MALVIDIN AND DELPHINIDIN EXHIBIT A DOSE-DEPENDENT EFFECT ON CELL VIABILITY AND APOPTOSIS IN HT-29 CELLS

by

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(Under the Direction of Joan G. Fischer)

ABSTRACT

Anthocyanidins induce apoptosis in some cancer cell lines, but studies describing the dose-dependent effects on cell populations are limited. My objective was to evaluate the dose-dependent response of malvidin and delphinidin, anthocyanidins with different chemical structures, on cell viability and apoptosis in HT-29 cells compared to curcumin, a known inducer of apoptosis. Lower concentrations of anthocyanidins increased cell viability by 12-16% compared to control. Higher concentrations of ≥80 umol/L of curcumin, malvidin, and delphinidin decreased cell viability by 16-44%. Apoptosis was assessed by measuring caspase-3 and caspase-8 activities, as well as mitochondrial permeability. Only high concentrations (80 umol/L) of anthocyanidins and curcumin increased caspase-3 and caspase-8 activity. Concentrations of 25, 50 and 80 umol/L malvidin and delphinidin significantly disrupted mitochondrial permeability, showing anthocyanidins may be more effective at inducing apoptosis intrinsically. There was a significant interaction between anthocyanidin concentration and anthocyanidin type on cell viability, caspase-8 activity, and mitochondria permeability.

INDEX WORDS: Anthocyanidins, Delphinidin, Malvidin, Curcumin, Apoptosis, Caspase-3, Caspase-8, HT-29 cells

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CHAPTER 1

INTRODUCTION

Currently, cancer is the second leading cause of death (American Cancer Society 2013). The development of cancer is characterized by sustained proliferative signaling, avoidance of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, and ultimately invasion and metastasis (Hanahan and Weinberg 2011). Normal cells are regulated by mechanisms that create a homeostasis between cell renewal and cell death. Apoptosis, known as programmed cell death, plays a crucial role in maintaining healthy multicellular organisms. Cancer is associated with an inhibition of apoptosis, which allows malignant cells to proliferate at an uncontrolled rate (Wong 2011). Many cancer treatments stimulate apoptosis through the intrinsic pathway. Anticancer agents may induce the mitochondrial pathway by increasing reactive oxygen species (ROS) within the cells or by oxidizing components of cancer cells which then favors the expression of proapoptotic proteins such as Bax (Hanahan and Weinberg 2011). Dietary factors have the potential to induce oxidative stress and ultimately induce apoptosis within cancer cells.

Consuming a diet rich in fruits and vegetables may decrease the risk of some cancers, especially those of the upper digestive tract (WCRF/AICR 2007). The health benefits from consuming high intakes of fruits and vegetables may be linked, in part, to their non-nutritive compounds. Anthocyanins, a subgroup of flavonoids, are responsible for the bright reds, blues, and purples that are found in a variety fruits and vegetables (Prior and Wu 2006). The average intake of anthocyanins in the United States is estimated to be 11.7 mg/d (Murphy et al 2012).

However intakes could be easily increased by the addition of anthocyanin-rich foods like berries, which contain 20-387 mg anthocyanins/100 g (Wu et al 2006). The bioavailability of anthocyanins is low, thus a high percentage can potentially reach the lower intestinal tract (He et al 2005; Kahle et al 2006).

Anthocyanins are formed from aglycone bases called anthocyanidins. *In vitro* studies have investigated the effect of anthocyanidin treatments on proliferation and apoptosis in various cancer cell lines. Anthocyanidins in high concentrations (ranging from 50-200 umol/L) have significantly reduced cell viability by 50-90% (Brown et al 2012). Several studies have shown anthocyanidins to induce apoptosis through both intrinsic and extrinsic mechanisms. However, in some studies anthocyanidins inhibited cell proliferation but did not induce apoptosis (Brown et al 2012). A remaining question is whether a feasible dose of anthocyanidins, from consumption of fruits and vegetables, can significantly impact cell proliferation and apoptosis (Brown et al 2012).

Differences in chemical structure between individual anthocyanidins may explain their varying anticancer properties (Prior and Wu 2006). Jing et al (2008) reported that an increase in free hydroxyl groups enhances antioxidant potential. A free hydroxyl group at the 3' position in anthocyanidins was also shown to contribute to cell proliferation and inhibitory activity (Zhang et al 2005). The two anthocyanidins of interest in this study are delphinidin and malvidin. Delphinidin contains three free hydroxyl groups on its peripheral phenol ring, while malvidin contains one hydroxyl group and two methoxy groups.

