Neuroprotective Effects of Anthocyanins on Neuronal Death Induced by Inhibition of Bcl-2 and Oxidative Stress

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NEUROPROTECTIVE EFFECTS OF ANTHOCYANINS ON NEURONAL DEATH
INDUCED BY INHIBITION OF BCL-2 AND MITOCHONDRIAL OXIDATIVE STRESS

A Thesis
Presented to
The Faculty of Natural Science and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Natalie A. Kelsey
August 2010
Advisor: Daniel A. Linseman Ph. D.
Abstract

Neurodegenerative diseases such as Parkinson's and amyotrophic lateral sclerosis have devastating consequences to the afflicted patients. A major cellular pathophysiology underlying these diseases is mitochondrial oxidative stress (MOS) leading to neuronal death. Here, we investigated the neuroprotective effects of a novel class of nutraceuticals, anthocyanins, against MOS-induced death in primary cultures of rat cerebellar granule neurons (CGNs). Anthocyanins are natural antioxidants whose neuroprotective potential has yet to be examined in detail. Kuromanin and callistephin are anthocyanins derived from black rice and strawberries, respectively. Glutathione (GSH)-sensitive MOS and intrinsic apoptosis were induced in CGNs by the Bcl-2 inhibitor, HA14-1. Callistephin and kuromanin each demonstrated significant neuroprotection from this MOS-induced death that was equal to that provided by the green tea polyphenol, epigallocatechin 3-gallate; however, neither anthocyanin was as effective as GSH at rescuing CGNs. Incubation with HA14-1 alone resulted in nearly 90% apoptosis of CGNs and either callistephin or kuromanin reduced this effect to approximately 20% cell death. Treatment with HA14-1 caused a marked depletion of mitochondrial GSH in CGNs to approximately 40% of the control level. Callistephin and kuromanin essentially prevented the reduction in this critical pool of endogenous antioxidant. These data indicate that callistephin and kuromanin represent a new class of neuroprotective compounds.
that warrant further study as possible therapeutic agents for the treatment of neurodegenerative diseases caused by MOS.
Acknowledgements

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Chapter One: Introduction

Neurodegenerative disease

There are a wide variety of neurodegenerative diseases with different symptoms and pathologies. For example, Parkinson's disease (PD) attacks dopaminergic neurons of the substantia nigra pars compacta while amyotrophic lateral sclerosis (ALS) manifests itself in the degeneration of motor neurons. Alzheimer’s disease (AD) hallmarks are amyloid plaques and neurofibrillary tangles in the brain and multiple sclerosis (MS) is a progressive demyelinating disease. All four of these diseases are prominent in the public eye and often portrayed as unrelated to each other, when in reality the cellular mechanisms behind them are likely the same. The strong majority of cases are sporadic, without a genetic link, so the challenge is to figure out what causes neurodegeneration so that it can be prevented or slowed. Oxidative stress is thought to be a common link in neurodegenerative disease and is a liable mechanism in aging as well [1]. Many studies have been conducted showing links between oxidative stress and PD [2, 3], AD [4-6], ALS [7, 8], and MS [9] to highlight a few.

Neurons are especially susceptible to oxidative stress because they lack the 6-phosphofructo-2-kinase pathway (PFK2) to regulate glycolysis [10]. Therefore, neurons are reliant on mitochondrial oxidative phosphorylation for energy because
they cannot up-regulate glycolysis in times when oxygen is scarce. Additionally, mature neurons no longer participate in the cell cycle, which makes them vulnerable to oxidative stress-induced apoptosis. Therefore, neurons cannot enter the cell cycle to replace neurons that have died. Since there are a fixed number of neurons, those that undergo apoptosis are lost forever. This, along with neuronal dependence on mitochondrial oxidative phosphorylation energy production creates a unique vulnerability to oxidative stress-induced apoptosis. The brain’s dependence on oxygen is even more apparent by the fact it uses approximately 20% of the body’s total oxygen while only consisting of 2% of total body weight [11].

**Oxidative stress**

Oxidative stress occurs as reactive oxygen species (ROS) accumulate in the cell, either from excessive production or insufficient neutralization, causing damage to DNA, lipids, and proteins. The Fenton reaction is a classic method by which toxic free radicals are created from hydrogen peroxide and iron as shown in figure 1. Hydrogen peroxide and iron are both readily available in neurons making this reaction relevant to neurodegenerative disease [12].

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH} + \text{OH}^*.
\]

*Figure 1: Fenton reaction.* This figure shows a schematic of how the Fenton reaction creates hydroxyl radicals.

Hydroxyl radicals are highly reactive and cause damage to DNA, lipids, and proteins as shown in figure 2. Oxidized DNA, lipids, and proteins all initiate localized damage
but eventually trigger downstream signaling cascades inducing apoptosis [13, 14]. Since reactive oxygen species (ROS) cause global damage to cells it is important for cells and especially neurons, to keep ROS in check with antioxidants.

Figure 2: *Cycle of lipid peroxidation*. This figure shows a schematic of hydroxyl radical propagating lipid peroxidation.

**HA14-1 mechanism of mitochondrial oxidative stress (MOS) induction**

HA14-1 is a unique organic compound designed to compete for Bcl-2 homology-3 (BH3)-domain binding pockets on pro-survival Bcl-2 protein family members (figure 3); essentially mimicking pro-apoptotic BH3-only family members [15-17]. The Bcl-2 protein family consists of pro-survival proteins and pro-apoptotic proteins; the latter are further classified as multi-domain or BH3-only as shown in table 1. BH3-only proteins provoke apoptosis by inhibiting the pro-survival Bcl-2 family members, like Bcl-2 and Bcl-xL, located on the outer mitochondrial membrane. Bax and Bak are the multi-domain pro-apoptotic Bcl-2 family members which Bcl-2 and Bcl-xL sequester [18]. The Bcl-2 protein family has complex
interactions regulating apoptosis as demonstrated in figure 4. For example, Bcl-2 inhibits the BH3-only protein tBid from inserting into mitochondrial membranes, inducing Bax translocation to the mitochondria, and creating Bax-Bak oligomers [19]. The roles of BH3-only proteins are divided into activators and inactivators in a highly regulated hierarchy with the purpose of inhibiting Bcl-2 and Bcl-xL to induce apoptosis [20]. As a BH3-only mimetic, HA14-1 is pro-apoptotic; more specifically, we have shown it to induce mitochondrial oxidative stress (MOS) in neurons [21].

