram Pandey, for giving me the oppourtunity to research in his laboratory and to learn about cell death. I would also like to thank the group members: Sudipa Chatterjee, Corey Scipione, Carly Griffin, Dennis Ma, Pamela Ovadje, Katie Facecchia, Krithika Kumaran, and Alyson Laframboise for all of their support. I wish to give a special thank you to Dennis Ma for reading my thesis and to Corey Scipione for his contribution to the stroke project, as well as Kevinjeet Manghar for his contribution to the Alzheimer's project. All the lab members, past and present, have been a delight to work with everyday and I wish them all the best in their future endeavours.

I wish to thank Dr. Donald Degracia at Wayne State University for his generous donation of his time and skills in performing the stroke surgery in an *in vivo* rat model. I would also like to give a special thanks to my committee members Dr. Lana Lee, Dr. Andrew Swan, and Dr. Otis Vacratis for their valuable insights to my project and the time spent reading my thesis.

I would like to thank Mrs. Marlene Bezaire for all of her assistance over the years. She never fails to ensure the best for all graduate students in the department.

Finally I wish to acknowledge my parents for their undying support throughout the years. Thank you all, it was a pleasure working with each and every one of you.

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LIST OF ABBREVIATIONS

Aβ	Amyloid Beta
AD	Alzheimer's Disease
ANT	Adenine Nucleotide Translocase
APAF-1	Apoptosis Protease Activating Factor-1
AP-1	Activator Protein-1
APP	Amyloid Precursor Protein
ATG-4	Autophagic-related Protein 4
BMSC	Bone Marrow Stromal Cells
C22	Compound 22
C9	Compound 9
CAD	Caspase Activated DNase
CypD	Cyclophillin D
DAPI	4',6-diamidino-2-phenylindole
DCFDA	2'-7'-dichlorodihydrofluoresceine diacetate
DISC	Death Inducing Signalling Complex
FADD	Fas Associated Death Domain
FasL	Fas Ligand
GSH	Glutathione
HRP	Horseradish Peroxidase

ICAD	Inhibitor of Caspase Activated DNase
ICH	Intracerebral hemorrhage
JNK	c-Jun N-terminal Kinase
LC-3	Microtubule Associated protein light chain
MAPK	Mitogen Activated Protein Kinase
MDC	Monodansylcadaverine
MnSOD	Manganese Superoxide Dismutase
MOMP	Mitochondrial Outer Membrane Permeabilization
$Oxo^{8}dG$	8-oxodeoxyguanosine
PE	Phosphotidylethanolamine
PEG	Polyethylene Glycol
PS-1	Presenilin-1
PS-2	Presenilin-2
PTP	Protein Transition Pore
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species
SIPS	Stress-Induced Premature cellular Senescence
SMAC/DIABLO	Second Mitochondria-derived Activator of Caspases/Direct IAP Binding protein with Low PI
TMRM	Tetramethyl Rhodamine Methyl Ester

TNF-α	Tumour Necrosis Factor-α
VDAC	Voltage Dependent Anion Channel
WS-CoQ_{10}	Water soluble Coenzyme Q10
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

CHAPTER I

INTRODUCTION

Part I: Cell Death and Pro-Death Proteins

Apoptosis

Apoptosis, or programmed cell death, is employed to maintain homeostasis within the body (Fadeel e*t al.,* 2005). Unlike necrosis which is a passive and unplanned form of cell death, cells that undergo apoptosis expend energy towards their own demise. Cells regularly undergo apoptosis as part of the normal developmental process (Adihetty *et al.,* 2003). Apoptotic cells display characteristic morphological changes such as membrane blebbing, cell shrinkage (Pollack *et al.,* 2001), nuclear condensation (pyknosis) and nuclear fragmentation (karyorhexis) (Kroemer *et al.,* 2005). Biochemical changes such as activation of caspases, membrane flipping, collapse of mitochondrial membrane potential, and DNA fragmentation are all characteristic of apoptosis (Spencer and Sorger, 2011; Heit *et al.,* 2011). Such changes can be brought upon by an increase in oxidative stress within the cell, DNA damage, or the activation of various death receptors and proteins responsible for initiating cell death (Pollack *et al.,* 2001). Apoptosis is a required process and must be properly regulated. If there is a lack of apoptosis, uncontrolled proliferation of damaged cells can occur resulting in cancer (Krishna *et al.,* 2011). However, if inappropriate initiation of apoptosis occurs, various neurodegenerative conditions, such as Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease could result (Kerr *et al.,* 1972). There are 2 main pathways, the extrinsic and the intrinsic pathways, of apoptosis.

1.2 Extrinsic Pathway of Apoptosis

In the extrinsic pathway of apoptosis, the initiation of cell death results when a series of death-inducing ligands, such as tumour necrosis factor-α (TNF-α) or the Fas ligand (FasL), bind to their appropriate death receptors (Adihetty *et al.,* 2003). For example, when FasL binds to the Fas receptor, the Fas Associated Death Domain (FADD) is recruited to the intracellular domain of the death receptor. Procaspase-8 then binds to the FADD protein resulting in the formation of the death inducing signalling complex, otherwise known as DISC (Kroemer *et al.,* 2007). It is the DISC complex that will then proceed to cleave procaspase-8 into its active form (caspase-8) that is then able to activate caspase-3 (Hsu *et al.,* 1996). Once caspase-3 is activated it is able to translocate to the nucleus and assist in DNA fragmentation through the use of various DNases, while inhibiting the DNA repair mechanisms (Pollack e*t al.,* 2001). The extrinsic pathway of apoptosis can be intertwined with the intrinsic pathway via activation of Bid and Bax. Caspase-8 activation can lead to the cleavage of the pro-apoptotic protein Bid to its truncated form of tBid. tBid is then able to oligomerize with Bax and initiate pore formation in the mitochondrial membrane, subsequently leading to mitochondrial permeabilization (Fig. 1.1) (Ghibelli and Diederich, 2011). Lymphocytes commonly undergo apoptosis via the extrinsic pathway as a result of Fas-mediated apoptosis activation (Otsuki e*t al.,* 2011).

1.3 The Intrinsic Apoptotic Pathway

Mitochondria play an integral role to the functioning of the cell. They are responsible for integral cell processes such as energy production and have been implicated in many diseases, such as cancer, Parkinson's Disease, Alzheimer's Disease, and stroke (Kroemer *et al.*, 2007). Mitochondria also play a large role in the initiation of apoptosis via the intrinsic apoptotic pathway.

One key aspect of the intrinsic pathway of apoptosis is mitochondrial outer membrane permeabilization, or MOMP. In this case, the mitochondrial outer membrane ruptures and releases cytotoxic factors such as cytochrome c or SMAC/DIABLO into the cytosol. Subsequent resulting events include loss of inner mitochondrial membrane potential, caspase activation, and an increase in oxidative stress (Newmeyer *et al.*, 2003). MOMP can be caused by two mechanisms: the first involving the Bcl-2 family of proteins, and the other focusing on the permeability transition pore (PTP) (Bouchier-Hayes *et al.*, 2005).

The Bcl-2 family of proteins contains both pro-death and pro-survival proteins whose balance affects the potential for the cell to undergo apoptosis. When the pro-death members (such as Bax, Bid, Bak, etc.) associate with the mitochondria, they are believed to form a pore spanning the mitochondrial membrane. When this happens, it allows for the release of cytotoxic factors into the cytosol. In the cytosol, APAF-1 (apoptosis protease activating factor-1) exists as a monomer. In the case of MOMP and the release

of cytotoxic factors, APAF-1 complexes with procaspase-9 and cytochrome c and is activated with the addition of ATP/dATP (Cain *et al.,* 2002). This allows for the formation of the apoptosome which can cleave procaspase-9 into its active form (caspase-9) which leads to further activation of caspase-3. Caspase-3 then acts to cleave ICAD (inhibitor of caspase-activated DNase), which then allows for CAD (caspase-activated DNase) to enter the nucleus of the cell and initiate DNA fragmentation, subsequently resulting in cell death (Fig. 1.1) (Enari *et al.,* 1998).

The PTP encompasses both the inner and outer mitochondrial membranes and is composed of three main components: the voltage dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophillin D (cyp D) (Zamzani *et al.,* 2001). In the case of increased oxidative stress, such as that which is experienced in ischemia/reperfusion (Crompton e*t al.,* 1999), the PTP becomes saturated with bound Ca^{2+}. This saturation results in an unregulated influx and efflux of water and ions into and out of the mitochondria, as well as a disruption in the mitochondrial membrane potential. The influx of water causes the mitochondrial membrane to burst, thus, releasing cytotoxic factors into the cytosol and initiating the caspase cascade (Bouchier-Hayes *et al.,* 2005).

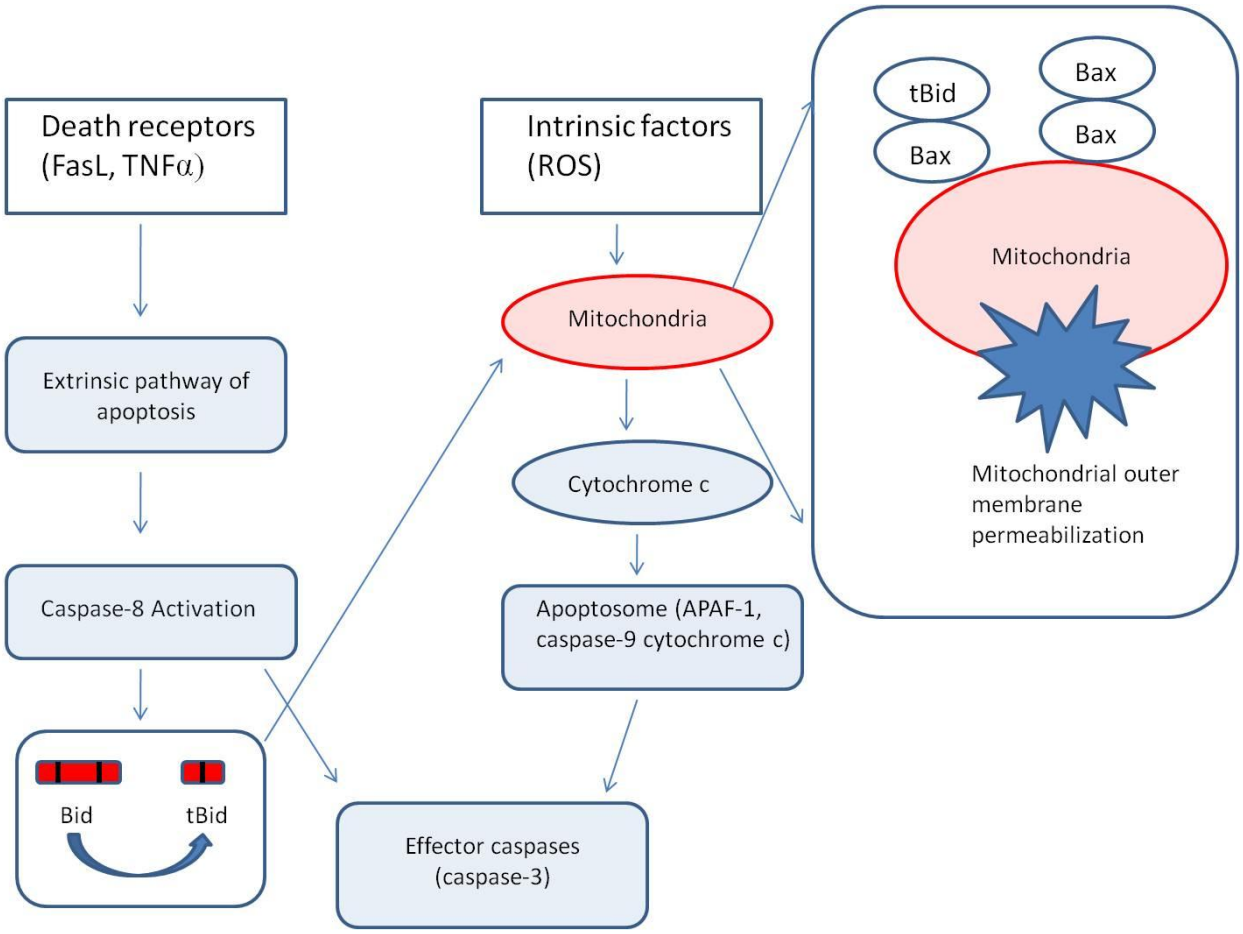

Figure 1.1: Extrinsic vs. intrinsic apoptotic pathways. This schematic representation illustrates the differences between the intrinsic and extrinsic pathway of apoptosis. In the extrinsic pathway, apoptosis is induced by the binding of a death ligand to its appropriate receptor. This leads to activation of effector caspases and inevitably cell death. In the intrinsic pathway of apoptosis, some intracellular stimuli (such as ROS) causes the migration of pro-apoptotic members of the Bcl-2 family of proteins to the mitochondria. This eventually leads to mitochondrial destabilization and the release of cytochrome c

into the cytosol where formation and activation of the apoptosome occurs. Subsequently, caspases are activated and cell death occurs. The extrinsic and intrinsic pathways are not entirely separate from one another. Interconnection between the two can occur when tBid (the truncated product of Bid cleavage by caspase-8) oligomerizes with Bax to cause pore formation to the mitochondrial membrane. This link from the extrinsic pathway can further propel the events of the intrinsic pathway of apoptosis, such as mitochondrial membrane permeabilization.

1.4 The Bcl-2 Family of Proteins

The Bcl-2 family of proteins contains both pro-apoptotic and anti-apoptotic members. They are composed of various arrangements of the homologous BH1-BH4 domains, which are independently folded parts of a protein that have their own specific function. Those members that have all 4 BH domains and a C-terminal hydrophobic tail used for mitochondrial membrane insertion include the anti-apoptotic Bcl-2 proteins. Those proteins that lack only the BH4 domain include the pro-apoptotic members Bax and Bak. BH3 only proteins are also pro-apoptotic and include Bid, Bad, and Puma (Hengartner *et al.*, 2000).

The balance between the pro-apoptotic and anti-apoptotic members of the Bcl-2 family is integral to whether apoptosis occurs (Park, 2009). The BH3 domain is an important part of the structure of these proteins, as it allows for the dimerization between

pro- and anti-apoptotic members to occur (Bax/Bcl-2), thereby halting apoptosis, or the dimerization of pro-apoptotic proteins (Bax/Bax) to occur resulting in apoptosis.

In particular, Bax plays a prominent role in the induction of apoptosis by dimerizing with itself or forming a heterodimer with other pro-apoptotic proteins. The resulting dimer is able to form a pore in the mitochondrial membrane, thus, causing release of cytotoxic factors into the cytosol.

Bax exists in 4 different isoforms: γ(5 kDa), β(24 kDa), δ(16 kDa), and α(21 kDa). Only the α and β isoforms are known to contain the BH3 domain and the hydrophobic C-terminal tail that is required for insertion into the mitochondrial membrane, and are thus the most detrimental to cell survival (Oltavai *et al.,* 1993). The β isoform of Bax is the one that will be discussed in this body of work. One important factor in determining the localization of Bax in the cell is the amino acid Ser184. If this serine is mutated to a Val or subjected to complete deletion, Bax becomes permanently associated to the mitochondrial membrane, even in healthy cells. However, if this serine is substituted for a His, Glu, Lys, or Asp, Bax becomes permanently located in the cytosol (Neuchastan *et al.,* 1999).

Bax is composed of 9 alpha helices, with 8 helices arranging themselves around one central helix. Helix 2 (α2) contains the BH3 domain responsible for facilitating the binding of Bax to other proteins, while helix 9 (α9) contains the C-terminal hydrophobic tail that allows Bax to insert into the mitochondrial membrane (Suzuki *et al.,* 2000).

Under normal conditions, the α2 BH3 domain is bound to the α9 C-terminal hydrophobic tail and is not able to dimerize nor is it able to insert itself into the mitochondrial membrane. Membrane permeabilization is able to take place when α2 and α9 are able to break free from each other to allow for dimerization and mitochondrial pore formation to occur (Suzuki e*t al.*, 2000).

1.5 Implications of Oxidative Stress in Stroke and Ischemic Related Brain Injury

In ischemic related brain injuries, one of the main perpetrators of cellular damage is oxidative stress. Ironically, the return of blood flow to the infarcted area of the brain causes harm along with its benefits due to the increase in oxygen availability and the increase in oxidative stress that reperfusion causes. In these situations, lactic acid accumulates in the affected neurons promoting pro-oxidant effects by increasing the H^+ concentration within the cells and generating more ROS (Allen *et al.*, 2009). The primary source of ROS is the superoxide anion radical (O_2^-), which is generated by leakage from complex III of the electron transport chain of malfunctioning mitochondria (Turrens *et al.*, 1985).

Oxygen is one of the main forms of oxidative stress but it is not the only one. Hydrogen peroxide is converted to the hydroxyl radical (OH^-), and the nitric oxide (NO) species that are produced can have extensive implications in neuronal signalling. DNA

fragmentation and lipid peroxidation can result when the O_2^- radical reacts with NO to produce the highly damaging peroxynitrite ($ONOO^-$) molecule (Bergendi *et. al.,* 1999).

The brain is the perfect environment for damage due to oxidative stress since it makes up only 2% of the total body weight but consumes nearly 20% of the total oxygen (Clarke *et al.,* 1999). The composition of the brain also makes it a target of oxidative damage. Its high level of polyunsaturated fatty acids and low level of antioxidant defence mechanisms makes the brain an excellent candidate for damage due to oxidative stress (Saeed *et al.,* 2007).

Stroke is a leading cause of death and long-term disability in industrialized nations (Alexandrova *et al.,* 2005; Moro *et al.,* 2005), and is known to induce impaired motor function and cognition, with nearly 40% of patients not expected to make a full recovery (Allen *et al.,* 2009). The production of free radicals and oxidative stress is the main culprit of stroke-induced damage.

Two types of stroke can occur, hemorrhagic stroke, and the more common, ischemic stroke. Hemorrhagic stroke is a result of a rupture of a blood vessel in the brain that leads to uncontrolled bleeding. On the other hand, ischemic stroke is a result of an obstructed flow of blood to the brain, usually caused by the formation of a blood clot. This deprivation of adequate blood to the brain results in the formation of the ischemic core. In this area, neurons die quickly via necrosis. The onset of lipolysis, protein

degradation, and the breakdown of ion homeostasis are some of the events responsible for the rapid death of these cells (Brouns *et al.*, 2009).