Other dietary compounds such as curcumin, the primary curcuminoid in the spice turmeric, have been investigated for anticancer potential. Curcumin induces intrinsic apoptosis through the mitochondrial-mediated pathway in HT-29 cells and extrinsic apoptosis through Fas-

associated death domain/ caspase-8 initiation (Wang et al 2009; Reuter et al 2008). It is unclear whether anthocyanidins are able to induce both apoptosis pathways to the same extent.

The objectives of this study were to investigate the dose-dependent response of malvidin and delphinidin on cell viability and apoptosis in HT-29 cells, and to compare the effects of these two structurally different anthocyanidins to curcumin. It was hypothesized that concentrations of 25-100 umol/L will decrease cell viability and induce apoptosis. Delphinidin will have a greater impact compared to malvidin due to the presence of three hydroxyl groups on the peripheral phenol ring. Also, curcumin will have similar antiproliferative and pro-apoptotic effects compared to the anthocyanidins.

Cells were treated with 0, 5, 10, 25, 50, 80, or 100 umol/L malvidin or delphinidin or 40 or 80 umol/L curcumin to evaluate antiproliferative effects. Further assessments investigating the effects of anthocyanidins on apoptosis used concentrations ranging from 25-80 umol/L. The lower concentrations anthocyanidins, 5 and 10 umol/L, increased cell viability by 12-16% compared to control, while higher concentrations of ≥ 80 umol/L of curcumin, malvidin, and delphinidin decreased cell viability by 16-44%. Apoptosis was assessed by measuring caspase-3, caspase-8, and mitochondrial permeability. Only 80 umol/L of anthocyanidins and curcumin increased caspase-3 and caspase-8 activity. However, concentrations of 25, 50, 80 umol/L malvidin and delphinidin significantly disrupted mitochondrial membrane potential, showing anthocyanidins may be more effective at inducing apoptosis through the intrinsic pathway.

CHAPTER II

LITERATURE REVIEW

Cancer

In the U.S., cancer accounts for every one in four deaths (American Cancer Society 2013). The National Cancer Institute (2013) defines cancer as a group of diseases in which abnormal cell division persists uncontrollably and results in molecular, cellular alterations, and the invasion of other tissues. Cancer development is classified into three phases. The transformation of a normal cell to cancer starts with the rapid initiation phase; carcinogen metabolism, gene mutation and abnormal DNA repair (Mehta et al 2010). The initiation phase is also associated with increased free radical generation, the activation of phase 1 metabolizing enzymes, and the inactivation of phase II metabolizing enzymes. Blocking initial genotoxicity has proven to be an effective way to prevent cancer. Chemopreventative methods at this stage include the activation of phase-II enzymes such as glutathione-S-transferase (GST), or scavenging reactive oxygen species to ultimately stimulate detoxification (Manson 2003). Environmental factors and dietary agents play a role in simple mutations or small deletions in genes during the initiation stage.

While the absolute cause of cancer is unknown, it is likely multiple factors play a role in DNA mutations. DNA damage can occur from endogenous and exogenous factors. Examples of endogenous factors are errors in DNA replication, oxidative stress, inherited genetic mutations, abnormal hormone production and chronic inflammation. Exogenous factors include radiation,

environmental chemicals, infectious agents, smoking, alcohol use and dietary components (WCRF/AICR, 2007).

Next, initiated cells undergo a longer promotion phase, where growth factors are enhanced, oncogenes are expressed, and cell proliferation is increased, contributing to tumor growth. During the promotion phase, cancer treatments slow or stop cell proliferation and induce apoptosis, with the goal to restore normal cell cycle arrest (Ramos 2008). Lastly, in the progression stage, cancer cell proliferation is increased, and premalignant cells turn to neoplastic cells, enhancing the tumor's invasive ability. Inflammatory markers such as cytokines and tumor necrosis factors are produced; cyclooxygenase-2 activity increases and adhesion molecules are expressed, leading to tumor metastasis (Ramos 2008). At this stage, the goal of treatment is to interrupt angiogenesis, contain the cancer to its original location, and generally decrease its invasive ability.