*Figure 3: HA14-1 binds Bcl-2.* This figure shows HA14-1 binding to the BH3 groove of Bcl-2. Image reproduced from Proc Natl Acad Sci USA 2000, 97:7124
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*Table 1: Bcl-2 protein family.* This table displays Bcl-2 family members categorized by function.

![Diagram](image)

*Figure 4: Pro-apoptotic and anti-apoptotic role of Bcl-2 proteins.* This figure shows Bim and Bax in their pro-apoptotic role inhibiting Bcl-2 as a part of the intrinsic apoptosis cascade.

**Mitochondrial oxidative stress (MOS) and dysfunction**

The mitochondria are the powerhouses of the cell. They have the duty of generating cellular energy in the form of ATP. Without ATP the cell will become energy deprived and eventually die. The most efficient way for a cell to create ATP is
through oxidative phosphorylation in the mitochondria, which uses the electron
transport chain (ETC). The ETC is not perfect so there is a basal level of electron leak
under the best of conditions. As shown in figure 5 the leaky electrons are the main
initiators of ROS. Moreover, ROS produced in mitochondria target the various
components of the ETC resulting in a feed forward cycle of further ATP depletion
and enhanced generation of ROS. As a result, mitochondria are both a major source
and target for ROS.

An oxidatively stressed mitochondrion is also a dysfunctional mitochondrion.
Mitochondria, under these conditions can repair through mitophagy [22], other
counteracting pathways [23], or can initiate the intrinsic apoptosis pathway.
Initiating apoptosis is a last resort for the cell, but there are only so many
compensatory mechanisms available. In addition to ROS production, mitochondrial
dysfunction also leads to alterations in mitochondrial membrane potential, ATP
production, and Ca^{2+} homeostasis. Changes in mitochondrial membrane potential
and calcium homeostasis can induce the mitochondrial permeability transition pore
(PTP) which is reversible but can also lead to cell death [24, 25]. It has been shown
by various research groups, through the use of knockout mouse models, that there
are many players in the PTP and disagreement exists as to which proteins are
required for the pore to be formed. However, it is clear that ROS sensitizes the PTP
to activation by Ca^{2+}, thus oxidative stress plays a key role in pore opening.
Figure 5: ETC generation of ROS. This schematic shows the ETC contributes to ROS by leaking electrons in their transport through the ETC. Reproduced from: Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J. Physiol. 552, 335-344.

If mitochondrial function deteriorates beyond repair the intrinsic apoptosis pathway will be initiated as in figure 4. This pathway often begins with the release of cytochrome C, one of many apoptosis-inducing factors. Cytochrome C is also a regular member of the ETC carrying electrons from complex III to complex IV. Cytochrome C release occurs when cardiolipin, its lipid tether, is oxidized making its release a direct effect of oxidative stress [26]. When cytochrome C is in the
cytoplasm, caspase 9 is activated followed by caspase 3, commonly known as the executioner caspase [27].

The complex role of MOS and dysfunction in apoptosis and neurodegeneration makes the mitochondrion an interesting organelle for investigation and its involvement in neurodegeneration has been thoroughly reviewed [28-31]. As its role is further understood, the pathways underlying MOS and intrinsic apoptosis will increasingly become promising therapeutic targets for neurodegenerative diseases.

There are few treatments currently available for neurodegenerative diseases. The treatments that are available only treat symptoms of the disease, not the pathology of the disease. For example, riluzole is the only drug available to treat ALS. Riluzole may delay the patient’s dependency on a ventilator. However, it is not effective in all patients and can only extend life by 3-5 months.

**Endogenous antioxidants**

ROS created during normal cellular respiration must be neutralized by a cell’s antioxidant defenses before these free radicals have the opportunity to damage the cell. As previously discussed, ROS become a major problem for the cell when there is an imbalance between ROS created and ROS neutralized. As the cell's balance of ROS and antioxidants become disparate, oxidative stress occurs which is a damaging phenomenon that will lead to apoptosis. The electron transport chain in the mitochondria is a major source of ROS in a cell. For this reason, it is important to have antioxidants, like glutathione peroxidase and superoxide dismutase, located within the mitochondria.
Glutathione, superoxide dismutase, and other endogenous antioxidants are important, but transcription factors for antioxidant genes, like nuclear factor-erythroid 2-related factor 2 (Nrf2) are also vital players because they initiate an antioxidant cascade. In response to oxidative stress, Nrf2 is known to induce a variety of antioxidant genes by recognizing an antioxidant response element (ARE) binding site within their promoter regions [32]. Some key antioxidant genes turned on by Nrf2 include γ-glutamylcysteine ligase (GCL), the rate limiting enzyme in the synthesis of GSH, MnSOD, and heme oxygenase, to name a few [33]. As a result this pathway has been identified as a promising therapeutic target for neurodegenerative diseases [34]. Nrf2 is normally sequestered in the cytoplasm by kelch-like ECH associating protein 1 (Keap1). These proteins must be dissociated in order for Nrf2 to move into the nucleus and promote gene transcription. The mechanism of activating Nrf2 is demonstrated in figure 6 and reviewed by Kobayashi and Yamamoto [35].

Nrf2 has been meticulously investigated in order to elucidate its role in antioxidant gene regulation. It has been shown to be neuroprotective in many cellular models. For example, an increase in Nrf2 activity protects SH-SY5Y human neuroblastoma cells from oxidative damage induced by the Parkinson's neurotoxin, 6-hydroxydopamine (6-OHDA) [36]. 6-OHDA was again used in both in vivo and in vitro neuronal models where Nrf2 was protective [37, 38]. Primary cortical neuronal cultures from mice are another popular model in which Nrf2 induction has been protective against oxidative stress [39, 40]. Finally, Nrf2 activation placated PD
symptoms in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD [41].