While neurons in the ischemic core die quickly due to necrosis, there is an area surrounding the ischemic core known as the penumbra. Here, these cells still suffer damage but they remain viable and are able to be saved. Neurons in the penumbra die via apoptosis and the full effects of this cell death may not be evident for up to a week following ischemia (Schaller, 2004).

The events that occur in the penumbra act as a double-edged sword. In order to survive, the neurons of the penumbra require the oxygenated blood delivered with reperfusion. However, this influx of oxygen does not come without its own problems. This large infusion of oxygenated blood brings with it a host of free radicals. These free radicals themselves pose a threat to the brain tissue by generating oxidative stress (Facecchia *et al.*, 2011).

When platelets are exposed to conditions of reperfusion they generate additional ROS in the form of O^{2-} and OH^{-}. Furthermore, the ROS that are produced during reperfusion are responsible for the activation and transcription of many proteins. For example, ROS stimulate the production of JNK and mitogen-activated protein kinase phosphorylation (p38 MAPK) which can regulate transcription and play a role in induction of apoptosis. During reperfusion, activator protein-1 (AP-1) binding is also enhanced (Schaller *et al.*, 2004). The activation of AP-1 is necessary for the induction of

apoptosis to occur (Chen *et al.,* 1999) as it controls the transcription of various apoptosis related proteins such as FasL and TNF-α (Karamouzis *et al.,* 2007). This action, along with the activation of caspase-3, are several examples of how reperfusion is responsible for initiating cell death within the neurons of the penumbra by controlling the expression of certain genes.

Along with their role in effecting the transcription of various proteins, ROS generated by reperfusion itself can cause direct cellular stress. The ramifications associated with reperfusion cannot be fully alleviated by the cell's normal radical scavenging mechanisms such as superoxide dismutase (SOD), glutathione peroxidase and catalase. These systems are overwhelmed and cannot adequately quench the multitude of free radicals introduced by reperfusion (Li *et al.,* 2002). The cells of the penumbra are already vulnerable to damage, and the generation of ROS exacerbates the damage that may have already occurred to these cells by lipid peroxidation. Phospholipid membrane degradation is one of the major concerns associated with increased oxidative stress levels. The larger number of PUFAs that compose the membrane are excellent targets for ROS. This leads to membrane instability that causes a disruption of receptor behaviour and ion channel activity. Along with its own deleterious effects, lipid peroxidation is also responsible for the inhibition of lipid repair enzymes such as lysophosphatidylcholine acyltransferase and fatty acyl CoA-synthase (Schaller *et al.*, 2004).

1.6 The Role of Pro-Apoptotic Proteins in Stroke

As previously mentioned, apoptosis is controlled by a wide range of proteins. Oxidative stress can cause the activation of the p53 tumor suppressor gene which in turn is responsible for the increased transcription of Bcl-2 associated X protein (Bax) and the p53-upregulated modulator of apoptosis (PUMA) (Nakano *et al.,* 2001). PUMA has been shown to be able to interact with the Bcl-2 family of proteins to assist in initiating apoptosis. PUMA has been known to work in conjunction with Bax to facilitate mitochondrial membrane permeabilization and the release of cytochrome *c* (Zinkel *et al.,* 2010).

The Bcl-2 family of proteins play a large role in maintaining the balance between life and death in the process of apoptosis. For example, under normal cell conditions, Bax is localized in the cytosol as a 24 kDa monomer. However, when increased oxidative stress is introduced Bax migrates to the mitochondria to initiate pore formation and apoptosis (Culmsee *et al.,* 2005)

Studies have demonstrated that Bax channel activity is necessary for apoptosis to occur since cell death was halted with the use of Bax channel blockers (Hetz *et al.,* 2005). Since Bax is an essential protein in the regulation of apoptosis, it is an excellent target candidate for therapeutic approaches. Not only does its extensive involvement in the process of cell death make Bax a good therapeutic target, its position in the apoptosis cascade does as well. While focusing on anti-oxidants may be a valid point of

investigation, bolstering of the anti-oxidant defense machinery still results in the permeabilization of the mitochondria. Blocking the initiation of apoptosis further up the chain by inhibiting Bax function may save the mitochondria and further halt the apoptosis cascade.

1.7 Therapeutic Approaches for Stroke

At this time the only known treatment for victims of stroke is the use of thrombolytics, such as tissue plasminogen activator. This is the most widely accepted treatment for stroke. In addition, other methods of treatment are being investigated (del Zoppo *et al.*, 1998). One example is the use of hypothermia as a means to combat stroke. It has been found that lowering the body temperature of a stroke victim may improve neurological outcome (Yenari *et al.*, 2010).

An emerging field of study for the treatment of ischemia includes the use of bone marrow stromal cells (BMSC). These cells can differentiate into neural and glial cells, both *in vivo* and *in vitro* after being transplanted into animal brains following neurological insult such as intracerebral hemorrhage (ICH) (Woodbury *et al.*, 2000). These neural stem cells localize to the injured section of the brain to regenerate damaged brain tissue in the appropriate locations. Recent studies have found that these BMSCs are able to generate functional recovery in Wistar rats following ICH (Otero *et al.*, 2011).

Recently, advances in treatments for stoke have been made by the use of low molecular weight compounds that inhibit proteins (such as Bax) that are critical in the apoptosis cascade. Bax channel formation is one of the key instigators of mitochondrial destabilization and blocking this action could halt apoptosis by preventing the release of cytotoxic factors into the cytosol, thus halting the caspase cascade (Hetz *et al.*, 2005). These inhibitory compounds were modeled after single domain antibodies that were able to bind specifically to Bax (Gueorguieva *et al.*, 2006). They are small enough to have the potential to cross the blood brain barrier and are not susceptible to proteolysis. Recent research completed in our laboratory indicates that these compounds show a high specificity towards the pro-apoptotic protein Bax, and are able to block its function and save cells *in vitro* (McGonigal *et al.*, 2009). These compounds are able to bind to Bax even in the presence of single domain antibodies that are specifically structured to bind to Bax. It is our hope that these compounds will be able to interfere with Bax activation and prevent its association to the mitochondria, thus preventing the Bax channel formation that leads to the leakage of cytotoxic factors into the cytosol (Fig. 1.2). These low molecular weight compounds would not be needed to be administered under such strict timeframes as thrombolytics and are not likely to cause the haemorrhaging associated with tissue plasminogen activator. With more investigation it is likely that the use of low molecular weight compounds will become valid treatment options for stroke patients.

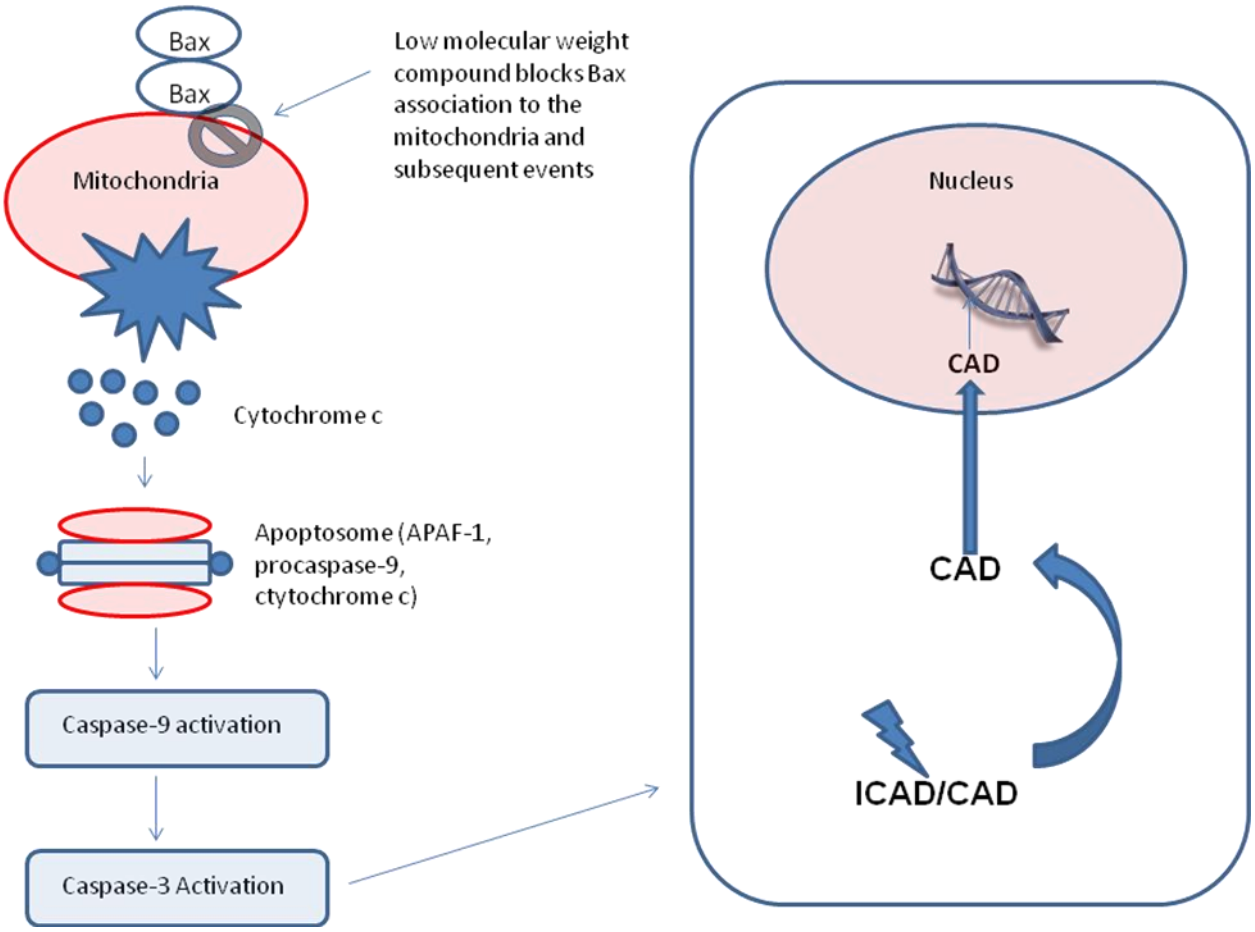

Figure 1.2: Target of low molecular weight compounds. A key aspect of apoptosis is destabilization of the mitochondrial membrane by the pro-apoptotic protein Bax. Mitochondrial pore formation results in release of cytochrome c into the cytosol. Bax-inhibiting low molecular weight compounds have the potential to bind to Bax and prevent its association to the mitochondrial membrane. If this can occur, Bax would no longer be

able to initiate the release of cytotoxic factors into the cytosol, resulting in the inhibition of subsequent apoptotic events.

1.8 Screening Pharmacophore Libraries for Low Molecular Weight Neuroprotective Compounds – Discovery of Compound 22

Low molecular weight compounds have the potential to be used as therapeutics in many conditions. They are generally stable, not susceptible to proteolysis, and small enough to cross the blood brain barrier. In 2001, a large library of 16, 320 low molecular weight compounds was screened to find compounds that would be able to block the Bcl-X_L/Bak BH3 interaction. Their intention was to find a compound that was able to disrupt the binding of these proteins by interfering with their BH3 domains. Previous studies in our lab have conducted a screen of 34 of these compounds that were found to exhibit binding to Bcl-2. Because Bcl-2 is close to Bax in structure, we hypothesized that a compound from this library could block the binding of Bax to other pro-apoptotic proteins, thus, neutralizing its role in initiating apoptosis. We found one compound, denoted as compound 22 (C22) that was able to competitively bind to Bax in the presence of single domain antibodies (sdAbs) specific to Bax.

Single domain antibodies differ from conventional antibodies, such as immunoglobulin, in the fact that they are derived from species of animals (such as the llama and camel) that produce antibodies that are absent of the variable light chain and contain only the variable heavy chain. The elimination of the light chain allows for a

more concise isolation of a smaller antigen binding site located on the variable heavy chain, known as a sdAb. These sdAbs are small in size (approximately 13 kDa) and consist of only 3 highly specific complementary determining regions that allow it to interact with a specific antigen (Tanha *et al.*, 2002). Since C22 was able to competitively bind to Bax in the presence of these sdAbs, it indicates that this compound is highly specific towards Bax and may be able to interfere with its apoptotic functions.

Previous work performed in our lab has found that C22 is able to lower the amount of ROS experienced by the mitochondria and prevented oxidative stress-induced apoptosis. The cells under oxidative stress that were treated with C22 were able to grow and divide normally and maintained mitochondrial membrane potential (Unpublished data; Katrina McGonigal, 2009). Compound 22 was studied as a potential neuroprotective agent in the hopes that it would be able to block the BH3 domain interaction that is essential for Bax dimerization and mitochondrial pore formation.

Part II: Oxidative Stress Induced Senescence

1.9 Senescence

Cellular senescence is a state where cells have reached a finite amount of replications and can no longer divide (Campisi, 2005). These cells are still metabolically active and are not destined to die via apoptosis, necrosis or any other form of cell death

(Campisi *et al.,* 2005). Senescent cells are instead removed from the cell cycle by being locked in the G_1 phase, and have reached the end of their replicative span, known as the "Hayflick Limit" (Dumont *et al.,* 2000). Senescence is a process induced to ensure that cells with defective or shortened telomeres do not enter the cell cycle and continue to divide.

Senescent cells display a variety of characteristics that distinguish them from their normal counterparts. Senescent cells display a larger, and more flattened morphology, in addition to possessing shortened telomeres (Sikora *et al.,* 2011). Senescent cells are also resistant to apoptosis (Guo *et al.,* 2010) and have increased metalloproteinase activity that has been shown to degrade the extracellular matrix (Campisi, 1999).

Senescence can be caused by a variety of factors. Due to the shortening of telomeres, senescent cells are subject to a higher level of DNA damage. Double stranded DNA breaks, histone deactylase inhibitors, and mitogenic signals can also be a cause of senescence (Campisi, 2005). Because of the increase in DNA damage, senescent cells have a higher level of endogenous oxidative stress and ROS production than their normal counterparts. Because of this, senescent cells also display differences in their genetic expression patterns and express higher levels of senescent-associated proteins such as p21, p16, and pRb (Sikora e*t al.,* 2011).

Senescent cells are relatively resistant to apoptosis and do not die, but instead have been found to contribute to the aging process. An accumulation of the defective

senescent cell phenotype has been shown to elicit changes in the tissue microenvironment. The affected tissues have been shown to suffer a loss of function and integrity due to the over expression of secreted molecules such as metalloproteinases, degrative enzymes, inflammatory cytokines and a variety of growth factors (Campisi *et al.*, 1996, Jennings *et al.*, 2000, Leung & Pereira-Smith *et al.*, 2001).

1.10 Mechanisms of Senescence

While senescence is a complex process, there are two main pathways involved in its initiation: the p53 pathway and the Rb pathway (Bringold and Serrano, 2000). The p53 protein is known to be a tumour suppressor and mediator of DNA damage, while the Rb protein is also a potent tumour suppressor and inhibitor of cell cycle progression (Wahl and Carr, 2001). Inactivation of one or both has been shown to increase cell replication and decrease the level of senescence (Itahana, 2001).

Because of shortened telomores, senescent cells are subjected to higher levels of DNA damage and oxidative stress, which results in the upregulation of p53 (Itahana, 2001). Once telomeric damage is identified, the ataxia telangiectasia mutated (ATM) pathway is activated and in turn activates p53 (Suzuki *et al.*, 2008). The ATM pathway is triggered under conditions of double stranded DNA breaks and results in cell cycle arrest (Li *et al.*, 2007). In senescent cells, p53 is also upregulated by the Ras oncogene.

In combination with ROS, Ras is able to activate MAPK-p38 kinase which in turn is able to upregualte the levels of p53 and the tumour suppressor p16 (Campisi, 2005).

In addition, p53 has also been shown to induce senescence by increasing the expression of other senescent associated proteins. For example, under conditions of increased oxidative stress, p53 has been shown to induce the expression p21 which is an inhibitor of the cell cycle (Campisi, 2005). Weeks after p21expression and senescence has occurred, there is a drop in the level of p21 present. Instead, the protein p16 is seen to increase (Campisi, 2005). p16 is an inhibitor of the cyclin D/Cdk4 and cyclin D/Cdk6 complex and plays a role in the maintenance of cellular senescence, while p21 and p53 are thought to be involved in the initiation of senescence (Campisi, 2005).

In addition to p53, activation of the Rb pathway was also shown to induce senescence. In this case, p16 and p21 are able to keep Rb in a hypophosphorlyated growth inhibiting state that prevents the binding of E2F transcription factor to its specific promoters (Chen e*t al.*, 2006). This Rb pathway has also been shown to be involved in senescence through chromatin disruption and re-organization (Zhang *et al.*, 2006), oncogenic signalling, and other stressors that lead to the expression of p16/p21 (Ben-Porath and Weinberg, 2004).

1.11 Stress Induced Premature Senescence (SIPS)

When cells encounter an increased, yet sub-lethal, level of ROS they can experience a phenomenon known as SIPS. In this case, the increased levels of ROS

cause the cells to withdraw from the cell cycle and remain halted in the G_1 phase of the cell cycle. These cells are locked in this phase and held there by various cell cycle inhibitors such as p21 and p16. Research has been conducted to experiment with the growth of cells under various levels of oxygen. It was found that when cells were placed in an environment of greater than 10% oxygen, they experienced a reduced lifespan and had a limited number of population doublings (Packer *et al.*, 1977).

Cells that have undergone SIPS were shown to have 30% more 8-oxodeoxyguanosine (oxo^8dG) in their DNA and possessed 4 times the level of free oxo^8Gua bases (Chen, 1995). This indicates that the level of oxidative stress associated with SIPS is potent enough to induce DNA damage (Chen, 1995). In addition to poor DNA repair mechanisms and low levels of telomere maintenance, oxidative stress has been found to be the main contributor to the induction of SIPS (Toussaint, 2000).

1.12 Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative disease that affects millions of people worldwide and is the most common form of dementia (Querfurth *et al.*, 2010). It is an age-related illness and with the average life span increasing, AD is becoming a more and more prevalent problem (Hampel *et al.*, 2003). AD is associated with mutations in the genes encoding for presenilin-1, presenilin-2 (PS-1 and PS-2), and amyloid precursor proteins (APP) (Cecchi *et al.*, 1999).