Homeostasis is maintained in normal tissues by a number of mechanisms including control over growth and division cycle, unlike cancer cells, which are able to sustain proliferation by producing their own growth factor ligands. Overall, the complex development of a malignant tumor is associated with six processes; sustaining proliferative signaling, avoidance of growth suppressors, resistance to cell death, establishing replicative immortality, inducing angiogenesis, and ultimately undergoing invasion and metastasis (Hanahan and Weinberg 2011). Cancer development is also characterized by increased expression of growth factor receptors, antiapoptotic members of the Bcl-2 family of genes, phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPKs), and nuclear factor-kappa B (NF-κB) or decreased expression of proapoptotic members of the Bcl-2 family of genes, and caspase activity (Ramos 2008).

Apoptosis

Cell death is vital part of any multicellular organism and occurs as a normal physiological process or under pathological conditions. However, cell death can occur through multiple mechanisms, which result in different morphological changes (Kroemer et al 2005). Necrotic cell death is considered detrimental because it affects groups of adjacent cells, and evokes an inflammatory response (Kanduc et al 2002). Unlike apoptosis, necrotic cell death is generally considered disordered because it is caused by external factors such as chemical and physical damage. There is new evidence, however, suggesting necrosis can be a controlled programmed process when cell death is unable to occur through apoptosis (Chaabane et al 2013). The morphology of a necrotic cell involves swelling of organelles, increases in volume, and plasma membrane rupture and lysis of the cell (Kroemer et al 2005).

Apoptosis, known as programmed cell death, plays a crucial role in maintaining healthy multicellular organisms. Apoptosis differs from necrosis in that it does not evoke an inflammatory response and occurs under normal physiological conditions. An apoptotic cell has a decrease in cell volume, blebbing plasma membrane, chromatin aggregation, nuclear condensation, and degrades DNA into nucleosomal fragments (Han et al 2008; Hu and Kavanagh 2003). ATP and UTP are released from the cell and the molecule phosphatidylserine is produced and is recognized by phagocytes. Phagocytes can then engulf the apoptotic cell; the cell particles are disposed by hydrolases or recycled. The phagocytes also release cytokines IL-10 and TGF-β, which inhibit inflammation (Wong 2011). Under normal conditions an interchanging balance of proapoptotic and antiapoptotic proteins regulates apoptosis. Apoptosis is induced by negative death signals, and/or a withdrawal of positive signals, such as growth factors and IL-2 (Hu and

Kavanagh 2003). The two major pathways for apoptosis are the extrinsic and the intrinsic pathway.

The intrinsic pathway, known as the mitochondrial pathway, is characterized by mitochondrial outer membrane permeabilization, which responds to triggers such as DNA damage (Kim et al 2006). Since mitochondria have an important function in aerobic respiration, damage to the organelle can trigger cell death (Jain et al 2013). The voltage-dependent anion channel (VDAC) on the outer mitochondrial member functions as a "gate-keeper" by facilitating ion and metabolite transportation between the mitochondria and the rest of the cell; it targets pro- and anti-apoptotic Bcl-2 family proteins and can release apoptotic proteins into the intermembrane (Shoshan-Barmatz and Golan 2012). The ratio of Bcl-2 family proteins, which includes proapoptotic and antiapoptotic proteins, decides the fate of the cell. The antiapoptotic proteins include Bcl-2, Bcl-x, Bcl-x_L, Bcl-w, Bcl-x_S, and BAG, while the proapoptotic proteins are Bax, Bak, Bcl-10, Bid, Bad, Bim, Bik, and Blk (Kim et al 2006). If the mitochondrial membrane is damaged, the synthesis of proapoptotic proteins are favored. Cytochrome c binds with ATP and apoptotic protease activating factor-1 (Apaf1) to form a proapoptotic complex, known as an apoptosome. The apoptosome activates caspase-9 causing the activation of effector caspase-3, -6, and -7 (Jain et al 2013). Inhibitors of apoptotic proteins (IAPs) prevent apoptosis by disrupting the apoptosome, ultimately blocking caspases. IAPs can be repressed by IAPs antagonists, small mitochondria-derived activators of caspases (SMAC), which are released by the mitochondria (Jain et al 2013). The tumor suppressor protein p53, stimulated by DNA damage or other stress agents, commonly activates apoptosis by stimulating the proapototic protein Bax (Hu and Kavanagh 2003). The intrinsic pathway is commonly targeted more by clinical treatments such as irradiation and chemotherapy (Jain et al 2013).