Figure 6: Nrf2/Keap1 antioxidant response element (ARE) pathway. This figure shows two ways the transcription factor, Nrf2, can be activated and one of many possible gene products and downstream effects.

**Nutraceutical antioxidants**

In addition to the cell’s endogenous antioxidant defenses, an organism can boost its antioxidant levels through a diet rich in antioxidants. Many foods in a common diet, like broccoli, berries, apples, and teas, are full of antioxidants. While every antioxidant rich food has a unique antioxidant profile there are a few main categories of antioxidants. The flavonoid polyphenols, non-flavonoid polyphenols,
phenolic acids, and organosulfur compounds are some of the most well known natural antioxidant families.

The flavonoid polyphenol family is typified by epigallocatechin 3-gallate (EGCG), the high profile antioxidant in green tea, and quercetin, abundantly found in the skin of apples. The chemical structures of these compounds are found in figure 7. EGCG has shown significant neuroprotective effects in a variety of in vitro paradigms. Previous work in our laboratory has that shown cerebellar granule neurons (CGNs) are selectively protected from oxidative stress by EGCG [42]. Other studies have reported similar results where EGCG relieved oxidative stress and lessened neuronal cell death induced by hydrogen peroxide in motoneurons [43], N18D3 mouse neuroblastoma x dorsal root ganglion hybrid cells [44], spiral ganglion cells [45], and RGC-5 retinal ganglion cells [46]. In addition to the various in vitro studies using EGCG, in vivo neurodegenerative disease models have also seen significant effects with EGCG. EGCG treatment prevents the MPTP-induced loss of dopamine neurons from the substantia nigra pars compacta and preserves striatal dopamine levels in mice [47]. Similarly, in the transgenic mouse model of ALS expressing human G93A mutant SOD1 (Cu, Zn-superoxide dismutase) EGCG significantly delays symptom onset and somewhat extends life span when compared to vehicle treated mice [43, 48].

Quercetin is a flavonoid found in many common foods like apples and capers. Like EGCG, quercetin has also been extensively studied in in vitro models of neuronal death and in vivo neurodegenerative disease models. In vitro studies showed that PC12 cells treated with quercetin displayed an increased survival
following exposure to hydrogen peroxide [49, 50], linoleic acid hydroperoxide [51], or tert-butyl hydroperoxide [52]. In an in vitro Parkinson’s disease model, 1-methyl-4-phenylpyridinium (MPP+)−induced toxicity of mixed ventral mesencephalon cultures were attenuated by quercetin treatment [53]. In vivo models of neurodegeneration with quercetin have mostly focused on cognitive impairments, ischemia, and traumatic injury. Quercetin improved memory, cognitive performance, and hippocampal synaptic plasticity in cognitive impairment paradigms using colchicine, lipopolysaccharide, or chronic lead exposure [54-56]. In an ischemia model, quercetin significantly decreased the size of the ischemic lesion [49] and suppressed hippocampal neuronal death [57]. Cumulatively, these studies show that quercetin has the potential, like EGCG, to be developed as a therapy for neurodegeneration and that flavonoid polyphenols as a family are potent antioxidants.
Non-flavonoid polyphenols are another distinguished group of antioxidants that includes curcumin and resveratrol shown in figure 8. Resveratrol, an antioxidant generally found in many kinds of grapes is known mostly for its cardiovascular benefits but it has also been used to reduce neuronal death in *in vitro* [58] and *in vivo* models [59, 60]. Research on the non-flavonoid polyphenol curcumin is less extensive than that of resveratrol. However, in Neuro2a cells infected with Japanese encephalitis, curcumin enhanced cell viability by decreasing ROS and pro-apoptotic signals [61]. Curcumin has also been demonstrated to
protect rats from brain ischemia [62] and mice from MPTP-induced neurodegeneration [63]. In the MPTP study, curcumin exhibited a capacity to block glutathione depletion and suppress lipid peroxidation stimulated by MPTP. These, the non-flavonoid polyphenols are another group of promising natural antioxidants.
Figure 8: Resveratrol and curcumin structures.

Phenolic acids are another family of antioxidants. These are found in the herb, rosemary. Rosmarinic acid and carnosic acid are the familiar compounds in this group (Figure 9). Rosmarinic acid has been shown to scavenge peroxynitrites and ROS [64, 65]. As a scavenger, rosmarinic acid has been effective at protecting SH-SY5Y human neuroblastoma cells from hydrogen peroxide-induced oxidative stress [66]. In vivo studies with mouse models of AD and ALS have shown that rosmarinic acid alleviates memory impairment associated with Aβ neurotoxicity and significantly delayed motor dysfunction and prolonged life in the G93A mutant SOD1 ALS mouse model [67, 68].
Carnosic acid, like rosmarinic acid, has been shown in both *in vitro* models of neuronal death and *in vivo* neurodegenerative disease models to be neuroprotective. *In vitro*, carnosic acid activates the Keap1/Nrf2 transcriptional pathway protecting neurons from oxidative stress [69]. Carnosic acid translocates to the brain and increases reduced glutathione levels protecting against middle cerebral ischemia [69].

*Figure 9: Phenolic acid structures.*
The last of the well known antioxidant groups to be discussed are the organosulfur compounds allicin and l-sulforaphane seen in figure 10. Allicin is found in garlic, and garlic extract is used more often than pure allicin in studies. A short study using garlic extract on young and old rat brain synaptosomes showed that production of 8-iso-prostaglandin F$_{2\alpha}$ (8-iso-PGF) was significantly decreased by treatment of garlic extract in the young rat brain under control and hydrogen peroxide-induced oxidative stress conditions. The aged rat brain only responded to the garlic extract at the highest dose studied and under oxidative stress [70]. 8-iso-PGF is a modified unsaturated fatty acid released from the plasma membrane under oxidative conditions when there are insufficient antioxidants available. In a cell free in vitro study, garlic extract inhibited caspase-3 activation; thus garlic would have neuroprotective potential because caspase-3 is the executioner caspase in the apoptosis cascade [71]. Chauhan [72] reviews the neuroprotective effects of garlic in the context of AD.