Behavioural and symptomatic characteristics of AD include a loss of word comprehension (aphasia), the inability to perform complex tasks (apraxia), and the inability to use common objects (agnosia) (Castellani et *al.,* 2010). Characteristics of AD on a cellular level include neuronal synaptic degeneration, accumulation of abnormal mitochondria, neuronal loss, and glial mediated inflammation (Palop and Mucke, 2011; Busciglio *et al.,* 2002; Castellani *et al.,* 2010). AD is most widely known to be a result of the formation of neurofibrillary tangles and plaques that are a result of improper processing of APP (Uchida, 2010). When APP is improperly processed, it results in an accumulation of amyloid β (Aβ) molecules that are predisposed to plaque formation ($A\beta_{1-42}$), while normal APP processing results in the formation of Aβ products that are not predisposed to plaque formation ($A\beta_{1-40}$) (Castellani e*t al.,* 2010). The accumulation of these plaques is detrimental because the Aβ molecules are likely to bind to the NMDA receptor and disrupt the proper flux of Ca^{2+}, thus decreasing neuronal plasticity. Aβ can also bind to the insulin receptor making the cells energy deficient (Palop and Mucke, 2010).

Another key element in the onset of AD is the hyperphosphorylation of the tau protein. Under normal conditions, the tau protein is responsible for stabilizing microtubules. When tau becomes hyperphosphorylated by either CDK5, ERK2, or GSK3β, it results in a self-assembly of straight and helical tangles; this produces an insoluble form of tau that forms aggregates in the brain (Ballard *et al.,* 2011).

Tau and Aβ accumulation can be a result of increased levels of ROS, impaired protein folding from defects in the endoplasmic reticulum, and the defective clearance of damaged proteins from the cell (Bi, 2010). Increased oxidative stress in AD can be a result of abnormal mitochondrial function, defective proteolysis, and activated microglia (Zhu e*t al.,* 2004). In addition, AD patients have an increased utilization of oxidative energy as oppose to glucose consumption, also increasing the amount of oxidative stress present in the brain (Fukuyama *et al.,* 1994).

1.13 Presenilin-1 Mutation

Mutations in the presenilin-1 (PS-1) gene are one of the most commonly associated mutations to the early onset familial AD, with the gene encoding for PS-1located on chromosome 14 (Cecchi *et al.,* 1999). PS-1 is a membrane protein and a major component of the atypical aspartyl protease complex that is responsible for the processing of APP (DeStrooper *et al.,* 1998). Abnormalities in the PS-1 component of this γ secretase responsible for cleaving APP into its amyloid beta (Aβ) products result in the production of plaque forming Aβ molecules ($A\beta_{1-42}$), as oppose to the non-aggregating $A\beta_{1-40}$ molecules.

PS-1 mutations can be any one of 170 possible missense mutations (van Tijn *et al.,* 2011). Cells that possess the PS-1 mutation are known to have higher than normal levels of endogenous ROS production. This higher level of ROS makes the cell

susceptible to undergoing SIPS, as oxidative stress is a main component of SIPS initiation (Toussaint, 2000).

In addition to improper APP processing, deletion of PS-1 has also been associated with disrupted Ca^{2+} homeostasis in the endoplasmic reticulum, resulting in a release of Ca^{2+} into the cytoplasm of the cell (Cook *et al.,* 2005). This results in a decrease in neuronal plasticity and makes the cells more susceptible to death via oxidative stress and metabolic or ischemic insults (Mattson *et al.,* 2003).

The effects of the PS-1 mutation is present in all cells, including neurons and fibroblasts. Because brain tissue from AD patients is unavailable to work worth, as biopsies of brain tissue of living AD patients is not possible, the effect of the PS-1 mutation can be studied in fibroblasts. Since the PS-1 mutation exists in all cells, information about this mutation gathered in fibroblasts cells can be extrapolated and applied to neurons with the PS-1 mutation.

1.14 Antioxidants

An overabundance of reactive oxygen species (ROS) has detrimental affects on protein function, stability, cell signaling, lipid peroxidation, and can cause disruptions to mitochondrial function. The brain is especially susceptible to ROS, as it consumes 20% of the body's oxygen while composing only 2% of the total body weight (Clarke, 1999). DNA and RNA are also prone to oxidation via ROS (Nunomura, *et al.,* 1999), making

ROS a key player in the onset of neurodegenerative conditions such as stroke and Alzheimer's Disease.

Cells combat ROS with a variety of different mechanisms, one being the use of anti-oxidants to quench the free radicals produced. Common anti-oxidant mechanisms employed to quench ROS include Cu/Zn/Mn superoxide dismutase (SOD), glutathione peroxidase, alpha-tocopherol, catalase, and ascorbate (Halliwell *et al.,* 1989) as well as the GSH and NADP/NADPH pathways (LeBras *et al.*, 2005). The maintenance of the oxidant-antioxidant balance of the cell is crucial to maintaining proper functioning and stability.

1.15 Coenzyme Q_{10} as a Neuroprotective Agent

Coenzyme Q_{10} (CoQ_{10}) is a naturally occurring, hydrophobic molecule that is responsible for shuttling electrons from complex I and complex II of the mitochondria to complex III. It is an integral member of the mitochondrial electron transport chain and plays an important role in free radical scavenging and the prevention of lipid peroxidation (Beal, 2003). CoQ_{10} is present in both its reduced and oxidized forms (Figure 1.3) in order to optimize its electron transport capabilities. CoQ_{10} has been found to be involved in disulfide bond formation, redox control, cell signalling, and gene expression (Jeya, e*t al.*, 2010). It elicits protective effects to the cells by regulating activity of the PTP,

activating mitochondrial uncoupling proteins and preventing the reduction of GSH and ATP levels (Jeya, *et al.,* 2010; Beal, 2003; Sandhu *et al.,* 2003).

CoQ_{10} has been tested in clinical trials for the treatment of neurodegenerative disorders, such as Parkinson's Disease, as well as various mitochondrial disorders (Shults *et al.,* 2004). One drawback to the use of CoQ_{10} as a therapeutic is the fact that it is lipophillic and its use in cell culture is very difficult and limited. In order to combat this problem, a water soluble version of CoQ_{10} (WS-CoQ_{10}) was developed at the National Research Council of Canada (Borowy-Boroski *et al.,* 2004). This formulation is conjugated to α-tocopherol and polyethylene glycol (PEG) to increase in solubility and uptake in the cell. Conjugation to PEG and the addition of a long aliphatic spacer (greater than or equal to 8 carbons in length) allowed for the formation of amphiphillic nanomicelles that facilitated the solubility of coenzyme Q_{10} in both water and lipids (Borowy-Boroski *et al.,* 2004). PEG was chosen as the hydrophilic component of this formulation while α-tocopherol was chosen as the hydrophobic component. Both PEG and α-tocopherol are commercially available and non-toxic (Borowy-Boroski *et al.,* 2004). This formulation has had great success in protecting neuronal cells as it has been found to prevent cell death induced by oxidative stress *in vitro* (McCarthy *et al.,* 2004; Somayajulu *et al.,* 2005). In addition, WS-CoQ_{10} has been found to prevent Bax-induced destabilization of the mitochondrial membrane, making it an attractive candidate for treating neurodegenerative diseases (Naderi *et al.,* 2006).

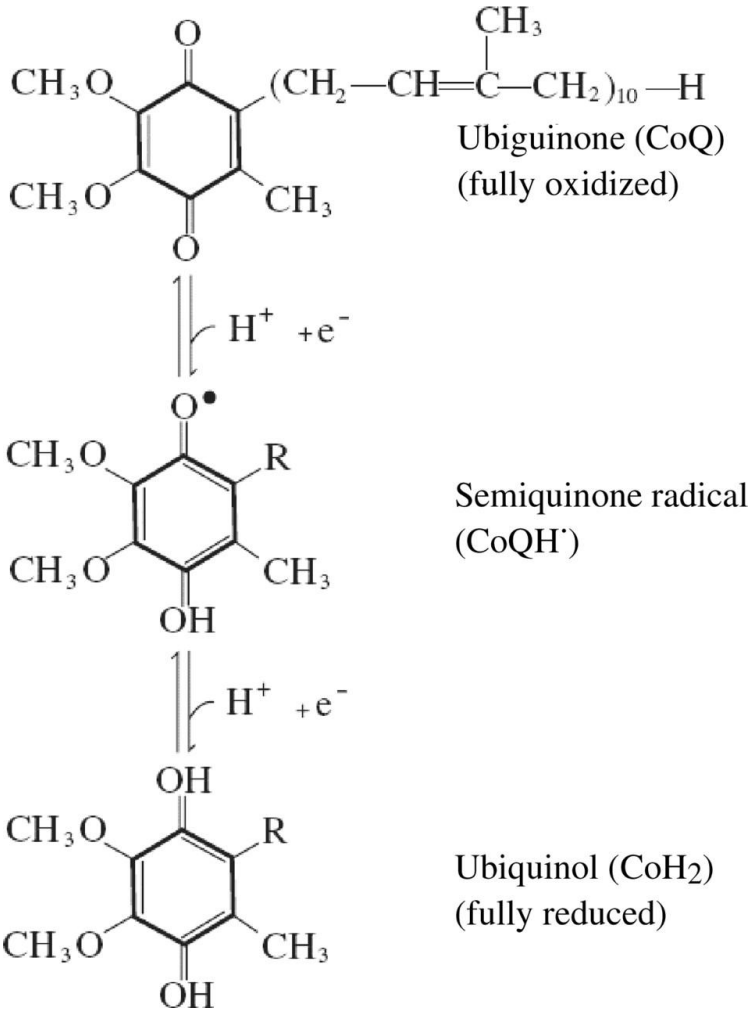

Figure 1.3: Oxidized and reduced forms of CoQ_{10}. Oxidized CoQ_{10} (ubiquinone) can accept an electron to form the semiubiquinone radical which is capable of accepting another electron to form the reduced form of CoQ_{10} (ubiquinol).

1.16 Objectives

Part I: Use of low molecular weight compounds for neuroprotection in preventing neuronal cell death in cases of stroke.

It has been well established that, in cases of stroke, Bax plays a large role in the destabilization of the mitochondrial membrane, eventually leading to apoptosis. We hypothesize that with the use of a low molecular weight compound, we can block and inhibit Bax activity and prevent neuronal cell death in stroke.

Objectives:

1.) Evaluate the efficacy of the anti-Bax inhibitor, Compound 22, in protecting neurons after the incidence of ischemic insult.
2.) Determine the mechanism of action by which Compound 22 acts in neutralizing Bax activity.
3.) Evaluate the ability of Compound 22 to protect neurons in an *in vivo* rat model of ischemia.

Part II: The use of WS-CoQ_{10} in Preventing the Induction of Cellular Senescence in PS-1 Mutated AD Fibroblasts

Oxidative stress is a major culprit in inducing SIPS in the fibroblasts of AD patients. Fibroblasts from AD patients are widely used to study familial forms of this

neurodegenerative disorder, as brain tissue from living patients is inaccessible. The PS-1 mutation in these fibroblasts results in an increased level of oxidative stress, making them more sensitive to oxidative damage, which may translate to neuronal cell death. In this part of my thesis, I have used PS-1 mutated AD fibroblasts obtained from an AD patient to study the effects of WS-CoQ_{10} in preventing the induction of cellular senescence.

Objectives:

1.) Evaluate the ability of WS-CoQ_{10} to prevent cellular senescence in PS-1 mutated AD fibroblasts.

2.) Preliminary studies into the mechanism of action by which WS-CoQ_{10} may be enacting its protective effects.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and Supplies

2.1.1 Chemicals

Most of the chemicals were purchased from Sigma Aldrich Chemical Company, Mississauga, ON, Canada. These chemicals include: Bacto yeast extract, Bacto tryptone, chloramphenicol, arabinose, IPTG, lysozyme, DNase, imidazole, BSA, CHAPS, HEPES, $MgCl_2$, H_2O_2, HRP, EDTA, EGTA, TRIS-HCl, Triton X-100, PHPA, PMSF succinate, trypsin-EDTA, KCl, mannitol, Tween 20, $CaCl_2$, BSA (bovine serum albumin) and horse radish peroxidise-conjugated anti-mouse and anti-rabbit antibodies.

NaH_2PO_4, Na_2HPO_4, DMSO, NaOH, NaCl, DTT, $NaHCO_3$, APS (ammonium persulfate), SDS (sodium dodecyl sulphate), citric acid, and sucrose were purchased from BDH, Toronto Canada. Protein assay reagent, protein marker, TEMED, and acrylamide were obtained from Bio Rad, Ontario Canada. Glycine was purchased from EM Sciences, NJ, USA. Hoechst 33342 and DCFDA (dichlorofluoroscein diacetate) was obtained from Molecular Probes, Eugene OR, USA.

Hematoxylin stain was obtained from Fischer Scientific and Eosin Stain was purchased from Harleco. Mounting media was purchased from Richard Allan Scientific and DAPI and NeuN was obtained from Santa Cruz.

2.1.2 Equipment

Fluorescence measurements were conducted in the SpectraMan Gemini XS multiplate reader (Molecular Devices, Sunnyvale CA USA). Cell culture was performed

under sterile conditions in the class-II type A/B3 Biosafety cabinet (Nuaire). Cells were maintained in an incubator with 5% CO_2 which used a HEPA filter (Thermo Forma). A Dounce homogenizer from Kontes Glass Company (NJ, USA) was used along with, freezer vials (VWR) and Eppendorf pipettes (Fisher Scientific). Phase contrast and fluorescent pictures were taken using an inverted stage fluorescent microscope (Leica DM IRB, Germany) and another fluorescence microscope (Zeiss Axioskope 2 Mot plus, Gottingen, Germany) and fluorescence pictures were taken using a camera (QImaging, Gottingen, Germany). The images were processed using Adobe Photoshop v8.0. Cell culture supplies included culture flasks and dishes, pipettes, freezer vials, and tubes were obtained from Sarstedt Inc, Montreal, Quebec, Canada.

Mini-Protean IV gel electrophoresis apparatus (Biorad Laboratories, Ltd., Mississauga Ontario) was used for protein gel electrophoresis. BioTrace®NT, pure nitrocellulose membrane (PALL Corporation, Pensacola, Florida) was used for immunoblotting.

A pH Meter model 8100 and buffer solutions (VWR) and an Adventurer balance (OHAUS) were used. Absorbance was measured by the Genesys 10 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and centrifugation was done using low speed centrifuge (Jouan) and DESAGA (Sarstedt-Gruppe). Vortex Jr. Mixer (Scientific Industries Inc), a heating block (Gibco BRL, VWR, Canada) and a rocking platform (VWR) were all used as well.

2.1.3 Antibodies

The following antibodies were purchases from Sigma (Mississauga, Ontario): anti-p21 WAF1/Cip1 antibody, anti-MnSOD, anti-p38, anti-actin, anti-mouse HRP, and anti-rabbit HRP. Anti-Bax antibody (Santa Cruz), anti-cytochrome c (Abcam), anti-LC3-II (Novus Biologicals), donkey-anti-rabbit HRP (Abcam), and anti-NeuN (Santa Cruz) were also used.

2.2 Methods

2.2.1 Cell Culture

SH-SY5Y human neuroblastoma cells were obtained from American Type Culture Collection (Manasas, VA) and grown in Dulbelcco's Modified Eagle's Medium with F12 HAM and supplemented with 10% FBS (Sigma, Mississauga), 20 μg/ml L-glutamine (Invitrogen, Burlington Ontario) and 10 μg/ml Gentamycin (Invitrogen, Burlington Ontario) in 5% CO_2 and 37˚C. Presenilin-1 mutated Alzheimer's Disease Fibroblasts (AG09035) were obtained from the Coriell Institute for Medical Research (New Jersey, USA) and grown in Dulbelcco's Modified Eagle's Medium supplemented with 15% FBS (Sigma, Mississauga) and 10 μg/ml Gentamycin (Invitrogen, Burlington Ontario) in 5% CO_2 and 37˚C. Those PS-1 mutated Alzheimer's Disease Fibroblasts grown with or without 50μg/ml WS-CoQ_{10} (Zymes) supplemented in their media.

2.2.2 H_2O_2 Treatment of SH-SY5Y Human Neuroblastoma Cells

SH-SY5Y cells were grown to 70% confluence and treated with 100 µM H_2O_2 for 1 h at 37º C. Following this, the media was replaced with fresh, complete media without H_2O_2 and the cells were incubated for varying time periods and monitored for apoptotic features and protein expression.

2.2.3 Cell Quantification with Trypan Blue Staining

To quantify the number of viable cells in a sample, Trypan blue staining was used. A 1:1 mixture of cell suspension and 0.4% Trypan blue dye (Sigma Chemical Company, Mississauga, Ontario, Canada) was loaded onto a haemocytometer (Hausser Scientific, USA) where blue stained cells were counted as non-viable cells while the unstained cells were counted as viable cells and expressed as cells/ml.

2.2.4 Preparation of Post-nuclear Cytoplasmic Fraction

Cells were first grown to 70% confluence and subjected to mechanical dislodging by scraping with a cell scraper. Cells that were mechanically removed were centrifuged at 500 g for 5min. The media was aspirated off and the cells (pellet) were washed three times in 1% PB (pH 7.4). After this, the pellet was resuspended in hypotonic Buffer (10 mM Tris HCl pH 7.2, 5mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100) and incubated on ice for 5min. The cell suspension was then mechanically homogenized and

centrifuged for 5 min at 500xg at 4º C, following which the supernatant was removed and kept and the pellet was discarded as it consisted of the nuclear fraction.

2.2.5 Isolation of Mitochondria and Post-mitochondrial Cytosolic Fraction

The post-nuclear supernatant that was isolated as described above was centrifuged again at13,000 g at 4º C for 20 min. The pellet obtained consisted of the mitochondrial fraction and the supernatant consisted of the post-mitochondrial cytosolic fraction. The crude mitochondrial pellet was resuspended in reaction buffer (250 mM Sucrose, 1 mM $MgCl_2$, 10 mM HEPES, 20 mM Succinate) and kept on ice and used within 2 h for experimentation.

2.2.6 Protein Estimation

Protein concentration in both cellular lysates and mitochondrial fractions were determined by combining the sample with BioRad reagent and water. The samples were vortexed and incubated for 10 min at room temperature. Absorbance was measured at 595 nm using a UV-Visible Spectrometer and compared to a BSA standard curve.

2.2.7 Measurement of Total Cell ROS

Cells were grown as stated above. ROS production was measured using dichlorofluorescin diacetate (DCFDA) (Sigma, Mississauga) using a modification of a previous procedure (Siraki *et al.,* 2002). At various time points, cells were incubated

with DCFDA to a final concentration of 10 μM for 20 min at 37˚C and fluorescence (Ex. 495 nm and Em. 530 nm) was measured using a Spectra Max Gemini XS multi-well plate fluorescence reader.