The extrinsic pathway is known as the death-receptor induced pathway. It is activated by the binding of death-receptors from the TNF gene family, such as the FAS ligand, TNF, and Apo2L/TRAIL, to death receptors on the cell surface (Hu and Kavanagh 2003). The death signal is received and forms the death-inducing signaling complex (DISC). DISC is made up of the fas-associated death domain (FADD), procaspase-8 and -10 (Ashkenzai 2008). Caspase-8 and -10 are consequently activated; caspase-8, an initiator protein, then goes on to activate caspase-3, an effector caspase. Caspase-3 then stimulates a cascade of proteolytic activity. If the death signal needs to be amplified the activated caspase-8 could go through the intrinsic pathway by attaching to BID, an apoptotic Bcl-2 protein, causing BID to split into tBID. tBID binds to the mitochondria and acts with other proapoptotic Bax/Bak proteins to stimulate the release of cytochrome c (Lin et al 1998). Cytochrome c triggers Apaf1, forming an apoptosome and caspase-9 is activated which then activates caspase-3, -7, and -6; proteolytic activity occurs and results in cell death (Cain et al 2002).

Both apoptotic pathways require the activation of cysteinyl-aspartases, or caspases. Caspases are proteases containing cysteine, which cleave proteins at an aspartate residue. In either pathway the activation of a multi-protein complex, DISC or apoptosome, stimulates initiator or apical caspases (caspase-8, -9, -10). Caspase-3, -7, and -6 are effector proteins, which commit the cell to apoptosis (Huai et al 2010). Once caspase-3 is activated it causes the proteolysis of poly-ADP ribose polymerase (PARP), causing the enzyme to be deactivated, and activating enzymes that cause DNA fragmentation (Shih et al 2005).

While apoptosis is an important physiological process it is commonly altered by pathological conditions. Degenerative diseases, like Alzheimer's disease, increase apoptosis and result in excessive neuronal cell death. Carcinogenesis is an example where apoptosis is

inhibited, which allows the malignant cells to continue to proliferate at an uncontrolled rate (Wong 2011). Normal cell replication is limited to a number of growth and division cycles because the cells undergo senescence or are damaged. Cancer cells are immortalized because their telomeres remained intact, allowing continuous replication. Carcinogenesis also persists because it causes mutations in p53, resulting in a decreased expression of the proapoptotic proteins Bax and Bak (Hanahan and Weinberg 2011). Apoptosis-inducing treatments may stop the progression of cancer, as well as eliminate the tumor cells (Pan et al 2007). Cancer treatments induce apoptosis through death signaling receptors or by favoring the ratio of Bcl-2 proteins towards being proapoptotic. Anticancer agents may induce the mitochondrial pathway by oxidizing the cancer cells which then favors a Bax/Bcl-2 ratio (Hanahan and Weinberg 2011). Dietary factors also have the potential to induce oxidative stress and ultimately induce apoptosis within cancer cells.

Dietary influences on colon cancer

Colorectal cancer is the third and second most commonly diagnosed cancer worldwide in men and women, respectively, and second leading cause of cancer death in the United States (CDC, 2013). The majority of the cases, about 60%, occur in developed countries (GLOBOCAN 2008). The colon and rectum are continually exposed to mutagens and carcinogens from food or products of digestion (Wark et al 2004). Colorectal cancer develops from precancerous lesions, and can take 10 to 14 years to develop into an adenocarcinoma; during that long period, environmental factors may influence rate of development and prognosis (Pusatcioglu and Braunschwieg 2011). Non-modifiable risk factors for colorectal cancer include being over 50 years old, personal history of adenomatous polyps, inflammatory bowel disease,

family history of colorectal cancer or adenomatous polyps, or having another inherited genetic risk (Haggar and Boushey 2009).

Colorectal cancer may be affected by modifiable and preventable environmental factors. Modifiable risk factors include life-style choices such as smoking, inadequate physical activity, excessive alcohol intake, and dietary factors. It has been estimated dietary factors may be responsible for 30-90% of colorectal cases (Vargas and Thompson 2012; Araujo et al 2011). The risk for developing colorectal cancer increases with the consumption of excess energy, fat, red meats, and ethanol. There also may be an inverse relationship between colorectal cancer risk and an individual's intake of fruits, vegetables, short chain fatty acids, and polyphenolic-rich beverages (Vargas and Thompson 2012). The effect of diet and nutrients on colorectal cancer potentially decreases as the cancer develops, with nutrition having the most influence during the initiation stages and little to no effect during metastasis (Vargas and Thompson 2012).