L-sulforaphane, an organosulfur compound found in broccoli and other cruciferous vegetables has also been used as a neuroprotectant. Dopaminergic neurons, which are effected in PD, can be induced to produce toxic dopamine quinone and ROS by 6-hydroxydopamine (6OHDA) and tetrahydrobiopterin [73]. Dopamine quinone-induced neuronal death can be inhibited by pretreatment with l-sulforaphane [74]. Additionally, neurons undergoing hydrogen peroxide-induced oxidative stress in an astrocyte-neuron mixed culture can be protected through the activation of the ARE by Nrf2, which l-sulforaphane has been shown to activate [39].
L-sulforaphane activating this pathway is shown in figure 6. In another primary coculture model, L-sulforaphane mitigated dopaminergic neuronal loss from 6-OHDA-induced death [38].

![Allicin](image1)

**Figure 10: Organosulfur compound structures.**

It is easy to see that natural antioxidants are gaining popularity and credibility. There are many more studies than the ones mentioned here. Natural antioxidants and nutraceuticals are increasing their momentum in neurodegeneration because these diseases are publicized more than others and “natural” is perceived as safer than other treatment plans. In addition, the only treatments available for neurodegenerative diseases are palliative; they do not attack disease progression, only symptoms of the disease.

**Anthocyanins**

Anthocyanins are a unique family of natural antioxidants found in many fruits and vegetables. Many delicious berries such as blueberries, cranberries,
chokeberries, and lingonberries, have incredibly different poly-phenolic profiles but all have consistently high anthocyanin content [75]. A structural comparison of antioxidant families is reviewed by Rice-Evans et al. [76] giving a hierarchy of antioxidants based on their trolox equivalent antioxidant activities. This ordering has many anthocyanins near the top along with other well-known antioxidants like EGCG, a potent polyphenolic antioxidant found in green tea. As can be seen in figure 11 the structures of the two anthocyanins, callistephin, pelargonidin 3-O-glucoside, and kuromanin, cyanidin 3-O-glucoside, are extremely similar with only one hydroxyl group difference. The ring structures also share some obvious similarities to EGCG. Few studies have been conducted to assess the neuroprotective potential of anthocyanins. Anthocyanins have been shown to prevent ROS-mediated neuronal death in an aging mouse model of D-galactose-induced oxidative stress [77]. Another study showed that the same purple sweet potato anthocyanin used in the previously mentioned study also improves learning and memory in mice [78]. A variety of other in vivo studies in rats have shown that anthocyanins can recover age related cerebellar motor function, β-adrenergic receptor function, heat shock protein 70 responses, and other age related neuronal insufficiencies [79-81]. However, the neuroprotective effects of callistephin or kuromanin have yet to be investigated.
Hypothesis and rationale

With the recent boom in natural antioxidant research as previously discussed, and the consensus that oxidative stress plays a major role in neurodegenerative diseases, we thought it would be informative to identify specific
nutraceutical antioxidants that are capable of protecting CGNs from MOS-induced apoptosis. Oxidative stress has global consequences for the cell, which makes it even more important to examine molecular endpoints other than apoptosis such as lipid peroxidation and mitochondrial structure and function. These other measures are important because a neuron may not have gone through apoptosis but if it still has high levels of lipid peroxidation or fragmented mitochondria then the inevitable death of the cell, by apoptosis or otherwise, may just be delayed.

Heightened antioxidant capacity is a way neurons cope with oxidative stress. Therefore, the identification of new compounds that are capable of preventing apoptosis in an in vitro neuronal model, as well as inhibiting lipid peroxidation, mitochondrial fragmentation, OPA-1 cleavage, and mitochondrial glutathione depletion, may provide a foundation for new therapeutic strategies employing nutraceutical antioxidants for neurodegenerative diseases.

**Summary of major findings**

Using CGNs as a model in vitro neuronal system and the Bcl-2 inhibitor, HA14-1, to induce MOS, we tested a variety of nutraceutical antioxidants for neuroprotection. Of the active compounds, the anthocyanins, callistephin and kuromanin, stood out as being powerful antioxidants and neuroprotectants. Oxidative stress induces the activation of many damaging pathways in a cell that eventually lead to apoptosis. This investigation began by establishing that callistephin and kuromanin were equally as good as EGCG at rescuing CGNs from MOS-induced apoptosis. When endogenous antioxidant levels were examined, mitochondrial glutathione levels were significantly reduced by HA14-1 and this
essential antioxidant pool was preserved by callistephin or kuromanin. Lipid peroxidation is a major consequence of oxidative stress so a thiobarbituric acid reactive substances (TBARS) assay was used to show that callistephin and kuromanin could reduce cellular lipid peroxidation. A more specific lipid peroxidation study on a key mitochondrial lipid, cardiolipin, was used to show that callistephin could prevent HA14-1 from inducing cardiolipin oxidation. Additional scrutiny of mitochondrial function was conducted through mitotracker green imaging of mitochondrial structure and OPA-1 cleavage. In summary, callistephin and kuromanin appear to inhibit apoptosis by preventing glutathione depletion in the mitochondria, scavenging lipid radicals, and preventing cleavage of the mitochondrial fusion GTPase, OPA-1, in order to maintain normal mitochondrial structure and function.
Chapter Two: Materials and Methods

Reagents

HA14-1 and glutathione monoethylester were obtained from Calbiochem (San Diego, CA). Callistephin chloride, kuromanin chloride, thiobarbituric acid, and monoclonal antibody against β-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Mitochondria/cytosol fractionation kits were obtained from BioVision (Mountain View, CA). GSH/GSSG assay kit was purchased from Oxford Biomedical Research (Oxford, MI). Acridine orange 10-nonyl bromide and mitotracker green were obtained from Invitrogen (Carlsbad, CA). Trichloroacetic acid was from Fisher Scientific (Pittsburgh, PA). Polyclonal antibody against β-actin was obtained from Cell Signaling (Beverly, MA). Horseradish peroxidase-linked secondary antibodies and reagents for enhanced chemiluminescence detection were from Amersham Biosciences (Piscataway, NJ). FITC-conjugated secondary antibody for immunofluorescence was from Jackson Immunoresearch Laboratories (West Grove, PA).