2.2.8 Mitochondrial ROS Measurement

SH-SY5Y cells were grown to 70% confluence in 10 mL Petri dishes and treated with 100 μM H_2O_2 and 20 μM compound 22 or compound 9 where appropriate, and mitochondria were isolated as previously described. These mitochondria were analyzed for their levels of ROS using an Amplex Red assay. Isolated mitochondrial pellets were re-suspended in the Amplex Red reaction buffer (2.5 mM malate, 10 mM succinate), Amplex reagent was added to a final concentration of 50 μM, and HRP was added in the ratio of 6 U/ 200 μL. The mixture was incubated at room temperature for 30 min prior to reading the fluorescence at 560 nm excitation and 590 nm emission.

2.2.9 Western Blot

Equal amounts of protein (20 μg) per lane were resolved on a 12% acrylamide gel, separating post-nuclear lysate proteins by SDS-PAGE. Separated proteins were electro-transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk under gentle agitation. The membrane was probed with the appropriate primary antibodies with an overnight incubation at 4° C. The blots were washed with 1 x

TBST (1 mL of Tween 20 (ACP Chemicals) per litre of solution, 1:10 vol. of 10xTBS; 24.2 g/L Tris base, 80 g/L NaCl, up to 1 L with ddH_2O adjusted to pH 7.6. 9:10 vol. of ddH_2O), exposed to HRP conjugated secondary antibodies (Sigma, Mississauga), and developed using a Chemiluminescence Peroxidase Substrate kit (Sigma, Mississauga). The membrane was visualized with Alpha Innotech Corporation Imaging System (San Leonardo, CA). Desitometry was performed using Image J Software and chemiluminescence levels of the sample were compared to those of the control.

2.2.10 WST-1 Cell Viability Assay

Cell viability was measured using the WST-1 Assay. Approximately 12 000 cells were plated on a clear bottom 96 well plate and grown to 70% confluence and treated as required. WST-1 dye was then added and incubated for 4 h at 37˚C. Absorbance at 280 nm was then measured using a Wallac Victor^3 1420 Multilabel Counter.

2.2.11 Bax Association to the Mitochondria

Mitochondria were isolated from SH-SY5Y cells by the methods described above. Samples of mitochondria were incubated with 10 µg of Bax, and 20 µM concentrations of compound 22 or non-specific compound 9 when appropriate at 37˚C for 20 min. The samples were resolved on a 12% SDS-PAGE and transferred to nitrocellulose for western blotting. The samples were probed with a monoclonal anti-Bax antibody in a 1:2000 dilution as previously described in section 2.2.9.

2.2.12 Evaluation of Cytochrome c Release

Cytosolic fractions were isolated from SH-SY5Y cells by the methods described above. The samples were incubated with 10 µg of Bax, and 20 µM concentrations of compound 22 or non-specific compound 9 when appropriate at 37°C for 20 min. The samples were resolved on a 12% SDS-PAGE and transferred to nitrocellulose for western blotting. The samples were probed with an anti-cytochrome c antibody in a 1:2000 dilution as previously described.

2.2.13 SA-β-galactosidase Stain

Treated cells were briefly washed in three times with 1 X PBS solution, fixed for 3-5 min (room temperature) in 3% formaldehyde, followed by three washes with PBS, and incubated at 37°C (no CO_2) with fresh senescence associated β-Gal (SA-β-Gal) stain solution (stock = 20 mg of dimethylformamide per mL), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM Sodium chloride, 2 mM Magnesium chloride. Staining was maximal in 12-16h.

2.2.14 Monodansylcadaverine (MDC) Stain for Autophagy

Cells were plated in a 6 well plate, grown to 70% confluence with or without the presence of 50 µg/ml WS-CoQ_{10}. MDC stain was added along with propidium iodide as

a counter stain and allowed to incubate for 20 minutes at 37˚C. The cells were observed under a fluorescent microscope (Leica DM IRB) for the presence of autophagic vacuoles.

2.2.15 Tetramethyl Rhodamine Methyl Ester (TMRM) Stain for Mitochondrial Potential

Cells were plated in a 6 well plate, grown to 70% and treated as mentioned above. TMRM stain was added along with Hoechst 33342 as a counter stain and allowed to incubate for 20 min at 37˚C. The cells were observed under a fluorescent microscope (Leica DM IRB) for the presence of punctate staining.

2.2.16 Brain Ischemia and Reperfusion Model

Global forebrain ischemia was induced in Long Evans Hooded rats using the bilateral carotid artery occlusion and hypovolemic hypotension (2VO/HT) model of Smith *et al* (1984). Male Long Evans rats between the weights of 250-300 g were used and those in the compound treated therapeutic group were injected intraperitonially (IP) with 15 mg/kg of compound 22. The rats were then anaesthetized using 5% halothane and anaesthesia was maintained using 2% halothane with a facemask through the duration of the surgery. Rectal temperature was maintained at 37 +/- 0.5˚C using a homeostatic temperature blanket (Harvard Apparatus) for the first hour of reperfusion. Blood gas measurements ensured a pH of 7.4 and pO2 of 80 mm Hg via arterial access. Blood was withdrawn into a 10-ml syringe to a MAP of 50 mm Hg, and carotids were

clamped by using microaneurysm clips. Blood was further withdrawn to maintain the MAP at 40 mm Hg for the10-min duration of ischemia. After ischemia, blood was re-infused at a rate of 5 mL/min. All cut down wounds were sutured, and anaesthesia and temperature control were maintained for 1 h after surgery.

2.2.17 Brain Tissue and Slide Preparation

Harvested brains were placed in 70% ethanol overnight. They were then subjected to the following dehydration solutions: 80% ethanol for 1 h, 95% ethanol for 1 h, 100% ethanol for 1 h (3X), 40 min of xylene (2X). The brains were then placed in paraffin wax and left to sit overnight in a 60°C water bath. The brains were then placed in metal moulds and covered with paraffin wax and left to cool for approximately 4 hours at room temperature. The moulds were then placed onto the microtome and cut at 10 micron sections. These sections were then placed into a 43.5°C water bath and then transferred to Superfrost slides and left to dry overnight at room temperature.

2.2.18 Hematoxylin and Eosin Staining

Slides containing the sectioned brains were subjected to deparaffinization in xylene for 14 min (2X) and then re-hydrated using 100% ethanol for 10 min, 95% ethanol for 5 min, 70% ethanol for 5 min, and dH_2O for 5 min. The slides were then placed in hematoxylin stain for 3 min and then washed with dH_2O for 5 min. They were then

rinsed in an acid alcohol bath (0.2 mL of 12 M HCl in 100 mL of 70% ethanol) twice and then rinsed with dH_2O. The slides were placed in eosin for 1 min and then rinsed in dH_2O and placed in 95% ethanol for 10 min, 100% ethanol for 10 min, and xylene for 10 min (2X). The slides were then coverslipped using Cytoseal mounting media.

2.2.19 NeuN Staining

Slides of brain sections prepared as described above were immersed in xylene for 7 min (2X), 95% ethanol for 5 min (2X) dH_2O for 5 min, and 50 mM TBS for 5 min. Antigen retrieval was performed by placing the slides in 70°C 10 mM sodium citrate buffer (pH 6.0) for 15 min. The slides were allowed to cool for 10 min at room temperature then placed in TBS for 5 min. The sections were incubated with Dako universal blocking solution for 30 min in a humid chamber at room temperature. Excess blocking solution was removed and the sections were incubated with NeuN antibody (1:100 dilution using Dako antibody dilutent) overnight at 4°C. The slides were then washed with 50 mM TBS for 15 min and dehydrated in 95% ethanol for 5 min (2X) and xylene for 10 min before being cover slipped using mounting media containing DAPI and sealed with clear fingernail polish. Slides were stored at -20°C.

2.2.20 Expression of Recombinant Bax

A culture of *E.coli* transformed with the gene for Bax protein harbouring a His_6-tag was obtained from Bruno Antonsson and used to inoculate a 100 mL of a 1 L stock of L.B. media (10 g NaCl, 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 1 L ddH_2O, and 30 µg/and additional 900 mL stock L.B. media. The culture was then placed in the 37º C shaker for an additional 3-4 h, following which the OD_{280} was ensured to be between 0.40-0.75 using UV/VIS before induction with Arabinose (1 g/L). This final culture was then incubated overnight on the shaker at 37º C before centrifugation at 12,500 x g for 15 min at 4º C. The supernatant was discarded and the pellets were stored at -20º C overnight for subsequent purification.

2.2.21 Purification of Recombinant Bax

Prior to purification, the *E. coli* pellets expressing Bax were re-suspended in a lysis/loading buffer (0.02 M phosphate buffer (NaH_2PO_4, Na_2HPO_4), 100 µg/mL lysozyme, 5µg/mL DNase, 350 µg/mL PMSF, 1% Triton X-100, and 0.05M imidazole) and incubated on ice for 45 min. Following the incubation, the re-supended pellets were sonicated at 4º C, centrifuged at 12,500 x g for 15 min at 4º C and the supernatant was kept as it contained the protein of interest. The culture was purified using a Hi-Trap nickel chelating affinity column (GE Healthcare, Baie d'Urfé, QC, Canada). The

column was prepared by running 20 mL of ddH_2O, followed by 4 mL of a 0.1 M Nickel Sulfate solution, 20 mL of ddH_2O, and lastly 30 mL of the loading buffer to equilibrate the column. The supernatant was then loaded onto the column and washed with the loading buffer to elute any non-specific proteins that may be bound to the column. The fractions were collected (5 mL/fraction) and the absorbance was determined using UV/VIS. This absorbance was monitored and once the values significantly decreased indicating the removal of the loosely-bound non-specific proteins, 10 mL of the elution buffer (0.02M Phosphate buffer, 0.5M imidazole) was added. Fractions were collected in 1 mL increments and the absorbance was read again to determine the fractions that contained the highest amounts of desired protein. These fractions were placed in dialysis tubing (MWCO 6000-8000 Da) overnight followed by lyopholization and re-suspension in 5 mL of ddH_2O and EDTA to a final concentration of 0.05 mM. The purity of Bax was confirmed via Western Blot (Antonsson *et al.*, 2001).

CHAPTER III

RESULTS

Part I: Use of low molecular weight compound 22 in preventing cell death in cases of stroke

3.1.1 C22 Blocks Bax Activity In Isolated Mitochondria

Part of Bax's activity is its ability to generate ROS. We wanted to determine whether C22 was able to lower the amount of Bax-induced ROS production. Purified Bax protein has been shown to destabilize isolated mitochondria leading to an increased production of ROS (Naderi *et al.*, 2006). C22's ability to inhibit Bax induced production of ROS was investigated. Isolated mitochondria from SH-SY5Y cells were incubated with Bax either in the presence or absence of different compounds and the ROS generated was measured as described in Material and Methods. The results shown in figure 3.1indicate that Bax addition caused an increase in the production of ROS when incubated with isolated mitochondria. Non-specific compound 9 (C9) did not show any significant effect on Bax activity. C22 on the other hand showed a decrease in the Bax-induced ROS production from the mitochondria, indicating C22 was able to block Bax activity (Figure 3.1). Since non-specific compound 9, did not show any drastic decrease in the amount of ROS production, the inhibitory effect of C22 was due, at least in part, to Bax inactivation.

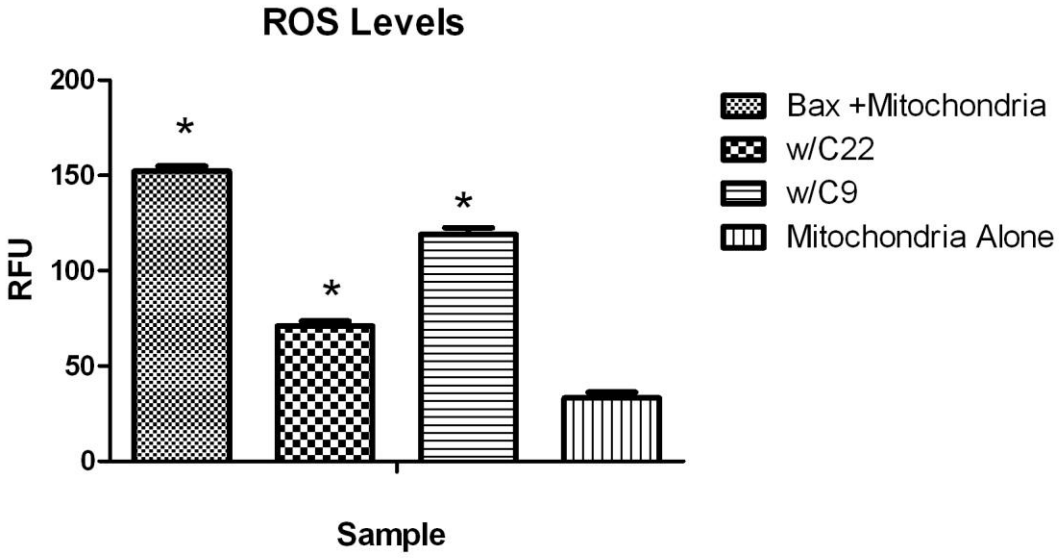

Figure 3.1 C22 inhibits Bax activity: Isolated mitochondria from SH-SY5Y cells were incubated with 10 μg of Bax in the presence and absence of 20 μM concentrations of Compound 22. The ROS production was measured using Amplex Red and HRP substrate at 560 nm excitation and 590 emission and measured at RFUs/150 μg of protein. Compound 22 was shown to decrease Bax activity by significantly decreasing the amount of ROS produced when combined with Bax and isolated mitochondria (*p<0.0001 vs. Mitochondria Alone).

3.1.2 C22 Prevents Association of Bax to the Mitochondria.

As previously shown, C22 is able to block Bax activity by significantly decreasing the level of ROS produced. Next we wanted to investigate the mechanism of this inhibition. Functional Bax oligomers can associate with mitochondrial membranes upon activation, causing destabilization of the mitochondria. In these cases, Bax is known to form a pore in the mitochondrial membrane and facilitate the release of cytotoxic molecules into the cytosol of the cell. We wanted to know if binding of C22 to Bax may inhibit its ability to associate with the mitochondria. Mitochondria isolated from SH-SY5Y cells were incubated with purified Bax for 20 min either with or without the presence of C22 and non-specific compound 9. Using western blot analysis, the samples were probed with anti-Bax antibody and it was found that the mitochondria combined with C22 resulted in a lower amount of Bax bound to the mitochondrial membrane as can be seen by figure 3.2. This finding indicates that C22 may be playing a role in preventing apoptosis by inhibiting Bax from associating to the mitochondrial membrane, and thus, preventing pore formation and mitochondrial membrane destabilization.

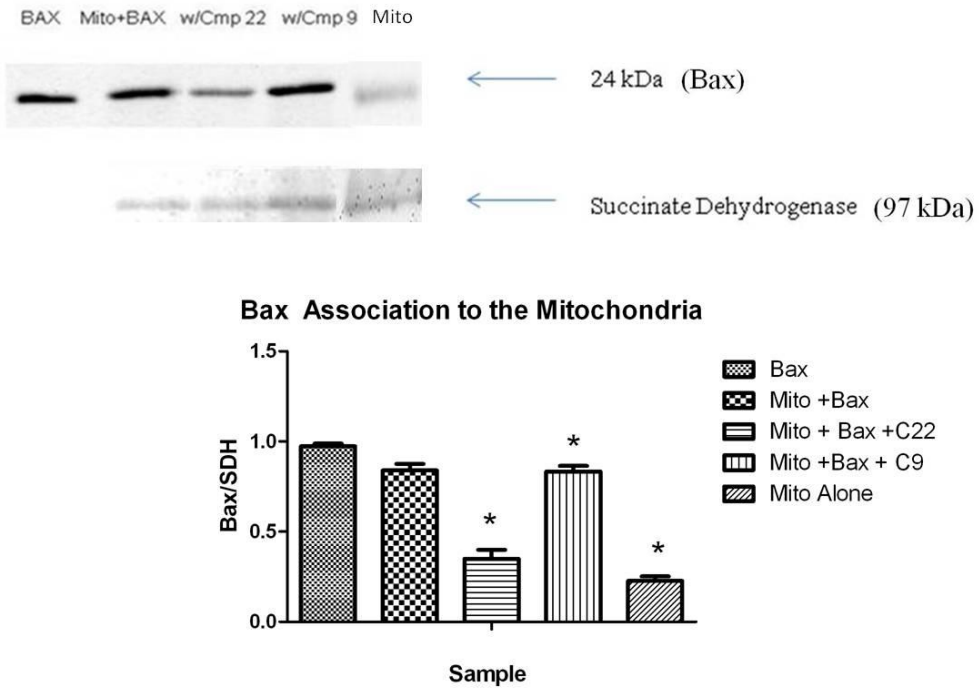

Figure 3.2 C22 inhibits Bax association to the mitochondria: Mitochondria from SH-SY5Y cells were isolated and combined with 10 μg Bax and 20 μM concentrations of C22 and compound 9 for 20 min. The samples were then resolved using SDS-PAGE and then probed with anti-Bax antibody for western blotting. It was found that C22 was able to prevent Bax from associating to the mitochondria. Non-specific compound 9 did not affect the association of Bax to the mitochondria. Succinate dehydrogenase was used as a loading control (*p<0.0001 vs. Bax + Mitochondria).

3.1.3 C22 Inhibits Cytochrome c Release From the Mitochondria

Since we found that C22 was able to prevent Bax association to the mitochondrial membrane we wanted to determine whether or not C22 was able to prevent the release of cytotoxic factors from the mitochondria into the cytosol. One potential factor released from the mitochondria is cytochrome c. Upon entrance into the cytosol of the cell, cytochrome c is able to complete the formation of the apoptosome, which subsequently leads to caspase activation, and ultimately apoptosis. Mitochondria isolated from SH-SY5Y cells were incubated with purified Bax for 20 min either with or without the presence of C22 and non-specific compound 9. Western blot analysis was performed with an anti-cytochrome c antibody to detect the amount of cytochrome c present in the supernatant. As can be seen in figure 3.3, C22 was able to lower the amount of cytochrome c present in the supernatant fraction. In fact, C22 was almost able to lower the amount of supernatant cytochrome c to levels comparable to the control where no additional Bax was added. This result indicates that C22 is able to prevent the release of cytochrome c into the cytosol of the cell, and thus, lowering the potential for apoptosis to occur.