Epidemiological studies have shown a diet rich in fruits and vegetables reduces the risk of some cancers, especially those along the digestive tract (Terry et al 2001). It has been estimated that diets with over 400 g/d of fruits and vegetables may prevent at least 20% of all cancers (Terry et al 2001). The American Institute for Cancer Research and World Cancer Research Fund examined the relationship between diet and cancer risk (WCRF/AICR 2007). They found convincing inverse associations between high intakes of fruits and vegetables and cancers of the lung, stomach, mouth, pharynx and esophagus, and probable inverse associations with colorectal cancers. Other studies have investigated whether specific foods or compounds have chemoprotective properties. For example, the Western Australian Bowel Health Study collected food frequency questionnaires from 834 colorectal cancer patients and 939 controls (Annema et al 2011). The results suggested intakes of different fruits and vegetables lower the

risks of selected regions in the colon. A higher intake of brassica vegetables was inversely associated with the development of proximal colon cancer. There was also an inverse relationship between distal colon cancer risk and the intake of dark yellow vegetables and apples. Fruits and vegetables may decrease the risk of cancer because of antioxidant activity, up-regulation of certain detoxification systems like glutathione (GSH) / glutathione S-transferases (GSTs), anti-proliferative and aninflammatory properties, and the ability to induce apoptosis (Wark et al 2004).

The nonnutritive compounds within fruits and vegetables are potential chemopreventative agents. Cancer treatments generally come with unwanted side effects, since healthy tissues are destroyed with the cancer cells. A compound may be labeled as chemopreventative if it can inhibit, suppress, or reverse the development and progression of cancer. The overall goal of chemopreventative agents is to use a compound that would cause little to no toxicity (Mehta et al 2010). The adverse side effects, high cost, and imperfect outcomes of conventional medicine, like chemotherapy for cancer treatment, increases the appeal of complementary medicine. Complementary medicine, such as the use of nutritional and botanical compounds, has been used throughout history and is increasing in today's culture, with 40% of Americans using alternative remedies for disease prevention (Gullet et al 2010; Eisenberg et al 1998). The safety and efficacy of these chemopreventative agents are not fully understood, however the chemical constituents of some natural compounds may have useful therapeutic benefits.

Polyphenols

Polyphenols aid in plant growth, protect cells from ROS and stress, and contribute to flavor, along with pigmentation (Aherne and O'Brien 2002). The beneficial bioactive activities of polyphenols may prevent chronic diseases like cancer, cardiovascular disease, diabetes, and

neurological disease (Gullet et al 2010). Polyphenols are a group of phytochemicals derived from phenylalanine and have at least one aromatic ring with a reactive hydroxyl group (Araujo et al 2011). Polyphenols are categorized as flavonoids, phenolic acids, stilbenes, coumarins and tannins, with flavonoids being the largest group. Subclasses of flavonoids, sorted by chemical structure, are flavonols, flavones, flavanones, isoflavones, flavan-3-ols, and anthocyanidins (Neuhouser2004). Polyphenols are abundantly found in plants but typically are not evenly distributed, with higher concentrations found in the skins of fruits, outer edges of vegetables, or within the plant leaves (Beecher 2003). The amount of polyphenols within a plant may vary depending on a climate, sunlight, season, food preparation, and processing (Aherne and O'Brien 2002). Important sources of polyphenols are found in fruits, vegetables, nuts, legumes, teas, coffee, cocoa, red wine, and beer. Various polyphenols are found ubiquitously within food, while others are limited to specific sources. For example, anthocyanins are mainly found in berries. The estimated intake of polyphenols has ranged from 0.1 to 1 g/d in Europe and U.S populations (Araujo et al 2011). The bioavailability of polyphenols is not completely understood but there is some research describing their digestion and absorption. Flavonoids may be hydrolyzed by two possible enzymes after ingestion, lactase phloridzin hydroase (LPH) or cystolic b-glucosidase (CBG) (Crozier et al 2010). LPH occurs at the brush border of the small intestine; after the aglycone flavonoid is released it becomes more lipophilic which may allow it to passively enter the epithelial cells (Day et al 2000). CBG hydrolysis requires the transportation of polar glucosides to the epithelial cells, most likely using the sodium-dependent glucose transporter SGLT1. The aglycones are metabolized even further before entering the circulation. Glucuronide, sulfate, or methylated metabolites are formed through the actions of uridine-5'-diphosphate glucuronsyltransferases (UGTs), sulfotranferases (SULT), or catechol-O-