CGN culture

CGNs were isolated from postnatal day 7 Sprague-Dawley rat pups of both sexes, as previously described [82]. CGNs were plated on poly-L-lysine coated 35mm diameter plastic dishes at 4.0 x 10^6 cells/well in Basal Eagle’s Medium
containing 10% fetal bovine serum, 25mM KCl, 2mM L-glutamine, and penicillin/streptomycin (100U/ml/100µg/ml). Cytosine arabinoside (10µM) was added to the culture medium 24h after plating to limit the growth of nonneuronal cells. This protocol produced cultures ~95% pure for granule neurons. Typically, experiments were performed after 6-7 days in culture.

**Treatments**

CGNs were treated with anthocyanins, or other antioxidants, and HA14-1 for 24 hours for immunocytochemistry and 4 hours for all other experiments.

**Immunocytochemistry**

CGNs were fixed in 4% paraformaldehyde for one hour followed by a blocking solution made of 5% BSA in .2% triton-X PBS for an hour. Once blocked, primary antibodies, β-tubulin and active caspase 3, were prepared at dilutions 1:200 and 1:1000, respectively, in 2% BSA .2% triton-X PBS then incubated overnight in the refrigerator. Secondary antibodies, conjugated to FITC and Cy3, were prepared at 1:250 dilution in 2% BSA .2% triton-X PBS with DAPI at 1:1000 and incubated for an hour at room temperature. 5 images per well were captured for apoptosis quantification.

**Cardiolipin oxidation assay**

CGNs were treated for four hours and then incubated at room temperature with 200µM acridine orange 10-nonyl bromide and Hoechst for 20 minutes then washed twice with PBS and imaged immediately under Cy3 and DAPI filters using a 40x objective.
Mitotracker Green

CGNs were treated for four hours and then incubated at 37 degrees Celsius for 20 minutes with mitotracker green and Hoechst in Hank’s balanced salt solution pH 7.4 and imaged immediately using FITC and DAPI filters with a 40x objective.

TBARS assay

This assay was performed as described below modified from [83-86]. Ferrous iron, thiobarbituric acid, and trichloroacetic acid solutions were prepared immediately before use with sterile, deionized water flushed with nitrogen gas for 30min. Seven day old rat pup brain was homogenized and diluted 1:10 (w:v) in Kreb’s buffer (pH 7.4). Samples were immediately prepared with 500µl of homogenate, callistephen (106µM, 213µM, and 426µM) and kuromanin (103µM, 206µM, and 412µM) in duplicate, so one sample would have 200µM, final concentration, ferrous iron added and one would not. All samples were qued to 750µl with Kreb’s buffer. Prepared samples were incubated at 37 degrees Celsius while shaking for 20 minutes. Then, 300µl of 12.5% trichloroacetic acid in .9N HCl was added to each sample to stop the reaction with 70µl of 10% thiobarbituric acid in .9N NaOH .9% NaCl solution. Then, samples were boiled for 20 minutes, cooled slightly, and centrifuged at 2000rpm for 15 minutes. The supernatant was transferred to duplicate wells on a 96 well plate and absorbance was read once per minute for ten minutes at 532nm. All readings for a sample were averaged together.
Corresponding without iron sample absorbance was subtracted from the with iron sample to account for the absorbance of the anthocyanins.

**CGN fractionation**

After treatment, CGNs were fractionated into mitochondrial and cytosolic fractions using a kit from BioVision (Mountain View, CA). Cytosolic and mitochondrial buffers were prepared per kit instructions. On ice, CGNs were washed once with ice cold 1X-PBS. Then, 200µl of cytosolic buffer was added to each well for 20 minutes. Wells were scraped and duplicate wells were transferred to microcentrifuge tubes. Samples were homogenized (about 40 strokes). In cold room, samples were spun at 720 rcf for 10 minutes. Supernatant is transferred to the “mitochondrial” tube and spun at 10,000 rcf for 30 minutes. Visible mitochondrial pellet should be present. Supernatant is transferred to “cytosolic” tube and pellet was resuspended in 100µl mitochondrial buffer.

**Glutathione assay**

Glutathione assay kit from Oxford Biomedical Research (Oxford, MI) was adapted from the manufacturer's instructions for cuvettes to be used in a 96-well plate by proportionally reducing the volume of reagents and sample from 1mL to 300µL.

**Cell lysis and immunoblotting**

After treatment, whole-cell lysates of CGNs for Western blotting were basically prepared as described previously [87]. Equal amounts of cell protein, as determined by a BCA protein assay kit (Pierce Chemical Co., Rockford, IL), were run
on a polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting. Membranes were blocked in PBS-T containing 1% BSA and .01% sodium azide for 1h at RT. Primary antibodies were prepared in blocking solution and incubated with the membrane for 1h. Membranes were next washed 5 times over 30min with PBS-T. Secondary antibody prepared in PBS-T was incubated with the membrane for 1h at RT. Then, membranes were washed again, 5 times over 30min. Finally, immunoreactive proteins were detected by chemiluminescence.
Chapter Three: Results