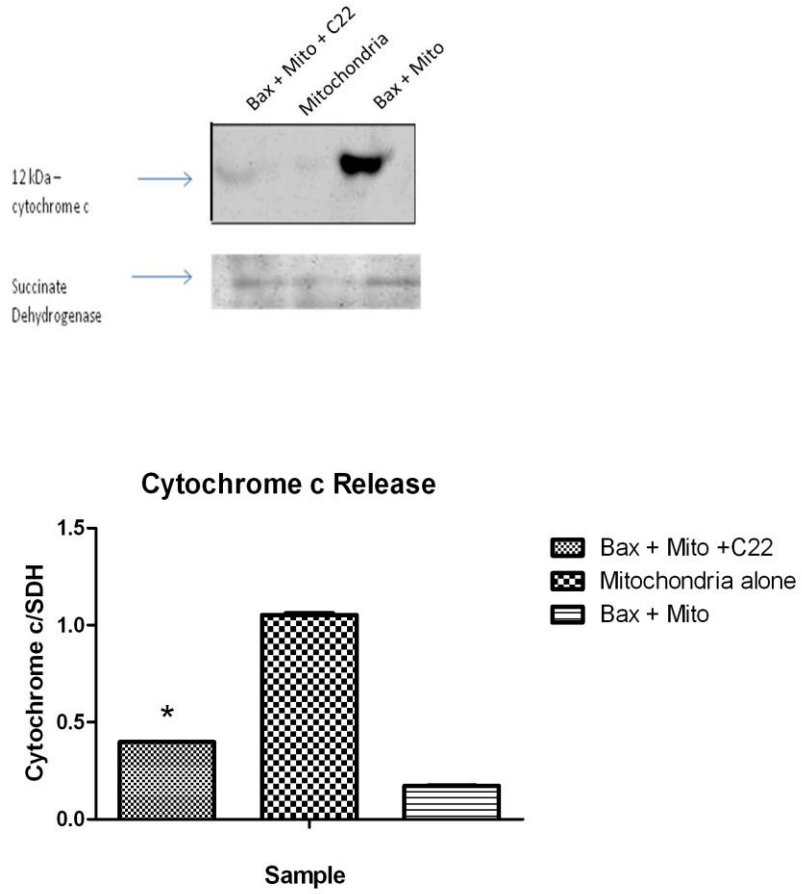

Figure 3.3 C22 inhibits cytochrome c release from the mitochondria: Mitochondria from SH-SY5Y cells were isolated and combined with 10 μg of Bax and 20 μM concentrations of C22 and compound 9 for 20 min as described in the methods section. The samples were then resolved using SDS-PAGE and then probed with anti-cytochrome c antibody. It was found that C22 was able to prevent cytochrome c from being released into the cytosol. Succinate dehydrogenase was used as a loading control to indicate equal loading of the mitochondrial reaction mixture (*p<0.0001 vs. Bax + Mitochondria).

3.1.4 C22 Maintains Cell Viability in Cells Undergoing Oxidative Stress

We have found that C22 was able to decrease the activity of Bax and prevent the release of cytotoxic factors from the mitochondria into the cytosol. We next wanted to investigate whether C22 is able to maintain cell viability after cells are subjected to oxidative stress. To accomplish this, the WST-1 cell viability assay was performed. In this assay, if the cells are viable, WST-1 dye is metabolized by cellular dehydrogenases to form formazen which is yellow in colour. In the case of viable cells, more yellow colour is seen as the cells have a greater ability to metabolize the WST-1 dye than those cells that are not viable. SH-SY5Y cells were treated with hydrogen peroxide with or without the presence of C22 or non-specific compound 9 as described in the methods. After incubation with the WST-1 dye, it was seen that C22 was able to increase the level of absorbance observed as compared to the hydrogen peroxide treated samples without compound treatment and those cells treated with non-specific compound 9 (figure 3.4). This indicates that under conditions of oxidative stress, C22 is able to maintain cell viability and preserve the metabolic ability of human neuroblastoma cells. C22 was also shown to increase viability in cells that were not subjected to hydrogen peroxide treatment. This could be due to C22 inhibiting any Bax-induced apoptosis that may be naturally occurring in these cells.

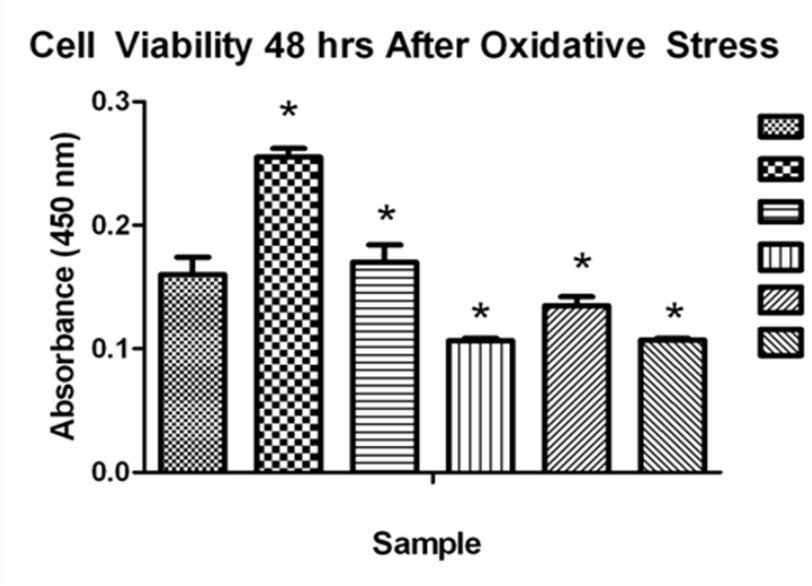

Figure 3.4 C22 maintains cell viability in cells undergoing oxidative stress: Approximately 1.20 x 10^4 SH-SY5Y cells were plated in a 96 well plate and treated for one hour with 150 μM hydrogen peroxide. Fresh media absent of hydrogen peroxide was added after an hour and the cells were allowed to grow for 48 hours. WST-1 dye was then added and allowed to incubate for 4 hours until absorbance was taken at 450 nm. It was found that C22 was able to maintain cell viability while non-specific compound 9 was not. The experiment was repeated 3 times, each time in triplicate (*p< 0.002 vs. Control).

3.1.5 C22 Protects Neurons in an *In Vivo* Rat Model of Stroke

Cell culture work done with C22 indicates that it is able to decrease the amount of Bax-induced oxidative stress in human neuroblastoma cells, and stabilize mitochondria to prevent the release of cytotoxic factors into the cytosol of these cells. In addition, C22 was shown to maintain cell viability in these cells placed under conditions of oxidative stress. Next, we wished to investigate whether C22 had neuroprotective effects in an *in vivo* model of stroke. In order to do this, Long Evans Hooded rats were subjected to global ischemia via the bilateral carotid artery occlusion and hypovolemic hypotension (2VO/HT) model. In this case the carotid arteries of these rats are clamped off for a duration of 10 minutes, effectively halting blood flow to the brain. This simulates the onset of stroke induced by a blood clot (please refer to the methods section for a more detailed explanation of the surgical procedure). One group of rats received no treatment, while another was administered 15 mg/kg of C22 via IP injection before induction of stroke. After 7 days the rats were sacrificed and their brains were harvested and sectioned for neuronal staining.

Brain sections of 10 microns were stained with Haematoxylin and Eosin (H&E) stain and NeuN stain to evaluate the ability of C22 to offer neuronal protection. H&E stain is a widely used stain to evaluate the damage done to cells (Schmued *et al.*, 1997). It distinguishes the nuclei from the cytoplasm by staining the nuclei blue and the

cytoplasm pink. It was found that rats that were administered C22 prior to stroke induction had more viable cells in the CA1 region of the hippocampus, (the area of the brain affected during stroke responsible for spatial awareness, memory, and informational output to other areas of the brain (Koehl &Abrous, 2011) compared to rats that were not administered C22 (figure 3.5). This indicates that C22 may have a protective effect in an *in vivo* model of stroke.

NeuN is a neuronal-specific marker used to detect viable neurons (Mullen *et al.*, 1992, Xu e*t al.*, 2002, Hassen *et al.*, 2004). NeuN stain was used to specifically detect viable neurons in the brain sections after stroke. It was found that those rats treated with C22 prior to stroke showed a more prominent NeuN staining profile, thus confirming the presence of viable and intact neurons (figure 3.6). Those rats without C22 treatment showed a prominent decrease in NeuN staining, indicating a decrease in the number of viable neurons. Taken together, the increased levels of cells present in the CA1 region, shown by H&E staining, and the amount of viable neurons preserved, as confirmed by NeuN staining, indicates that C22 may have the ability to protect neurons in an *in vivo* model of stroke.

Untreated C22 Treated

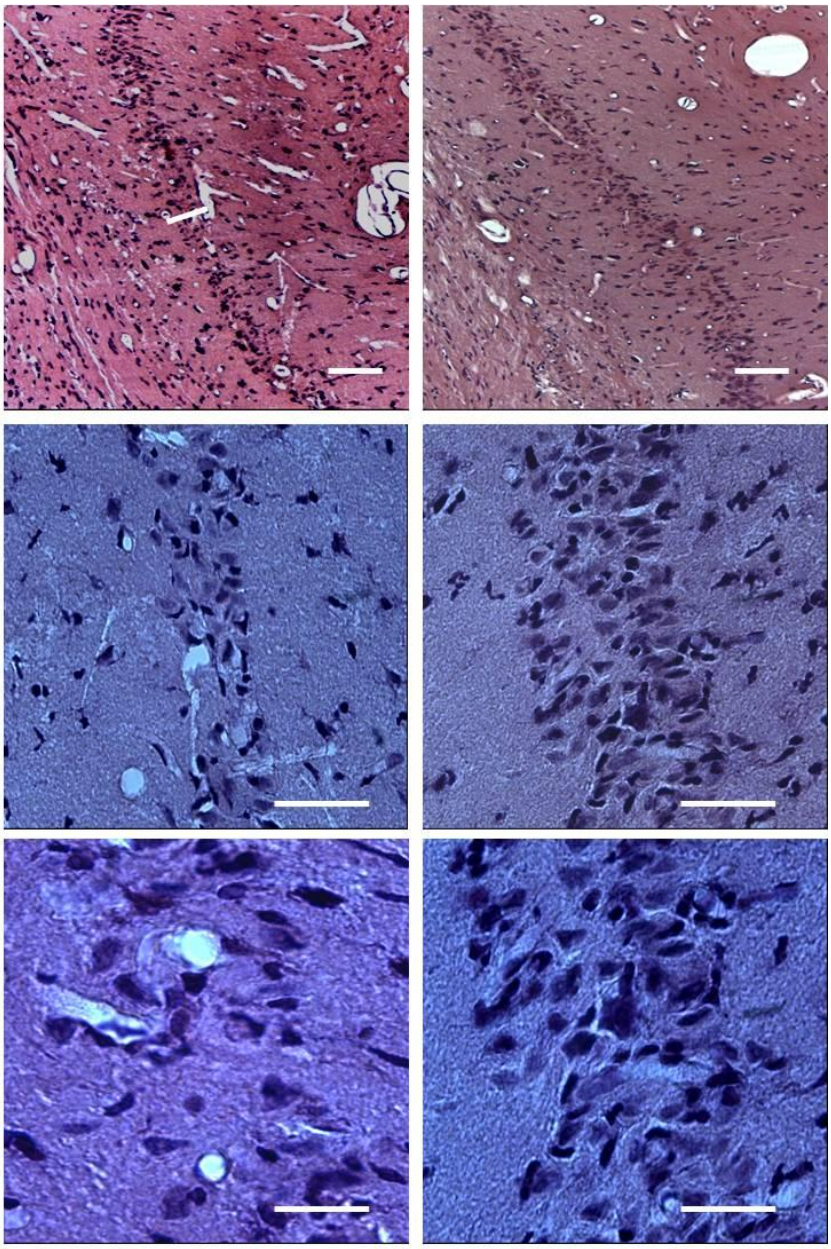

Figure 3.5 H&E staining shows that C22 results in neuroprotection in an *in vivo* model of stroke: Brains were harvested from Long Evans Hooded rats that were

subjected to the 2VO/HT model of ischemia. The brains were sectioned into 10 micron slices and placed on slides and then underwent H&E staining. Images were obtained at 10X, 40X, and 63X magnification of the CA1 region of the hippocampus. It was found that rats that were injected intraperitonially with C22 prior to induction of ischemia appeared to have more viable cells than those that did not receive the C22 treatment (3 rats per group were used in this study; scale bars are 170 microns in length).

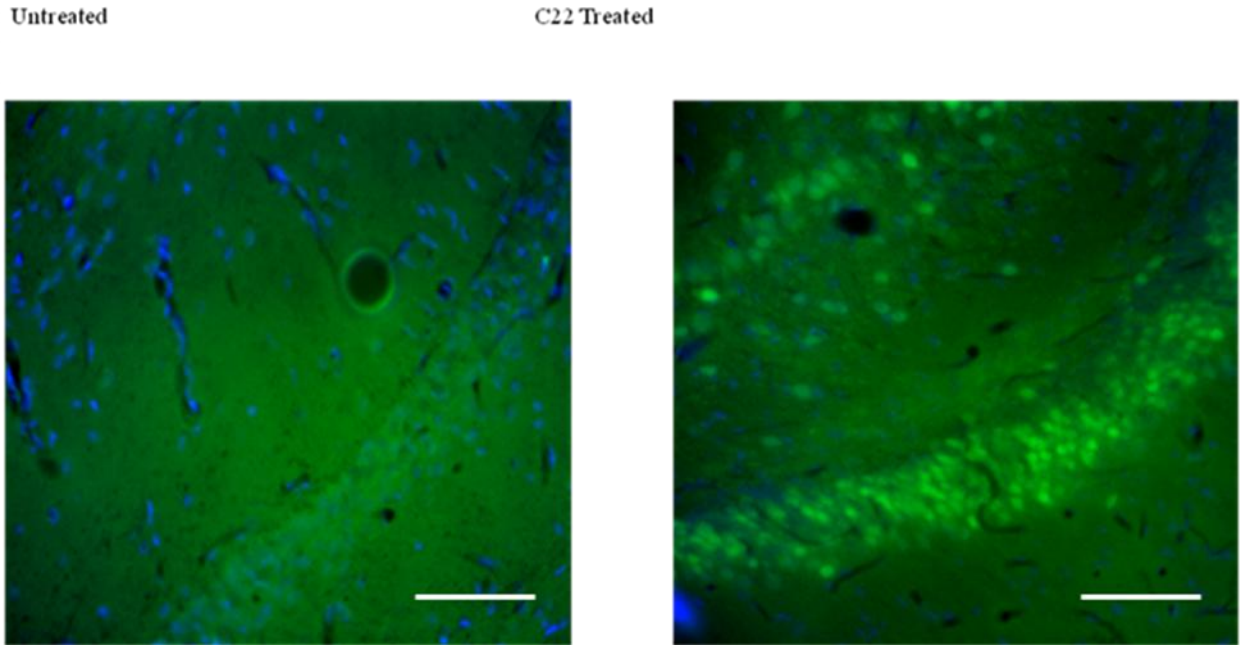

Figure 3.6 NeuN staining shows that C22 protects neurons in cases of stroke: Brains were harvested from Long Evans Hooded rats that were subjected to the 2VO/HT model of ischemia. The brains were sectioned into 10 micron slices and placed on slides and then stained with NeuN. Images were obtained at 10X magnification in the CA1 region of the hippocampus. It was found that those rats that were treated with C22 prior to induction of ischemia appeared to have more viable neurons, as indicated by an increase in NeuN stain, than those that did not receive the C22 treatment (3 rats per group were used in this study; scale bar is 70 microns).

Part II: The use of WS-CoQ_{10} to prevent cellular senescence in presenilin-1 mutated Alzheimer's Disease fibroblasts

3.2.1 WS-CoQ_{10} Decreases ROS Generation in AD Fibroblasts

In the case of PS-1 mutated AD fibroblasts, the cells have an increased level of ROS production which eventually results in them entering a state of premature cellular senescence (Toussaint, 2000). It is not known by what exact mechanism the PS-1 mutation causes this increase in oxidative stress, but it is this increased level of endogenous oxidative stress that causes the AD fibroblasts to undergo SIPS (Toussaint, 2000). If this oxidative stress could to be lowered, the PS-1 mutated fibroblasts would have a lower probability of being pushed towards a state of cellular senescence and could instead continue to divide. To investigate whether WS-CoQ_{10} is able to lower the amount oxidative stress, the presence of ROS was measured. Cells grown with and without the presence of WS-CoQ_{10} were measured for ROS generation using DCFDA as described in the methods section. It was found that WS-CoQ_{10} was able to lower the amount of ROS produced in these AD fibroblasts, thus lowering the burden of oxidative stress induced by the PS-1 mutation (Fig. 3.7). This indicates that WS-CoQ_{10} is able to lower the increased levels of oxidative stress.

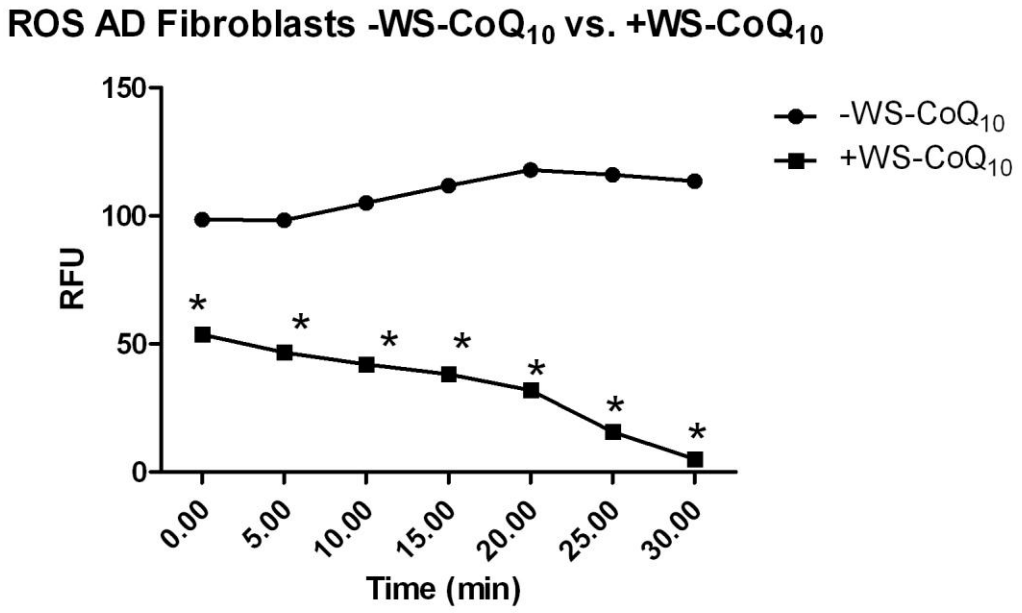

Figure 3.7 WS-CoQ_{10} decreases ROS levels in PS-1 mutated AD fibroblasts: PS-1 mutated AD fibroblasts were grown with and without the presence of WS-CoQ_{10} and ROS production (RFU = relative fluorescence units) was measured using DCFDA for a period of 30 minutes. It was found that WS-CoQ_{10} resulted in a decrease in the amount of ROS generated by the PS-1 mutated AD fibroblasts (*p<0.0002 vs. –WS-CoQ_{10}).