methyltransferases (COMT). Some metabolites may migrate back to the small intestinal lumen by ATP-binding cassette transporters (Crozier et al 2010). Once in circulation the flavonoid metabolites reach the liver and undergo phase II metabolism and reenter the enterohepatic circulation or the small intestine through bile excretion (Donovan et al 2006). Flavonoid absorption occurs in the small intestine and colon, but colonic microbiota cleaves the aglycones into smaller molecules (Kahle et al 2005). The rate and extent of absorption of polyphenols depends on chemical structures and the type of the sugar in the glycoside. For example, the peak plasma level for anthocyanidins is 114 nmol/L versus proanthocyanidins at 40 nmol/L (Aiyer et al 2012). In addition, bioavailability is greatly affected by interactions with other compounds.

It was previously difficult to conduct epidemiological studies examining flavonoid consumption and disease risk because the flavonoid content of U.S fruits and vegetables was only first published by the Nutrient Data Laboratory at USDA in 2003 (Harnly et al 2006). However, several epidemiological studies have shown dietary flavonoid intake is inversely associated with cancer risk and other morbidities. The Zutphen Elderly Study collected data for 20 years to distinguish chronic disease risk factors among elderly men in the Netherlands. The study found a nearly statistically significant reduction of 43% in all cancer risk between men with high and low intakes of flavonoids and a 50% decrease in risk for gastrointestinal and respiratory cancers (P=0.06;Hertog et al 1994). Wedick et al (2012) followed up 70,359 women of the Nurses' Health Study (NSH), 89,201 women of the NHS II, and 41,334 men of the Health Professionals Follow-Up Study to investigate if specific flavonoid intake was associated with type 2 diabetes risk. All participants were free of diabetes, cardiovascular disease, and cancer at baseline. At the follow up there were 12,611 documented cases of type 2 diabetes. Higher intakes of anthocyanidin-rich foods, in particular consuming 2 ≥ serving/wk of blueberries and ≥

5 servings/week of apples/pears, were associated with a reduced risk of type 2 diabetes compared to consuming 1 ≤ serving/month ($P<0.001$;Wedick et al 2012).

Polyphenols have antioxidant, pro-oxidant, pro-apoptotic, antiproliferative, antiangiogenic, and anti-inflammatory properties, and increase phase I and II enzyme activities (Asensi et al 2011; Crozier et al 2009; Das et al 2010; Pan et al 2009; Ramos 2008). Hydroxylation status on polyphenol rings determines antioxidant potential. Polyphenols generally work as antioxidants by directly scavenging ROS, chelating and stabilizing divalent cations, or altering endogenous antioxidant/detoxification enzyme activities (Araujo et al 2011). The flavonol quercetin is a prime example of a polyphenol with antioxidant ability because it possesses the ortho-diyhydroxy structure on the B ring, a 2,3 double bond with a 4 oxo function in the C ring, 3- and 5- hydroxyl groups with 4-oxo function in the A and C rings, and a 3 position-hydroxyl group (Dai and Mumper 2010).

Polyphenols have a biphasic effect; acting as antioxidants as well as pro-oxidants, promoting apoptosis and inhibiting cell proliferation. The pro-oxidant status of phenolic compounds is modulated by pH, the presence of oxygen molecules and transition metal ions. Smaller compounds like gallic acid are more easily oxidized, unlike hydrolysable tannins. In vitro antioxidant tests may also harbor more extreme oxidized conditions compared to in vivo systems, though (Dai and Mumper 2010). The biphasic nature of polyphenols is cell and dose-dependent. Polyphenols exhibit more cytotoxic effects on cancer cells, yet normal cells are generally spared. After treatment with a tea polyphenol, epicatechin gallate (ECG), apoptosis was induced in carcinoma HSC-2 cells but not normal HGF-2 fibroblasts (Babich et al 2004). Park et al (2005) had similar results demonstrating the selectivity of polyphenolic effects. Green tea polyphenols were examined in normal rate osteoblasts (NRO) and human osteosarcoma

(MG-63 and Saos-2) cells at 0.1, 1, 10, and 100 umol/L for 24 hours. A dose-dependent response in inhibition of cell growth, alkaline phosphatase activity, morphological alteration, G0/G1-phase arrest of the cell cycle, and induction of apoptosis was observed in the osteosarcoma cell lines but not in NRO. At higher concentrations polyphenols increase ROS production in cancer cells (Neuhouser 2004).