Antioxidant screen in MOS-induced death of CGNs

As there are multitudes of naturally occurring antioxidants it was necessary to perform an initial screen in order to determine those that might provide robust neuroprotection in our CGN MOS model. As shown in table 2, about half of the antioxidants used did not provide any significant protection against MOS-induced CGN death. Of the compounds that provided protection, we chose to concentrate on callistephin and kuromanin because they are from the same chemical family, anthocyanins. Additionally, we have already thoroughly investigated EGCG in the CGN model [42], while callistephin and kuromanin have not previously been tested in any neuronal model.
<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Protection against 15µM HA14-1 (MOS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callistephin (106µM)</td>
<td>++</td>
</tr>
<tr>
<td>*Curcumin (10µM)</td>
<td>-</td>
</tr>
<tr>
<td>*EGCG (25µM)</td>
<td>++</td>
</tr>
<tr>
<td>Kuromanin (206µM)</td>
<td>++</td>
</tr>
<tr>
<td>*Quercetin (20µM)</td>
<td>-</td>
</tr>
<tr>
<td>*Resveratrol (100µM)</td>
<td>-</td>
</tr>
<tr>
<td>*Rosmarinic acid (50µM)</td>
<td>++</td>
</tr>
</tbody>
</table>

*Maximum nontoxic concentration in CGNs

**Table 2: Antioxidant Screen.** CGNs co-treated with 15µM HA14-1 and listed natural antioxidants for 24 hours. Protection is indicated with (+) or (-) (for comparison GSH=+++) determined by percent fragmented nuclei using DAPI staining, n=2 independent experiments.

**Anthocyanins protect CGNs from MOS-induced apoptosis**

There are many compounds in the anthocyanin family that have radical scavenging abilities [88] which is why we chose this family to investigate.

Callistephin and kuromanin are the two anthocyanins we chose because they are commercially available in their pure forms. This makes it easier to compare their specific effects, whereas extracts and juices contain many additional unknown compounds. Shown in figure 12A, MOS induced by the Bcl-2 inhibitor, HA14-1, caused a complete disassembly of the microtubule network in CGNs. This is
visualized through immunocytochemistry of β-tubulin, shown in green. Also in figure 12A, active caspase-3 is shown in red. Active caspase-3 is the executioner caspase in the intrinsic apoptosis pathway. The natural antioxidants, EGCG (25µM), callistephin (106µM and 213µM), and kuromanin (103µM and 206µM), preserved the essential microtubule base of the cytoskeleton and neurites that were lost during MOS. Apoptotic nuclei were scored as fragmented or condensed using de-saturated DAPI images like the ones shown in figure 12B. De-saturated, or uncolored, images are used because it is easier to see morphology of the nuclei in a black and white image than a colored one. The data from all experiments, n=4, are combined and shown in figure 12C as the mean +/- SEM. Callistephin and kuromanin each significantly reduced MOS-induced CGN apoptosis to levels comparable to that observed for EGCG. However, neither the anthocyanins nor EGCG were as effective as GSH at protecting CGNs from MOS.
Figure 12A: CGNs co-treated with antioxidants (concentrations indicated), and 15μM HA14-1, with 15μM HA14-1 alone, or no treatment (control) for 24 hours. Immunocytochemistry shows β-tubulin, green; active caspase 3, red; DAPI, blue.
Figure 12B: Images from Figure 12A showing desaturated DAPI fluorescence.
Figure 12C: Percent apoptosis for combined experiments from Figure 1A. Apoptotic cells are those with condensed or fragmented nuclei. Results are shown as mean +/- SEM, n=4.

*** indicates p<.001 compared to all, ### indicates p<.001 compared to HA14 treatment, and ++p<.01 compared to other antioxidant treatments. All determined by one way ANOVA with post hoc Tukey’s test.
Mitochondrial Glutathione plays an essential role in CGN survival

After determining that the anthocyanins, callistephin and kuromanin, significantly protected CGNs from MOS-induced apoptosis, we next investigated the mechanism of this neuroprotection. First, we determined whether glutathione, a powerful endogenous antioxidant, was being maintained in the mitochondria by anthocyanin treatment during MOS. As shown in figure 13, MOS (HA14-1) significantly reduces mitochondrial glutathione content. All of the nutraceuticals used sustained glutathione levels in the mitochondria above that observed with HA14-1 alone with callistephin and kuromanin being slightly more effective than EGCG. The data presented in figure 13 represents the mean +/- SEM, n=8. Thus, the anthocyanins preserved the essential pool of mitochondrial GSH during MOS in CGNs.
Figure 13: CGNs co-treated with antioxidants (concentrations indicated) and 15μM HA14-1, 15μM HA14-1 alone or no treatment (control) for 4 hours. Post treatment, cells were fractionated using differential centrifugation into mitochondrial and cytosolic fractions. Data represented as percent glutathione concentration of control in mitochondrial fractions, mean +/- SEM, n=8. * indicates p<.05 when compared to HA14 by a two-tailed T-test. ** indicates p<.01 when compared to HA14 by a two-tailed T-test. *** indicates p<.001 when compared to HA14 by a two-tailed T-test.

**Anthocyanins reduce lipid peroxidation in vitro**

Lipid peroxidation occurs when there are unsaturated fatty acids available to hydroxyl radicals as previously discussed and shown in figure 2. Hydroxyl radicals
are highly reactive and readily available when a cell is undergoing oxidative stress, such as that induced by iron. The thiobarbituric acid-reactive-substances (TBARS) assay is a classic way to approximate lipid peroxidation in biological systems [89, 90]. Therefore, we initially tested the anthocyanins for their ability to inhibit lipid peroxidation in a cell free system of rat brain homogenate. As shown in figure 14, TBARS are measured by the absorbance of a sample’s supernatant at 532nm. Anthocyanins are highly pigmented compounds so it was necessary to adjust the assay to account for their interfering absorbencies. Samples of rat brain homogenate were prepared in duplicate; one was reacted with iron and one without iron as a control for the anthocyanin absorbance at each concentration. The anthocyanin absorbance at each concentration was subtracted from the corresponding sample that was reacted with iron. Figure 14 shows the mean +/- SEM, (n=4) of the calculated absorbencies. Both callistephin and kuromanin each caused a dose dependent reduction in lipid peroxidation induced by iron. The approximately 400µM dose for each anthocyanin reduced TBARS to essentially non-detectable levels.
Figure 14: Rat whole brains were homogenized and treated with callistephin, kuromanin (at the concentrations indicated) or no treatment (control). Lipid peroxidation was induced using 200µM ferrous iron and assayed for TBARS absorbance at 532nM. Higher absorbance indicates increased levels of lipid peroxidation. Data represented as mean +/- SE, n=4. ** indicates p<.01 post hoc Tukey’s test. ### indicates p<.001 post hoc Tukey’s test.