3.2.2 WS-CoQ_{10} Prevents Senescence in PS-1 Mutated AD Fibroblasts

Since WS-CoQ_{10} was able to lower the unusually high level of endogenous ROS generated by the PS-1 mutated AD fibroblasts, we wanted to investigate whether WS-CoQ_{10} was able to also inhibit SIPS from occurring. In order to evaluate the ability of WS-CoQ_{10} to prevent PS-1 mutated AD fibroblasts from entering a premature state of senescence, senescence-associated β-galactosidase expression was measured using an X-gal stain; only those cells in a state of senescence will stain blue. As can be seen in figure 3.8A, those cells without WS-CoQ_{10} have more cells stained blue, indicating senescence. However, when WS-CoQ_{10} is added to the PS-1 mutated AD fibroblasts there is a marked decrease in the number of cells with positive x-gal stain, indicating that WS-CoQ_{10} has the ability to prevent senescence from occurring. Senescent cells have a larger, and more flattened morphology than their normal counterparts. WS-CoQ_{10} was also found to decrease the size of the cells and resulted in cells with normal morphology. This indicated that WS-CoQ_{10} is able to decrease the amount of cells that undergo SIPS (figure 8B), thus allowing these cells to still maintain their replicative potential.

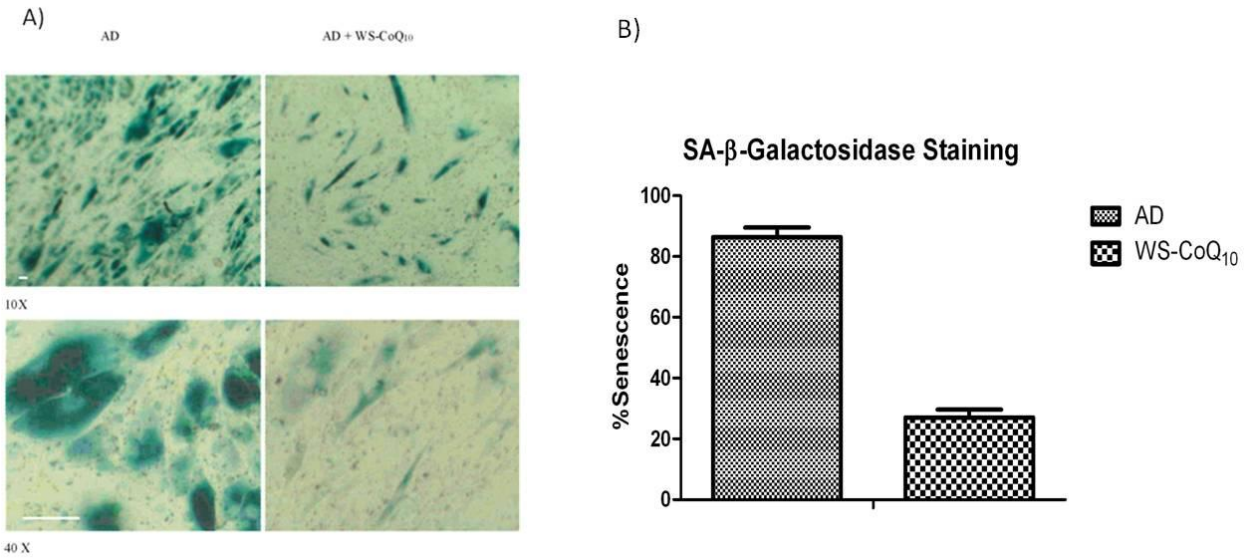

Figure 3.8 WS-CoQ_{10} decreases senescence in PS-1 mutated AD fibroblasts: A) PS-1 mutated AD fibroblasts were grown with or without the presence of WS-CoQ_{10} and senescence-associated β-galactosidase expression was evaluated using an X-gal stain. Images were obtained at 10X magnification (top panel) and 40X magnification (lower panel). It was found that those cells grown in the presence of WS-CoQ_{10} showed less X-gal (blue) stain and an absence of a larger morphology characteristic of senescence, indicating a decreased level of cellular senescence. B) Graphical representation of the amount of senescent cells observed in cells grown with and without the addition of WS-CoQ_{10}. Results were based on 2 separate experiments with 5 views/experiement. Scale bars represent 15 microns.

3.2.3 Treatment with WS-CoQ_{10} Increases Cell Proliferation in PS-1 Mutated AD Fibroblasts

Since we determined that WS-CoQ_{10} was able to have a significant impact in preventing cellular senescence and decreasing ROS production, we next investigated whether WS-CoQ_{10} has a positive effect on cell proliferation in PS-1 mutated AD fibroblasts when compared to those cells grown in the absence of 50μg/ml WS-CoQ_{10}. Approximately 6.0 x 10^5 cells were grown with and without the addition of WS-CoQ_{10}. Cells counts were performed using Trypan blue after 48 hrs, 72 hrs, and 96 hours. As can be seen in figure 3.9, it was found that the addition of WS-CoQ_{10} resulted in an increased cell proliferation rate as compared to those cells grown in the absence of WS-CoQ_{10}, indicating WS-CoQ_{10} is able to increase the population doubling of PS-1 mutated AD fibroblasts and prevent the induction of premature cellular senescence.

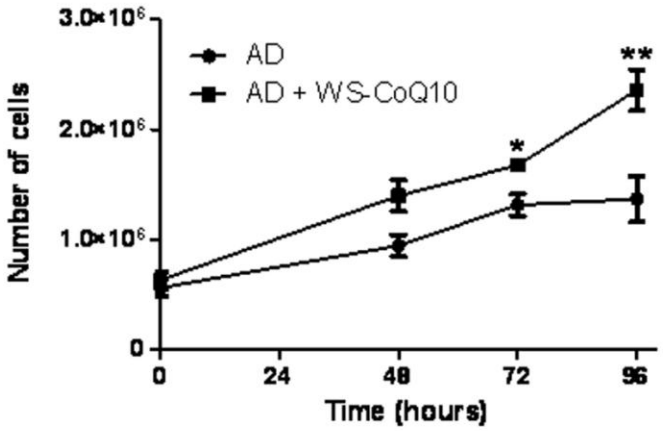

Figure 3.9 WS-CoQ_{10} increases population doubling in PS-1 mutated AD fibroblasts: PS-1 mutated AD fibroblasts were grown with or without the presence of WS-CoQ_{10}. At 48 hours, 72 hours, and 96 hours, the cells were counted using Trypan blue to determine the population doubling potential of these PS-1 mutated AD fibroblasts. It was found that those cells grown in the presence of WS-CoQ_{10} were able to maintain their population doubling potential
(* $p<0.05$, ** $p<0.01$ vs. AD).

3.2.4 WS-CoQ_{10} Maintains Cell Viability in PS-1 Mutated AD Fibroblasts

We found that WS-CoQ_{10} is able to maintain cell proliferation and inhibit senescence. What we next wanted to investigate was whether WS-CoQ_{10} had any positive effect on maintaining cell viability. We used the WST-1 based colorimetric cell viability assay, measuring cell viability as a function of cell metabolism, to measure the viability of these cells that were grown with and without the presence of WS-CoQ_{10}. It was found that those cells grown in the presence of WS-CoQ_{10} showed a higher level of absorbance than those not exposed to WS-CoQ_{10}. Since in this assay, absorbance is directly related to viability, WS-CoQ_{10} was found to maintain the metabolic activity of the cells and allow them to remain viable (figure 3.10).

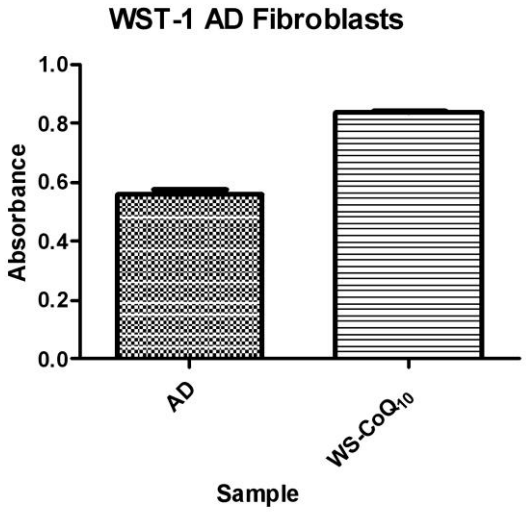

Figure 3.10 WS-CoQ_{10} increases cell viability in PS-1 mutated AD fibroblasts: Approximately 1.2 x 10^4 PS-1 mutated AD fibroblasts were plated in a 96 well clear bottom plate and grown with or without WS-CoQ_{10}. WST-1 dye was then added and allowed to incubate for 4 hours until absorbance was taken at 450 nm. It was found that WS-CoQ_{10} was able to maintain cell viability in these PS-1 mutated AD fibroblast.

3.2.5 WS-CoQ10 Decreases the Levels of p21 and p38 and Increases the levels of MnSOD in AD Fibroblasts

Since it was evident that WS-CoQ_{10} was able to inhibit cellular senescence and maintain a cellular growth rate higher than those cells grown in the absence of WS-CoQ_{10}, we investigated the effects that WS-CoQ_{10} had on the expression of the senescence-initiating proteins p21 and p38, and well as the radical quenching molecule manganese superoxide dismutase (MnSoD). In PS-1 mutated fibroblasts there is an increased level of oxidative stress that causes these cells to become blocked in the G_1 phase of the cell cycle and experience an increased expression of the cell cycle inhibitor CDKI $p21^{Waf\text{-}1}$, effectively halting any further replication (Dumont *et al.*, 2000). In addition, MnSOD plays an important role in the replicative ability of these cells by quenching the excess oxidative stress produced, thus decreasing the potential for them to undergo SIPS. In order to investigate whether WS-CoQ_{10} has an effect on the expression of these proteins PS-1 mutated AD fibroblasts were grown with or without WS-CoQ_{10} and after 24 hours cellular lysates were isolated and the presence of p21, p38, and MnSOD were assessed using western blot analysis. It was found that WS-CoQ_{10} was able to decrease the levels of p21 in both young and old AD fibroblasts (figure 3.11) and decrease the level of phosphorylated p38 in young AD fibroblasts (figure 3.12). These preliminary results again indicating that WS-CoQ_{10} was able to play a role in preventing senescence from occurring by down regulating the expression of these senescence-

initiating proteins. On the other hand, WS-CoQ_{10} was able to cause an increase in the expression of MnSOD in passage 31 ("old") PS-1 mutated AD fibroblasts, but had no significant effect on the levels of MnSOD in passage 16 ("young") PS-1 mutated AD fibroblasts (figure 3.13).

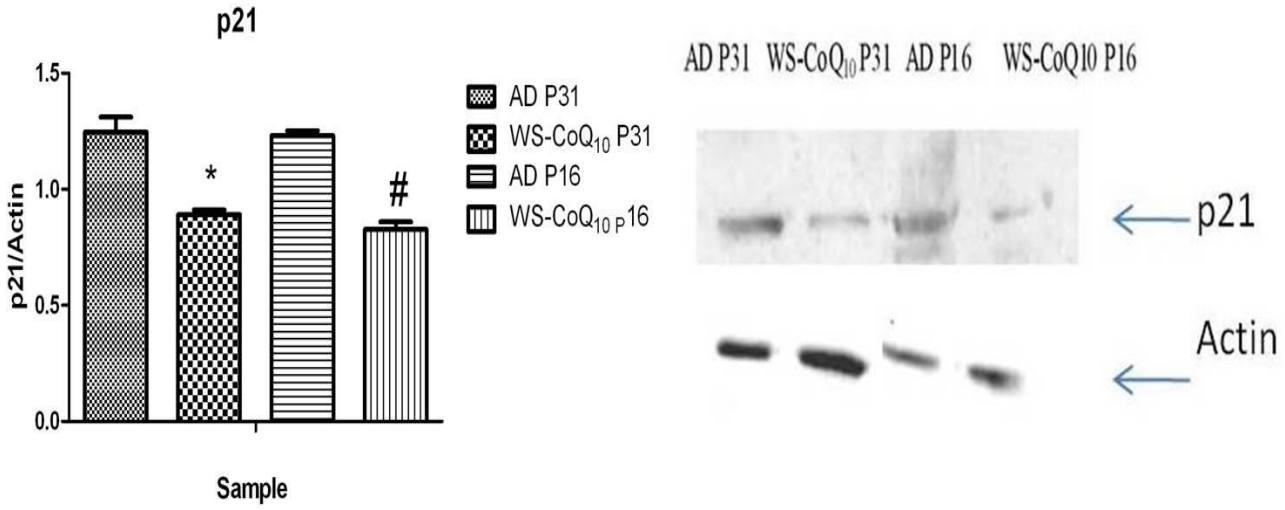

Figure 3.11 WS-CoQ_{10} decreases levels of p21: PS-1 mutated AD fibroblasts (population doubling 31 (P31) referred to as 'old' and population doubling 16 (P16) referred to as 'young') were grown both in the presence and absence of WS-CoQ_{10}. Cell lysates were analyzed for the presence of the p21 protein via western blot analysis. p21 is up-regulated in cellular senescence. Actin was used as a loading control (*p<0.01 vs. AD P31, #p<0.0001 vs. AD P16).

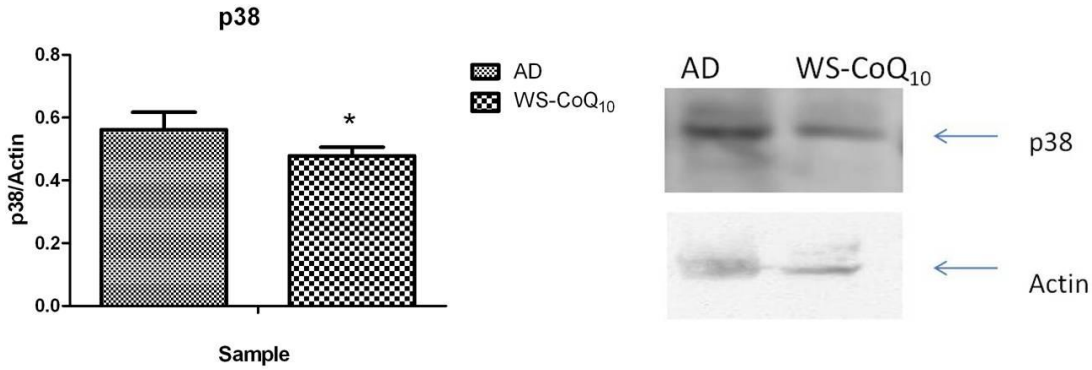

Figure 3.12 WS-CoQ_{10} decreases the level of p38: PS-1 mutated AD fibroblasts (population doubling 18) were grown both in the presence and absence of WS-CoQ_{10}. Cell lysates were analyzed for the presence of the phosphorylated p38 protein via western blot analysis. p38 is up-regulated during the occurrence of senescence. Actin was used as a loading control (*p<0.0001 vs. AD).

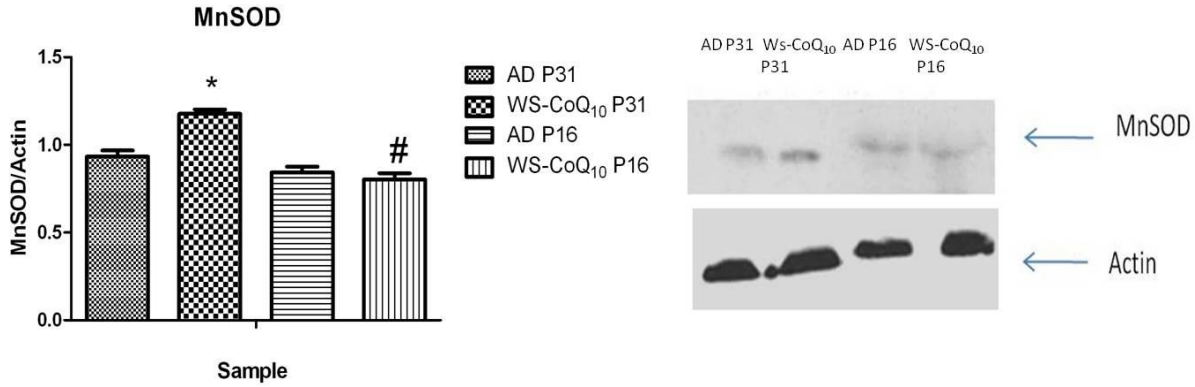

Figure 3.13 WS-CoQ_{10} increases levels of MnSOD: PS-1 mutated AD fibroblasts (population doubling 31 (P31) referred to as 'old' and population doubling 16 (P16) referred to as 'young') were grown both in the presence and absence of WS-CoQ_{10}. Cell lysates were analyzed for the presence of MnSOD via western blot analysis. Actin was used as a loading control (*p<0.0009 vs AD P31, #p<0.0001 vs. AD P16).

3.2.6 Mitochondrial Membrane Potential Remains Intact With and Without WS-CoQ_{10} Treatment

In order to determine whether the mitochondrial membrane remains stable in PS-1 mutated AD fibroblasts TMRM stain was conducted. We wished to determine whether the PS-1 mutation had any destabilizing effects on the mitochondrial membrane and whether or not WS-CoQ_{10} may be eliciting its protective effects by maintaining the mitochondria membrane potential. TMRM stain accumulates in mitochondria if the mitochondrial membrane potential is intact. Positive staining is seen by red punctate stain accumulating within the cells. We grew PS-1 mutated AD fibroblasts both with and without the presence of WS-CoQ_{10} and subjected them to TMRM stain to assess mitochondrial membrane potential. As can be seen in Figure 3.14, there is no significant difference in TMRM staining between the WS-CoQ_{10} treated group and the untreated cells. This indicates that the PS-1 mutation is not creating a detrimental effect on the mitochondrial membrane, as the mitochondrial membrane potential remains intact. Hoechst was used as a counter stain to determine if any apoptosis was occurring. Hoechst staining confirmed that neither groups of these cells were undergoing apoptosis as both groups were absent of brightly stained, condensed nuclei that is indicative of apoptosis.