Polyphenols have shown to impact important proliferative signaling pathways like PI3K, MAPK, NF-κB, growth factor receptors, and protein kinase B (Ramos 2008). Das et al (2010) found flavonoids induced pro-apoptotic caspases in human glioblastoma T98G and U87MG cells but not in human normal astrocytes. EGCG and genistein triggered ROS production by phosphorylating p38 MAPK, increased intracellular free Ca^{2+} and induced caspase-4 activity. Chronic inflammation has been significantly associated with carcinogenesis. Polyphenolic compounds have demonstrated anti-inflammatory activity by inhibiting cyclooxygenase-2 (COX-2), lipooxygenase, phosphoinositide 3-kinase (PI3-kinase), tyrosine kinases, inducible nitric oxide synthase (iNOS) enzymes, and NF-κB (Pan et al 2009; Guo et al 2009).

The interest in polyphenols will only keep increasing because of their potential to reduce chronic diseases, including the number 1 and 2 killers, cardiovascular disease and cancer. Numerous studies have demonstrated that polyphenols affect multiple cellular pathways, and are cell selective. While the toxicity of polyphenols appears to be lower than conventional chemotherapy they still have the potential to become toxic at higher concentrations. Additional trials are needed to assess if feasible doses of polyphenols can reduce the risk of these diseases (Vauzour et al 2010).

Anthocyanins

Anthocyanins, a subgroup of flavonoids, are water-soluble pigments responsible for the bright reds, blues, and purples are found in a variety of fruits and vegetables. Anthocyanins have the skeleton of C6-C3-C6 but are unique because they are positively charged at an acidic pH. High concentrations of anthocyanins are found in berries, like blueberries and blackberries, which contain around 365 and 245 mg/100g,respectively (**Table 2.1**). In general fruits are the main source of anthocyanins but they are also found in a variety of leafy and root vegetables and some grains (Wu et al 2006). The average intake of anthocyanins in the United States is estimated to be 11.7 mg/d (Murphy et al 2012). Murphy et al (2012) reported the 6% of the participants who met the dietary recommended intake for fruits and vegetable had the highest intakes of anthocyanins. It was estimated women who met the recommendations consumed 20 mg anthocyanins/d (per 1000 calories) versus 6.2 mg anthocyanins/d for the women who did not while intake was 15 mg/d for men who met the recommendations versus 4.5 mg/d for the men who did not ($P<0.05$). About 70% of the anthocyanins consumed came from grapes, blueberries, strawberries, bananas, and oranges.

Anthocyanin concentrations are influenced by many factors, including different processing techniques. Juice pressing decreased original anthocyanin concentration by 15%, and an additional 10% was reduced after the sediment was removed during the juice clarification step. Products (canned blueberries in syrup, canned in water, pureed, and juiced) stored at 25°C for 6 months lost 62-85% of their total anthocyanin content. Anthocyanin concentration was reduced due to specific processing techniques; 8-55% of the total anthocyanin content was reduced during the juice process, canning decreased total anthocyanin content by 28-34%, and processed purees resulted in a 43-80% decrease depending on storage temperature (Brownmiller et al 2008). Growing conditions, storage time, and preparation methods can also affect

anthocyanin concentrations, making it difficult to assess one's intake. Degradation of anthocyanins even occurs in human saliva; however this is dependent on structure and the individuals' oral microbiota (Kamonpatana et al 2012).