**Cardiolipin oxidation induced by MOS in CGNs is reduced by callistephin**

The TBARS assay showed that the anthocyanins are capable of inhibiting lipid peroxidation *in vitro* in rat brain homogenates. In the neuronal cell model used, CGNs and HA14-1, mitochondria are specifically targeted so an assay to specifically
assess the oxidation of mitochondrial lipids will yield more relevant information.

Cardiolipin is a lipid found nearly exclusively in mitochondrial membranes [91]. It plays an important role in tethering cytochrome C to the inner mitochondrial membrane and preventing its release from mitochondria. Indeed, cytochrome C release in apoptosis is linked to cardiolipin peroxidation [26, 92, 93]. Peroxidation of cardiolipin can be assessed through binding of the fluorescent dye acridine orange 10-nonyl bromide (AO) [94]. AO binds to reduced cardiolipin and the dye is released when cardiolipin is oxidized making it easy to visualize. Untreated (control) CGNs have a very bright AO fluorescence as do the (HA14-1 + callistephin)-treated CGNs indicative of substantial amounts of reduced cardiolipin in the mitochondrial membranes. CGNs treated with HA14-1 and therefore, undergoing MOS, fluoresce very dimly in comparison at the same exposure time. The dim fluorescence indicates that most of the cardiolipin has been oxidized (figure 15). The images presented in figure 15 are representative images of all experiments performed, n=3. These data indicate that MOS-induced CGN death is associated with cardiolipin oxidation and this effect is mitigated by the anthocyanin callistephin.
Figure 15: CGNs co-treated with 426µM callistephin and 15µM HA14-1, 15µM HA14-1 alone, or no treatment (control). Cells were stained with acridine orange 10-nonyl bromide (AO). AO binds exclusively to cardiolipin and is released when cardiolipin is oxidized. AO fluorescence shown in red and Hoechst nuclear staining shown in blue, n=3. Scale bar = 10µm.
**Mitochondrial disassembly induced by MOS in CGNs is prevented by callistephin**

After seeing a difference in cardiolipin peroxidation between HA14-1 and HA14-1 with callistephin, mitochondrial morphology was investigated using Mitotracker Green. Mitotracker is accumulated selectively in the mitochondria of the cell and allows visualization of mitochondrial structure. In the control CGNs and (HA14-1 + callistephin)-treated CGNs, the mitochondria appear long tubular, and connected. In contrast, CGNs treated with HA14-1 alone have mitochondria that appear punctate and disconnected. Both of these morphologies are shown in figure 16A. These images are representative of all experiments performed, n=3.

Mitochondria are dynamic organelles, constantly undergoing fission and fusion. A key fusion protein, OPA-1, has a unique, nonfunctioning, cleavage product present during apoptosis[95]. Figure 16B shows a western blot of OPA-1. The cleavage product is present in the HA14-1 lane and is reduced or eliminated in the lanes where the CGNs had been treated with HA14-1 and either callistephin or kuromanin. Below the OPA-1 blot, a blot of β-tubulin is shown to ensure equal protein loading in all lanes. Collectively, these data suggest that MOS induces cleavage of OPA-1 and enhanced mitochondrial fission or fragmentation; both of these effects are largely prevented by anthocyanins.
Figure 16A: CGNs treated as in Figure 15. Cells were stained using mitotracker green fluorescence indicating mitochondrial morphology, n=3. Scale bar = 10µm.
Figure 16B: CGNs co-treated with callistephin and 15µM HA14-1, kuromanin and 15µm HA14-1, 15µM HA14-1 alone, or no treatment (control) for 4 hours. Post treatment cells were lysed using 1% Triton X-100 and western blots were completed for OPA1. Asterisk indicates presence of OPA1 cleavage product. β-tubulin indicated loading control, n=3. * indicates cleavage product.
Chapter Four: Discussion

In the beginning of these experiments it was frustrating to find so many natural products that did not provide protection to the CGNs against HA14-1 (MOS-induced) death when they had been shown to work so well in other in vitro neurodegenerative models. Our model targets the mitochondrial oxidative stress specifically; whereas other in vitro models of oxidative stress are less organelle-specific. Also, the previous studies were not done in CGNs. These differences in neurodegenerative models are probably why some of the antioxidants protected well and others including, curcumin, quercetin, and resveratrol, did not protect the CGNs from mitochondrial oxidative stress. It was determined that the anthocyanins provided strong, consistent neuroprotection in the CGN model. Once we were satisfied that the anthocyanins rescued CGNs from HA14-1 (MOS-induced) apoptosis, we were able to look into the cellular mechanisms that are disrupted under oxidative stress.

Glutathione (GSH) is a prevalent antioxidant in mitochondria and its availability is key to keeping up with the necessary neutralization of ROS. For this reason, we looked specifically at the mitochondrial GSH levels of CGNs after 4 hours of treatment. As expected, HA14-1 treatment reduced the amount of GSH in the mitochondria. Since HA14-1 binds to the BH3-only domain on Bcl-2 and GSH also is
predicted to bind to that same pocket [21], it is not surprising that HA14-1 would have a negative effect on the mitochondrial pool of GSH. It is encouraging that callistephin and kuromanin were able to partially restore this key pool of mitochondrial GSH. MOS is an underlying problem in neurodegenerative disease so it is imperative that potential nutraceutical therapies be able to preserve or restore the mitochondrial pool of GSH, as observed for callistephin and kuromanin.