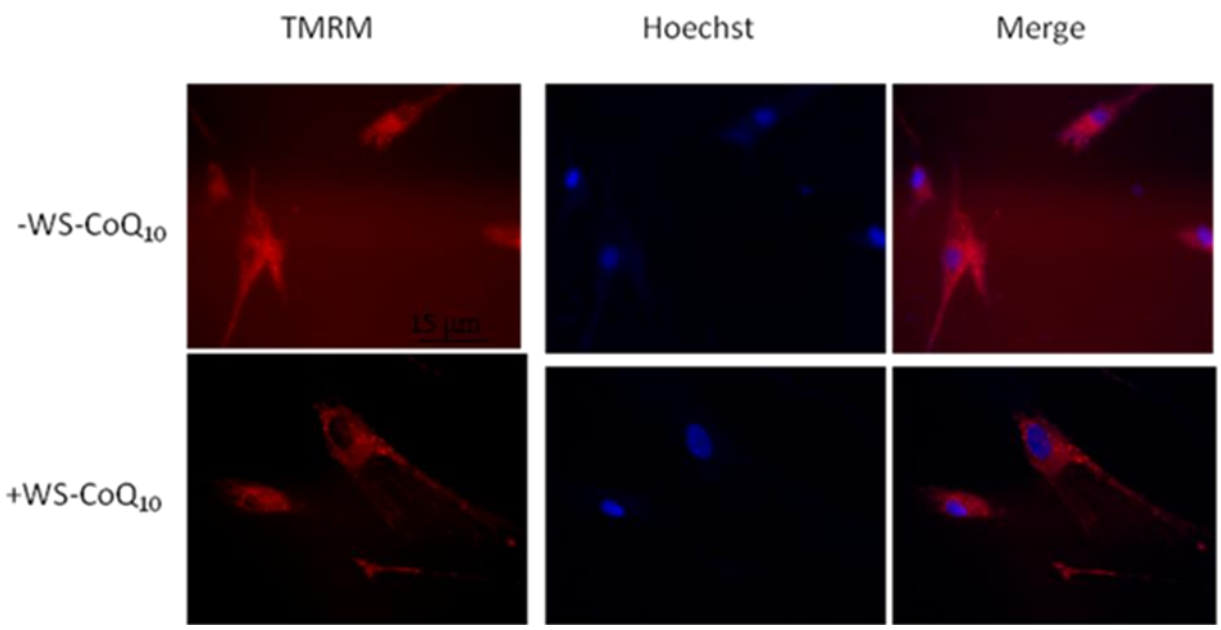

Figure 3.14 Mitochondrial membrane potential remains intact with or without WS-CoQ_{10} Treatment: PS-1 mutated AD fibroblasts were grown in the presence and absence of WS-CoQ_{10} and then evaluated for mitochondrial membrane potential using TMRM stain. Hoechst was used as a counter stain to detect apoptosis. The two images (TMRM and Hoechst) are merged in the far right panel. No significant difference in membrane potential was observed between those cells grown with WS-CoQ_{10} and those grown without.

3.2.7 WS-CoQ_{10} May Induce Protective Autophagy in PS-1 Mutated AD Fibroblasts

To gather some insight as to how WS-CoQ_{10} is protecting these cells against senescence and ultimately cell death, the avenue of autophagy was explored. Autophagy can be both detrimental and protective depending on how salvageable the cells are after damage has been induced (Dalby *et al.,* 2010). In order to investigate whether WS-CoQ_{10} is able to induce autophagy the cells were cultured with and without the presence of WS-CoQ_{10} (50 μg/ml). Mondansylcadaverine stain (MDC) was used to detect the presence of autophagic vacuoles. It was found that in the cells treated with WS-CoQ_{10} there was a significant amount of punctate MDC staining , indicating the presence of autophagic vacuoles. On the other hand, those cells void of WS-CoQ_{10} showed no accumulation of MDC stain, indicating an absence of autophagic vacuoles and a lack of protective autophagy (figure 3.15). Accompanying lack of positive propidium iodide staining indicates that the cells are not dying, thus any autophagy that is occurring is protective and not lethal. Another method to detect autophagy is to discern the presence of the autophagic protein LC3-II. Upon induction of autophagy cytoplasmic LC3-I is recruited to autophagosomes where it undergoes lipidation to be converted to LC3-II (Fulda *et al.,* 2010). Thus, the accumulation of LC 3-II can be used as an indicator of autophagy. Cells were grown with and without the presence of WS-CoQ_{10} and then

subjected to western blot analysis to probe for the presence of LC3-II. It was found that WS-CoQ_{10} was able to induce the conversion of LC3-I of LC3-II (figure 3.16). Those cells that were not grown in the presence of WS-CoQ_{10} did not show expression of LC3-II, thus indicating that WS-COQ_{10} may elicit its protective effects through the induction of protective autophagy.

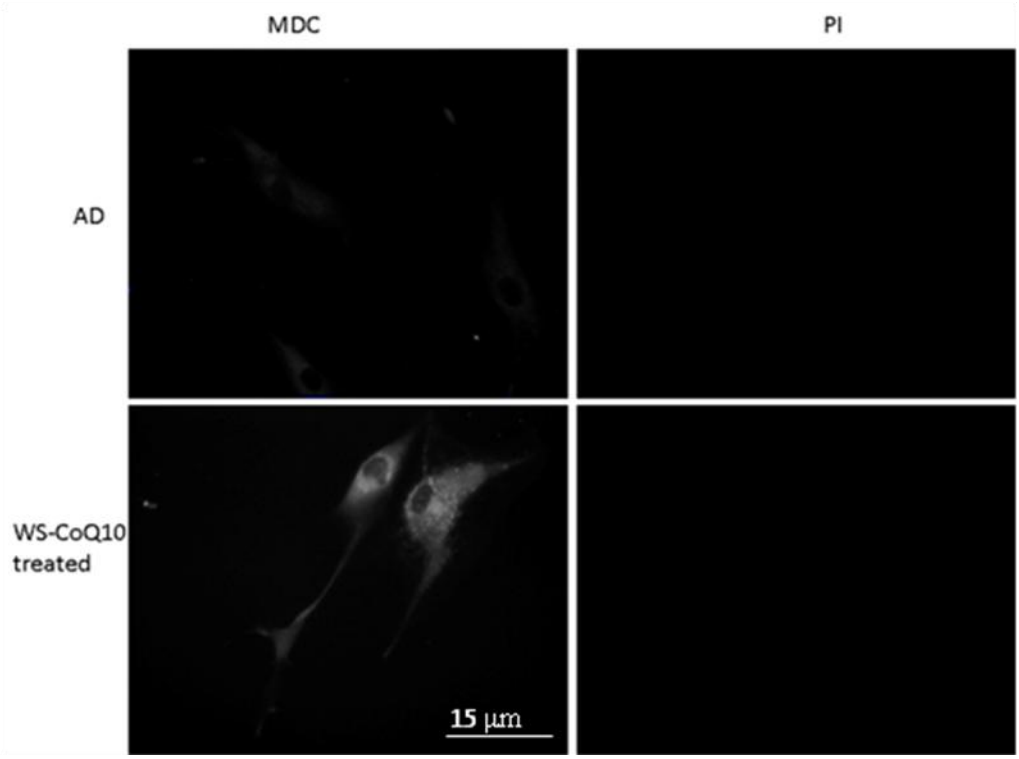

Figure 3.15 WS-CoQ_{10} induces formation of autophagic vaculoes: PS-1 mutated AD fibroblasts were grown both in the presence and absence of WS-CoQ_{10}. The presence of autophagic vacuoles was stained for using Monodansylcadaverine (MDC) stain, which shows positive autophagy by concise punctate staining. Cells were also counterstained with propidium iodide (PI) to verify if cells were undergoing cell death

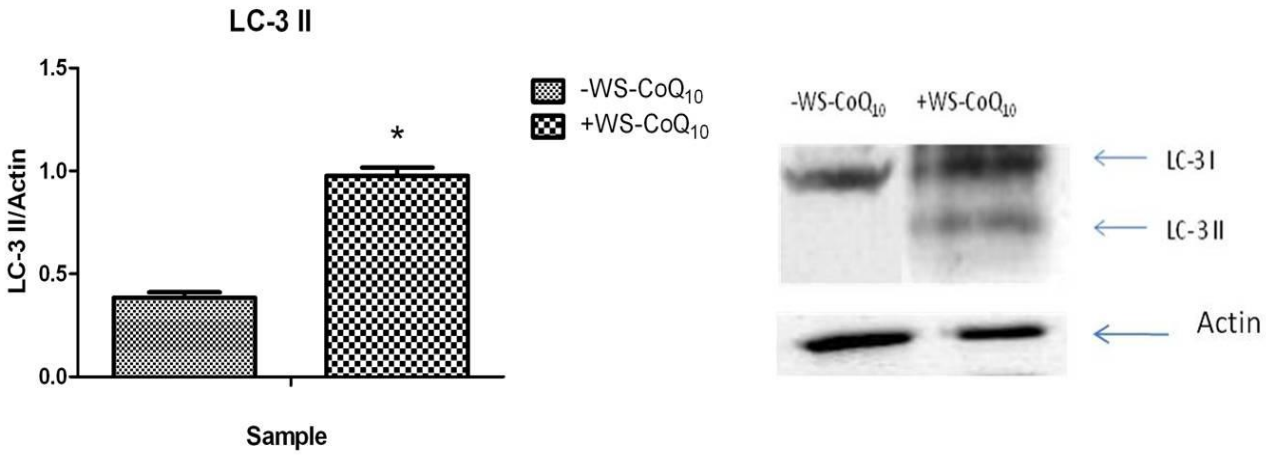

Figure 3.16 WS-CoQ_{10} results in increased levels of LC-3 II: PS-1 mutated AD fibroblasts were grown both in the presence and absence of WS-CoQ_{10} and cell lysates were analyzed for the presence of the autophagic marker LC-3 II. Actin was used as a loading control (*p<0.0001, vs. AD). (LC3-I has a molecular weight of 18kDa, LC-3 II has a molecular weight of 16 kDa).

CHAPTER IV

DISCUSSION

4.1 Compound 22 Prevents Bax-Induced Apoptosis

There are a limited number of treatment options available to victims of stroke. To date, only the tissue plasminogen activator is an accepted treatment for stroke. The most common form of tissue plasiminogen activator administered is Alteplease. This drug enacts its clot-breaking action by binding to the fibrin present in a clot and converting plasminogen to plasmin via breaking of the plasminogen Arg/Val bond (Ouriel *et al.,* 2004). The downfall to the use of this drug is the fact that it must be administered within 3 hours of the onset of stroke to be effective. This is relatively hard to accomplish since most stroke victims are not diagnosed with stroke, nor are they transported to hospital within this three hour window. Another downside to thrombolytics is that they have the potential to cause hemorhaging in the brain (del Zoppo *et al.,* 1998). Hypothermia has also been studied as a method of stroke treatment. It was found that lowering the body temperature may have a beneficial neurological outcome, however this technique remains highly experimental as many variables, such as temperature, duration, and optimal method of induction still remain in question (Yenari e*t al.,* 2010).

In order to find an alternative means of treatment, our lab has investigated the use of low molecular weight compounds to inhibit the pro-apoptotic protein Bax. The protein Bax plays a prominent role in the induction of apoptosis (Kroemer *et al.,* 2007).

If its function is able to be blocked, levels of apoptosis could be significantly decreased. In the present study, we have used an anti-Bax compound (Compound 22) and evaluated its ability to prevent Bax-induced apoptosis and mitochondrial destabilization.

Bax was chosen as a target for this study due to its role in apoptosis. Bax plays a key role in the initiation of apoptosis and is one of the first proteins involved in the apoptosis cascade. Because its role in apoptosis occurs so early in the process, and is heavily involved in mitochondrial membrane permeabilization, Bax is an excellent protein to target to inhibit apoptosis. If apoptosis was attempted to be blocked by targeting a protein further down the apoptosis cascade, the mitochondria may already be permeablized, thus already compromising the viability of the cell.

Bax has such capabilities as to form a homodimer and insert itself into the mitochondrial membrane forming Bax channels. Such channels cause mitochondrial membrane permeabilization that inevitably leads to the leakage of apoptotic factors such as cytochrome c, AIF, and SMAC/DIABLO into the cytosol which in turns causes activation of the apoptosis cascade (Kroemer *et al.*, 2007). Inhibiting this detrimental function of Bax can have practical applications in cases of ischemia caused by stroke. By inhibiting the function of Bax, neurons that are subjected to oxidative stress can be prevented from undergoing apoptosis when stroke occurs. Apoptotic cell death has been shown to be a major contributor in tissue damage pathology associated with ischemia (Grahm and Chen 2001, Gottlieb and Engler, 2005). Studies regarding the role of Bax in

apoptosis have shown that Bax knockout mice are more resistant to ischemia induced neuronal death (Gibson et al 2001), indicating Bax's critical role in neuronal cell death following ischemia. Also, Bax channel inhibiting compounds have been shown to protect neurons in a global model of ischemia (Hetz *et al.*, 2005).

Previous work conducted by our laboratory has identified and characterized six Bax-specific single domain antibodies (sdAbs) that were shown to block its activity and protect mammalian cells against oxidative stress-induced apoptosis when expressed intra-cellularly as intrabodies (Gueorguieva *et al.*, 2006). Despite their specificity for Bax, these sdAbs are not appropriate therapeutic agents due to their large size (13 kDa) and susceptibility to proteolysis. In order to overcome this problem, a targeted library of small molecular weight compounds was screened to discover a compound that could bind to Bax and inhibit its function. Previous work conducted by Degterev and co-workers screened a library of 16000 compounds to discern if any were able to bind to Bcl-2, a member of the same protein family as Bax and similar in structure (Degterev *et al.*, 2001). It was found 2 compounds showed the ability to bind to the BH3 domain of Bcl-2. There were an additional 40 compounds that were similar in structure and could have the potential to interact with other members of the Bcl-2 family of proteins. We screened this targeted library of 40 compounds in an effort to determine if any were able to bind to Bax . We found one compound (Compound 22) that was able to competitively bind to Bax in the presence of the Bax-specific sdAbs and displace these sdAbs.

In order to investigate the mechanism of inhibition of Bax by C22, we looked at the association of Bax to isolated mitochondria in the presence of absence of C22. We have found that C22 was able to prevent Bax from being associated to the mitochondria. In addition to preventing Bax association to the mitochondria, C22 was also able to inhibit the release of cytochrome c into the cytosol. This finding indicates that C22 is able to stabilize the mitochondrial membrane by inhibiting the release of cytotoxic factors into the cytoplasm of the cell. Because mitochondrial membrane permeabilization is a critical step in the induction of apoptosis, stabilization of the mitochondrial membrane increases the potential to inhibit cell death. This indicates that C22 has a protective role in maintaining mitochondrial stabilization, and plays a role in inhibiting Bax activity.

Oxidative stress is a contributor to apoptosis as it is one of the factors that initiates the migration of Bax to the mitochondria. It is implicated in many detrimental events such as altered protein function, susceptibility to proteolysis, altered cell signalling, lipid peroxidation, and DNA/RNA modifications (Davies *et al.*, 1987; Droge *et al.*, 2002; Nunomura e*t al.*, 1999). In cases of ischemia, ROS plays a large role in the death of neurons. When blood flow is returned to the brain (reperfusion) there is a large influx of oxygenated blood that is taken up by the neurons. The return of oxygen is accompanied by the addition of free radicals to the penumbra region of the brain (Li e*t al.*, 2002). This increase in oxidative stress is a contributor to cell death in ischemia, where cells can experience apoptosis up to seven days following the initial ischemic attack (Schaller *et*

al., 2004). The cell death occurring in the penumbra after reperfusion is largely Bax dependent (Hetz *et al.*, 2005). If its activity can be inhibited, subsequent apoptotic events triggered by Bax can be halted.

In this study we have found that when isolated mitochondria were combined with Bax there was an increase in the levels of ROS that was generated. However, when C22 was added there was a significant decrease in the level of ROS that can lead to cell death. This indicates that C22 is able to neutralize Bax activity by inhibiting its ability to cause mitochondrial dysfunction, and thus prevent apoptosis from occurring. This effect of Bax inhibition is not widespread amongst similar compounds from this library. Another similar compound, compound 9 (C9), showed no effect in lowering the amount Bax-induced oxidative stress.

Oxidative stress is a key component in the disruption of normal cell functions (Bergendi *et al.*, 1999). These events triggered by oxidative stress can have a dire effect on the viability of the cell. If the ROS levels present in the cell can be lowered to manageable levels, the cells in the penumbra region of the brain would have an increased potential for survival. Bax plays a critical role in inducing apoptosis in the penumbra. The increased levels of oxidative stress are responsible for the dimerization and migration of Bax to the mitochondria, which can inevitably result in mitochondrial destabilization. Human neuroblastoma cells challenged with hydrogen peroxide treatment showed a significant decrease in their viability, as shown by the in the WST-1 assay (figure 3.4).

On the other hand, when these same cells were placed under similar conditions of oxidative stress with the addition of C22, there was a marked increase in the level of their viability. This indicates that C22 has the ability to maintain the metabolic activity of the cell under oxidative stress and protect against oxidative stress-induced apoptosis. Non-specific compound 9 did not have a positive effect on maintaining cell viability, once again indicating C22's specificity towards Bax.

Cell culture work convincingly showed that C22 was able to prevent Bax-induced destabilization of the mitochondria and lower the levels of oxidative stress in human neuroblastoma cells. C22 was able to maintain cell viability and inhibit apoptosis from occurring.

The challenge with any new therapeutic is to create a compound that is not toxic to the organism when it is administered. We found that C22 displayed no characteristics of toxicity when it was administered to a Long Evans Hooded rat. The rats subjected to C22 injection did not show any characteristic symptoms of toxicity such as weight loss, change in behaviour, or death. All animal subjects remained healthy after injection with C22.

In order to induce stroke in a Long Evans Hooded rat, a procedure known as the bilateral carotid artery occlusion and hypovolemic hypotension (2VO/HT) model, originally performed by Smith and co-workers was generously conducted by Dr. Donald DeGracia at Wayne State University (Roberts *et al.*, 2007). In this model, the carotid

arteries of the animal are occluded for a period of 10 min to mimic blood loss to the brain experienced in cases of ischemia. Thirty minutes before the induction of stroke, 15 mg/kg of C22 was administered to the animals via IP injection to determine whether it was able to preserve neurons after ischemia has occurred.

Seven days following the procedure the brains of the rats were analyzed for the presence of viable neurons. Together, hematoxylin and eosin staining along with NeuN staining indicated that C22 was able to preserve the number of neurons present in the CA1 region of the hippocampus in the brains of the study animals. Those animals that did not receive C22 treatment prior to the induction of stroke had a lower amount of neurons present than those that did receive C22 treatment (figure 3.5 and figure 3.6). These preliminary results indicate that C22 may have a protective effect on preserving neurons in an *in vivo* model of stroke. Since C22 did have an effect on neuron number in the brain, this shows that C22 was able to cross the blood brain barrier to have a positive effect on neuron protection. In addition, the rats that were used in this study did not experience any toxic effects of C22 administration. The rats did not experience a drop in weight or any significant alteration in behaviour or eating habits that would indicate exposure to a toxic compound.

The pore forming activity of Bax has been well established as a central part of apoptosis. Other groups have focused on using Bax channel blockers as a method of inhibiting apoptosis and have found that the use of these compounds have had an effect at

inhibiting apoptosis (Hetz *et al.,* 2005). Other Bax-inhibiting compounds have had problems with administration and crossing of the blood brain barrier (BBB) due to their hydrophobic nature or large size. Compound 22 combats these issues by being water soluble and our preliminary data indicates that C22 is small enough to effectively cross the BBB.

Exactly how C22 is inhibiting the apoptotic activity of Bax is unknown. Docking studies to determine how C22 is ineracting with Bax were performed with this compound show that C22 may inhibit the dimerization abilities of Bax by binding to its BH3 domain located on α2 (Unpublished data; Katrina McGonigal, 2009). The BH3 domain has been shown to be an essential part of the Bcl-2 family of proteins function, by allowing for these proteins to bind to one another or other members of the Bcl-2 family (Suzuki *et al.,* 2000). If this binding can be inhibited, Bax would no longer be able to dimerize into its active form that is responsible for mitochondrial destabilization. Size-exclusion chromatography would be required to determine if C22 has any effect on the ability of Bax to dimerize. SDS-PAGE gel electrophoresis is not able to discern between the dimerized and un-dimerized form of Bax, as Bax is always present in the dimerized form when run on a gel due to the reducing conditions of the gel.

Another characteristic component of Bax that allows for its ability to form pores in the mitochondria is the C-terminal domain located on α9. This domain is responsible for anchorage to the mitochondria to allow for pore formation to occur (Suzuki *et al.,*

2000). In order for Bax to anchor to the mitochondria and form a pore, energetic conditions must exist to allow for α2 of the BH3 domain and α9 of the C-terminal domain to break away from each other partake in their respective events (Suzuki *et al.*, 2000). C22 was found to significantly decrease the amount Bax that was able to associate to the mitochondria, thereby limiting the possibility of Bax-induced mitochondrial destabilization. C22 may be preventing this action by stabilizing the bond between the BH3 and C-terminal domains and inhibiting insertion into the mitochondrial membrane. These results indicate that on a cellular level, C22 is able to prevent Bax-induced apoptosis by inhibiting the association of Bax to the mitochondria and thus decreasing Bax destabilization and the release of cytotoxic factors into the cytosol of the cell. C22 also had beneficial effects by decreasing the level of Bax-induced oxidative stress while maintaining cell viability. In addition, preliminary *in vivo* results indicate that C22 may have a protective effect on preserving neurons in a rat model of ischemia, thus making it a potential therapeutic to treat ischemia related injuries.

4.2 WS-CoQ_{10} Protects Cells Against SIPS

Mutations of the presenilin-1 gene are known to be a cause of familial Alzheimer's Disease (Cecchi, e*t al.*, 1999). The PS-1 mutation has been shown to increase oxidative stress in fibroblasts obtained from subjects expressing this mutation. There are over 170 possible autosomal mutations in the PS-1 gene that have been shown

to contribute to the onset of familial AD. The majority of these mutations are missense mutations that result in a single amino acid alteration in the PS-1 gene (van Tijn *et al.*, 2011). Loss of PS-1 activity in a knockout mouse was shown to result in neurodegeneration, synaptic loss, and neural death (Saura *et al.*, 2004). It was also reported that the PS-1 mutation in mice resulted in an increase in ROS production (Schuessel *et al.*, 2006). The PS-1 mutation was also found to increase oxidative damage in primary neurons in APP/PS-1 knockout mice (Sompol e*t al.*, 2008). The exact mechanism by which PS-1 mutation increases oxidative stress levels is unknown. One hypothesis to this mechanism suggests that it may cause mitochondrial instability since presenilin-1 is a membrane protein. In addition, the PS-1 mutation is thought to potentially contribute to apoptosis via Aβ formation and trophic factor withdrawal (Guo *et al.*, 1998). Others have suggested that the PS-1 mutation may enact its detrimental effects by inducing a calcium imbalance if it were to associate to the endoplasmic reticulum (Mattson, 2011). In this case, the PS-1protein localized in the ER of neurons may cause an increase flux of Ca^{2+} across the ER membrane, resulting in a disruption of synaptic signalling (Mattson, 2011). Cells that express the PS-1 mutation have developed an ability to survive in an environment with sub-lethal ROS levels. Due to these increased ROS levels, PS-1 mutated cells are susceptible to undergoing stress induced premature senescence (SIPS) which is usually accompanied by senescence-associated β-galactosidase activity (Dmiri *et al.*, 1995) and shortened telomere length

(Bodnar *et al.,* 1998*).* For the first time, we have shown that WS-CoQ_{10} is able to protect against SIPS while decreasing oxidative stress levels and increasing population doubling.

Previous work conducted in our laboratory found that a variety of AD cells lines displayed increased levels of endogenous oxidative stress (Naderi *et al.,* 2006). This increased level of oxidative stress allowed the cells to become pre-conditioned and resistant to external sources of oxidative stress. These AD cells were found to show an increase in the expression of p21 and instead of undergoing cell death, they entered a state of senescence (Naderi, *et al.,* 2006). Similarly, when external oxidative stress was placed upon these AD cells in a confluent G_0 stage, they once again displayed increased p21 levels and entered a state of senescence as oppose to undergoing apoptosis (Domazet-Damjanov e*t al.,* 2009). Both of these studies indicate that increased levels of oxidative stress contribute to the induction of SIPS.

The induction of SIPS begins with an increased level of ROS, facilitated by the PS-1 mutation. This increased level of oxidative stress causes DNA damage which elicits a response by p53 (Itahana *et al.,* 2001). This leads to a subsequent activation of p21 which is responsible for exerting a G_1 cell cycle arrest, effectively removing the replicative ability of these cells (Waldman *et al.,* 1995). Manganese superoxide dismutase (MnSOD) is essential for neurons to combat against oxidative damage (Sompol *et al.,* 2008). It is a highly important member of the anti-oxidant defence system and is responsible for converting the superoxide radical to molecular oxygen and

hydrogen peroxide which can then be further metabolized to water and molecular oxygen. The oxidative stress generated in these PS-1 mutated fibroblasts is not enough to induce Bax initiated apoptosis, but it is sufficient enough to result in p21 induced senescence.

We have investigated the possibility of using WS-CoQ_{10} to reduce oxidative stress and inhibit senescence in PS-1 mutated AD fibroblasts. An oil soluble version CoQ_{10} is currently being tested in clinical trials for the treatment of Parkinson's Disease (Shults *et al.,* 2004). Unfortunately this formulation cannot be used in cell culture studies due to its lack of solubility in water, thus the water soluble CoQ_{10} that we used in this study could serve as an effective alternative. Current work being conducted in our lab shows that WS-CoQ_{10} is able to protect dopaminergic neurons in cases of Parkinson's Disease, and is able to do so with a dosage much lower than those levels being prescribed with studies involving the oil soluble version of CoQ_{10}.

WS-CoQ_{10} was found to protect neurons under oxidative stress in a variety of instances (Somayajulu *et al.,* 2005; McCarthy *et al.,* 2004). In these cases, differentiated human neuroblastoma cells and human tetracarcinoma cells were protected from hydrogen peroxide induced apoptosis by the addition of WS-CoQ_{10}. WS-CoQ_{10} was able to lower the amount of ROS produced in these cells, as well as decrease the mitochondrial ROS generated in differentiated human neuroblastoma cells. In addition to decreasing levels of oxidative stress in these cells, WS-CoQ_{10} also played a role in

decreasing the caspase 3 activity in these cells and preventing the collapse of the mitochondrial membrane potential (Somayajulu *et al.,* 2005). When differentiated human neuroblastoma cells were subjected to oxidative stress via paraquat treatment, WS-CoQ_{10} was also able to induce its protective effects and prevent apoptosis from occurring (McCarthy *et al.,* 2004). In addition, it has also been found that WS-CoQ_{10} was able to block Bax disruption of the mitochondria (Naderi *et al.,* 2006) and stabilize the permeability transition pore. By blocking Bax association to the mitochondria and preventing the release of cytochrome c into the cytosol, WS-CoQ_{10} was able to inhibit Bax-induced apoptosis as well as the Bax-induced generation of ROS (Naderi *et al.,* 2006). CoQ_{10} is known to be a potent anti-oxidant and able to increase ATP production levels through fast electron transport (Sikorska *et al.,* 2003). Taken together, the myriad of protective effects WS-CoQ_{10} has on neuronal protection and apoptosis prevention makes it an excellent candidate to be used as a therapeutic to treat neurodegenerative diseases. More specifically, because of these abilities, WS-CoQ_{10} could be used as in PS-1 mutated AD fibroblasts to down regulate PS-1 induced ROS levels and inhibit senescence. To study the effects of WS-CoQ_{10} on the PS-1 mutation in AD neurons is not practical, as brain tissue from living AD patients is not procurable. Instead PS-1 mutated fibroblasts are a good substitution as they still display the effects of the PS-1 mutation, and any data gathered can be extrapolated to be applied to neuronal models.

We have found that WS-CoQ_{10} was able to lower the unusually high endogenous levels of ROS seen in PS-1 mutated AD fibroblasts and decrease the senescence associated β-galactosidase activity of PS-1 mutated fibroblasts (figure 3.8). WS-CoQ_{10} was also shown to increase the population doubling of these PS-1 mutated AD fibroblasts, indicating they were no longer arrested in the cell cycle (figure 3.7). The dominating characteristic of SIPS is the fact that cells are able to be removed from the cell cycle and thus can no longer replicate. By observing that WS-CoQ_{10} is able to increase the population doubling of PS-1 mutated AD fibroblasts, we can conclude that these cells are no longer arrested in the cell cycle and once again have replicative abilities. In addition, we observed that WS-CoQ_{10} was able to significantly decrease the level of p21 expressed in these PS-1 mutated AD fibroblasts, while increasing levels of MnSOD present in later passage numbers of these cells. Taken together, this is the first report to indicate that WS-CoQ_{10} is effectively able to neutralize the detrimental effects that the PS-1 mutation has on these AD fibroblasts. By decreasing the levels of ROS production in these cells, WS-CoQ_{10} is able to significantly lower the levels of p21 which leads to an inhibition of senescence, while increasing the radical scavenging potential by upregulating the levels of MnSOD.

Under normal circumstances, WS-CoQ_{10} has no effect on the radical scavenging ability of MnSOD. However, in this study we observed that WS-CoQ_{10} is able to increase the level of MnSOD present in later passage PS-1 mutated AD fibroblasts when

compared to untreated fibroblasts. One possible explanation for this finding could be that WS-CoQ_{10} is able to maintain the stability of MnSOD under oxidative stress while having no appreciable effects on its anti-oxidant activity. It was observed that under conditions of ischemic/oxidative stress, coenzyme Q_{10} was able to restore the protein levels of MnSOD in gastrocnemius muscle cells to a level comaparable to non-ischemic models, but did not appear to have any effect on the activity of MnSOD (Tran *et al.*, 2011). This suggests that while coenzyme Q_{10} does not have an effect on the anti-oxidant activity of MnSOD, it may play a role in stabilization of the protein as it is able to affect its level of expression under oxidative stress.

This indicates that WS-CoQ_{10} is able to lower the unusually high sub-lethal levels of endogenous ROS produced in the cells. Since this increased level of ROS is one of the major factors responsible for the induction of SIPS, the ability of WS-CoQ_{10} to lower these levels allows these PS-1 mutated AD fibroblasts to continue replicating, significantly lowering the degree to which senescence occurs. Since WS-CoQ_{10} was able to neutralize the effect of the PS-1 mutation in a genetic model of Alzheimer's Disease, it can be reasonably extrapolated that WS-CoQ_{10} would have the same beneficial effects in AD neurons affected by the PS-1 mutation. As with all potential neuroprotective agents, the question of whether the compound is able to be made available to the brain when administered through intraperitonial or intravenous injection is always in consideration. Another concern for any therapeutic compound is whether or not it will elicit a toxic

response from the organism to which it is being administered. Previous work has shown that WS-CoQ_{10} is able to cross the blood brain barrier and remain non-toxic (Unpublished results; Mallika Somayajulu-Nitu, 2009) and thus would be a beneficial therapeutic to patients suffering from PD (Somayajulu-Nitu *et al.*, 2009).

We have found that WS-CoQ_{10} was able to decrease the levels of ROS and expression of p21 generated by PS-1 mutated AD fibroblasts, thereby effectively inhibiting senescence and increasing the population doubling of these cells. Because of these findings, WS-CoQ_{10} could be used as a preventative treatment option for AD. If taken before cells have been removed from the cell cycle, WS-CoQ_{10} could be able to prevent SIPS from occurring by lowering the high levels of oxidative stress that is responsible for inducing senescence.

CHAPTER V

SUMMARY AND FUTURE PROSPECTS

5.1 Summary

In the first part of the study we reported the use of a novel low molecular weight compound to inhibit apoptosis in cells exposed to increased levels of oxidative stress. Compound 22 was identified by a novel screening process, where low molecular weight compounds were screened against Bax specific sdAbs in order to discover if any had the ability to bind to Bax. We demonstrated that C22 was able to block Bax-induced oxidative stress and prevent destabilization of the mitochondrial membrane and the release of cytotoxic factors into the cytosol. A low dose of 20 μM of C22 significantly lowered the ROS generated in human neuroblastoma cells and preliminary data suggests that C22 may protect neurons in an *in vivo* rat model of stroke.

Compound 22 was shown to inactivate Bax and prevent its association to the mitochondria and Bax induced apoptosis. Because of this, it is our hope that C22 could be used as a potential neuroprotective agent in stroke.

C22 was injected intraperitonially into a rat at a dose of 15 mg/kg and no effects of toxicity were observed. Because preliminary results indicated C22 may be able to have a protective effect on neurons, it has the potential to be able to cross the blood brain barrier. This compound was also shown to be Bax-specific.

While the induction of apoptosis is detrimental in neurodegenerative diseases, it is an event required to prevent uncontrolled cell proliferation and to remove damaged cells. While inhibition of Bax may cause improper regulation of apoptosis in areas other than the penumbra, this inhibition would not last long enough to have any detrimental effects.

Compound 22 is a temporary treatment and is only required to be administered within a short time after stroke has occurred. Any extraneous apoptosis inhibition would only be for a short period of time, and not long enough to cause complications associated with a lack of apoptosis, such as cancer.

In the second part of the study, we investigated the ability WS-CoQ_{10} to lower the unusually high levels of endogenous oxidative stress generated by PS-1 mutated AD fibroblasts and its effect on cellular senescence. Our laboratory has had success with the use of WS-CoQ_{10} in protecting cells in culture from oxidative stress and in preserving neurons in rat models on Parkinson's Disease. In addition to it being a potent anti-oxidant, we have also found that WS-CoQ_{10} works to stabilize the protein transition pore and block Bax-induced destabilization of the mitochondria. This is the first study that has used WS-CoQ_{10} to prevent stress-induced premature cellular senescence.

It was found that WS-CoQ_{10} was able to lower the levels oxidative stress produced by these cells and inhibit the induction of cellular senescence and increase the population doubling potential of PS-1 mutated AD fibroblasts. It was also found that WS-CoQ_{10} was able to decrease the expression of senescence associated proteins p21 and p38, while increasing the expression of the radical scavenging protein MnSOD. In addition, studies into the mechanism of action of WS-CoQ_{10} indicate that it is able to induce the formation of protective autophagic vacuoles and increase the expression of the autophagic marker LC-3 II.

The protective effects of WS-CoQ_{10} seen in PS-1 mutated AD fibroblasts can be extended to neurons as well. In fibroblasts with the PS-1 mutation, this increased level of endogenous oxidative stress inevitably leads to SIPS. In the case of neurons, increased oxidative stress as a result of the PS-1 mutation results in cell death. If WS-CoQ_{10} is able to inhibit SIPS from occurring in PS-1 mutated AD fibroblasts, it is our hope that it may be able to be used as a neuroprotective agent to prevent cell death in neurons of patients that possess the PS-1 mutation and are predisposed to Alzheimer's Disease.

5.2 Future Prospects

We have seen that C22 is able to prevent Bax-induced cell death in cases of stroke and preliminary results indicate that C22 may be able to prevent neuronal cell death in an *in vivo* rat model of stroke. Future work that can be done with this compound is to optimize the dosing amount and time of administration that is required to achieve maximum neuronal protection following stroke. Also to be studied is the effect different modes of administration (oral, intravenous, intracranial etc.) have on the protective ability of C22 and whether multiple dosings would be of benefit. In terms of understanding the mechanism of action of C22, it can be radiolabelled and its progress in the cell can be tracked to determine where it localizes in the cell. In addition, studies using size exclusion chromatography to determine whether C22 has an effect on Bax dimerization

can be conducted as well as protein NMR to determine what effect C22 binding has on the overall Bax conformation.

WS-CoQ_{10} was found to prevent the induction of cellular senescence and decrease the expression of senescence-related proteins in PS-1 mutated fibroblasts. Future studies to be conducted with WS-CoQ_{10} include investigating whether WS-CoQ_{10} to prevent the toxicity associated with Aβ accumulation. The ability of WS-CoQ_{10} to inhibit SIPS in other PS-1 mutated cell lines can be investigated. The mechanism of action of WS-CoQ_{10} is still unknown and additional studies regarding the effects of p38 and MnSOD on the function of WS-CoQ_{10} can be investigated using appropriate knockout studies. Investigations as to whether or not WS-CoQ_{10} has a protective effect in an *in vivo* model of AD can be conducted using transgenic mice that possess the PS-1 mutation.

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Publications

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Fochesato LA., Manghar K., Ma D., Tremblay P., Sikorska M., Pandey S. 2011. Water soluble coenzyme Q10 inhibits senescence in presenilin-1 mutated Alzheimer's Disease Fibroblasts. (Submitted to Journal of Gerontology).

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