TBARS is a classic assay used to measure membrane lipid peroxidation, which is why we chose it to begin the investigation into the state of the lipids in our model MOS system. Anthocyanins are a highly pigmented family and they are what give berries and other red foods their color [86]. As a result, callistephin and kuromanin have a large amount of interfering absorbance at 532nm. To account for this interference, we simply had a non-reacting sample for each concentration of anthocyanin used to subtract from the reacting sample. We found that both callistephin and kuromanin were excellent at preventing thiobarbituric acid reactive substances from being created in rat brain homogenates. This is important for the stability of the neurons because lipid peroxidation can change mitochondrial membrane potential inducing PTP activation.

After seeing that both callistephin and kuromanin could prevent lipid peroxidation in a global assay like TBARS, we narrowed in on a mitochondrial lipid, cardiolipin. Cardiolipin is an integral part of the inner mitochondrial membrane and is key in tethering cytochrome C to the ETC. Cardiolipin’s role in cytochrome C release makes its oxidation specifically important in assessing neuronal health and
as a therapeutic target [96]. Therefore, the fact that callistephin was able to block cardiolipin oxidation is important to its potential future use therapeutically. As seen in figure 15, kuromanin is notably absent from the panel. Kuromanin did not necessarily fail to prevent cardiolipin oxidation, but the color of kuromanin seemed to interfere with the AO fluorescence so images could not be collected. This may indicate that kuromanin somehow quenched the fluorescence of AO.

Similarly to looking at cardiolipin oxidation, Mitotracker Green focuses on the mitochondria. It is used to examine mitochondrial structure, which is critical to well functioning mitochondria. The images show a distinct difference between the mitochondria of HA14-1 treated CGNs and the mitochondria of CGNs co-treated with callistephin and HA14-1. The co-treated CGNs show mitochondria that are long and tubular which is the expected structure. Proper mitochondrial structure can be indicative of overall neuronal health. Since neurons are more sensitive to MOS induced death than other cell types, mitochondrial structure is an important index of health. Analogous to the cardiolipin assay, kuromanin imaging was not possible due to color interference. OPA-1 cleavage products are another way to assess mitochondrial structure and function because OPA-1 is necessary for mitochondrial fusion. The cleavage product in the HA14-1 treatment is a non-functioning product because the GTPase domain is absent [95]. When mitochondria are only able to undergo fission, they become smaller and smaller until the mitochondria are inefficient fragments. These fragmented mitochondria are not able to support the
cellular energy needs in addition to the fact that inefficient mitochondria have excessive ETC leak, increasing ROS.

In summary, the anthocyanins, callistephin and kuromanin, have proven to be effective nutraceutical antioxidants in our MOS-induced CGN apoptosis model. Figure 17 shows that these nutraceuticals block mitochondrial glutathione depletion and cardiolipin/lipid peroxidation, prevent OPA-1 cleavage and maintain mitochondrial structure. These nutraceuticals have an overall effect of preventing MOS and therefore MOS-induced apoptosis. The multitude of palliative effects that the anthocyanins have on MOS-related cellular deficiencies will make them excellent candidates for future use with in vivo neurodegenerative disease models.

Figure 17: Summary schematic. This schematic shows how callistephin and kuromanin act as nutraceutical antioxidants to block MOS-induced death, mitochondrial GSH depletion, and lipid peroxidation.
In the future, we would like to use these compounds in a glutathione
peroxidase assay to see if the increase in the mitochondrial glutathione pool is a
result of increased turnover of GSSG/GSH. Glutathione must be recycled back to its
reduced form in order to continue its antioxidant effects. This assay will provide
important information as to the mechanism of neuroprotection of the anthocyanins.
Glutathione reductase is used to convert GSSG back to functional GSH. GSH
peroxidase activity is measured in an activity assay coupled to GSH reductase
function. Thus, an increase in GSH peroxidase activity in the presence of
anthocyanins will indicate an enhancement of GSH recycling.

Glutathione peroxidase, a phase II enzyme, is not the only way to increase the
antioxidant capacity of a cell. There are many phase II enzymes whose genes have
an antioxidant response element (ARE). Many endogenous antioxidants and phase II
enzymes, like γ-glutamylcysteine ligase, MnSOD, and heme oxygenase, are regulated
by an ARE on their genes. Nrf2 is a transcription factor that regulates ARE genes. A
study investigating the activation of phase II enzymes by anthocyanins showed that
anthocyanins, including kuromanin, up-regulated the production of
NAD(P)H:quinone oxidoreductase 1 (NQO1), a phase II enzyme [97]. This up-
regulation demonstrates that anthocyanins can activate Nrf2 because Nrf2 is a
necessary transcription factor for the nqo1 promoter. Nrf2 activation can have huge
effects on the antioxidant capacity of a cell because it is a transcription factor for
many genes coding for antioxidant enzymes. The more antioxidant functions a
compound has the greater the effect it will have on reducing oxidative stress in the
cell. If the anthocyanin family can both scavenge free radicals and induce antioxidant enzyme up-regulation, they have a greater potential to be transitioned into nutraceutical use for neurodegenerative diseases.

We also would like to take these compounds into an *in vivo* model of neurodegeneration, which involves MOS, like the G93A mutant SOD1 transgenic mouse model of ALS. In a study using acai berry pulp, which includes anthocyanins, phenols, and flavonoids, Swiss albino mice were given between 3.33 g/kg b.w. and 16.67 g/kg b.w. of the pulp and doxorubicin, to induce DNA damage [98]. For a person to consume this amount, they would have to eat about a pound of acai pulp. This study did see a significant decrease in DNA damage in blood, liver, and kidney cells post euthanasia of the mice. It will be exciting to see if nutraceuticals can have significant effects in an ALS mouse model by delaying onset and extending life span of the mice. Nutraceutical antioxidants have a promising future in the treatment of neurodegenerative diseases because of the relationship between these diseases and mitochondrial oxidative stress. Anthocyanins and other nutraceuticals that mitigate MOS-induced neuronal death will hopefully have a place one day in the treatment and prevention of neurodegenerative diseases.
References