The differences in chemical structure between individual anthocyanidins may explain the varying bioavailability and anticancer properties, however this is not fully understood (Prior and Wu 2006). Anthocyanins exist as glycosides of their aglycone-anthocyanidin base. Glycosylation typically occurs at the 3-position on the C-ring or the 5, 7- position on the A-ring. There are mono-, di-, or tri-saccharide forms of anthocyanins; the attached sugar moieties are commonly glucose, galactose, arabinose, rhamnose, or xylose (Prior and Wu 2006). About 17 anthocyanidins are found in nature but the 6 most common forms are cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg), and malvidin (Mv) (**Figure 2.1**). Chemical structures of anthocyanins differ due to varying amounts of glycosylation and acylation. Structural differences between the anthocyanidins occur at the 3' and 5' positions, with combinations of hydrogen, hydroxyl, or methoxyl groups at R1, R2, or R3. For example, delphinidin contains three free hydroxyl groups, while malvidin only contains one and two methoxyl groups. There are about 600 anthocyanins, differing by the number and position of hydroxyl and methoxyl groups on their aglycone (anthocyanidin) structure, type and position of sugar(s) attachment, and acylating agent and extent of sugar acylation (Prior and Wu 2006). The size of the anthocyanins may play a role in their bioactivity. Anthocyanins come in varying sizes, an example is pelargonidin 3-arabinoside (from a strawberry) has a molecular weight of 403 compared to cyanidin 3-(sinapoly)diglucoside-5-(sinapoyl)glucoside (from red cabbage) has a molecular weight of 1185(Prior and Wu 2006).

Jing et al (2008) reported an increase in free hydroxyl groups aids in absorption in the gastrointestinal tract and increases antioxidant potential, and an increase in o-methylation in the B ring decreased antioxidant ability. A free hydroxyl group at the 3' position in anthocyanidin has also shown to contribute to cell proliferation and inhibitory activity (Zhang et al 2005). In an ex vivo study delphinidin and petunidin were more susceptible to saliva degradation compared to the other anthocyanidins. Delphinidin and petunidin experience about a 60-100% and 65-100% degradation in the salvia, compared to malvidin, peonidin, and cyanidin, which only had about a 5-55%, 5-60%, and 7-60% degradation respectively (Kamonpatana et al 2012). The stability of the anthocyanidins may be inversely correlated to superoxide radical scavenging ability, Dp > Pt > Cy = Mv > Pn > Pg (Rahman et al 2006).

Studies have found confounding results about the bioavailability and absorption rates of anthocyanins. Most flavonoids, due to their hydrophilic nature, are thought to require a transporter, such as SGLT1, to be absorbed in the small intestines. After ingestion anthocyanins may be hydrolyzed to alglycone forms by the enzyme beta-glucosidase from intestinal bacteria (Zhang et al 2005). However anthocyanins with intact glycosides can also be absorbed and detected in plasma circulation and urine excretion (Prior and Wu 2006; Nurmi et al 2009). In animal and human studies only about 0.1% of the anthocyanins ingested are detected in urine, however the rate of absorption and excretion depends on the sugar moiety and aglycone structure (Wu et al 2005).

The stomach's acidic environment may help stabilize the anthocyanins (Passamonti et al 2003). Anthocyanins have the capability of permeating through the gastric mucosa and being absorbed by human gastric cells (Shih et al 2005; Passamonti et al 2003). In laboratory conditions anthocyanidins are produced from glycosylated anthocyanins by acid hydrolysis. The

hydrolysis of the glycosidic bond may be critical for flavonoid digestion because the aglycone, the anthocyanidin form, is more readily absorbed (He et al 2010).

In gastric absorption studies, anthocyanins were detected in the blood after oral consumption; the anthocyanins could potentially be absorbed in the stomach through a bilitranslocase-mediated mechanism (Passmonti et al 2003). Bilitranslocase is an organic anion carrier that is expressed in gastric epithelium. This carrier potentially binds to the anthocyanin in the stomach and allows it to penetrate through the gastric mucosa; however saturation occurs at high concentrations (Talavera et al 2003). The maximum absorption of anthocyanins in gastric cells occurs after 12 hour of incubation and after 4 hour in rodent models (Shih et al 2005; Passamonti et al 2003). In animal and human colostomy studies phytochemicals from berry extracts have shown to survive digestion and reach the colon (He et al 2005; Kahle et al 2006). *In vivo* studies have found up to 80% of blueberry anthocyanins reach the colon unmetabolized, depending on the degree of methoxylation and sugar moiety (Esselen et al 2011). In one study rats were fed anthocyanin-rich extracts from either chokeberry, bilberry or grape (3.85 g monomeric anthocyanin per kg diet), or a control diet for 14 weeks. Intense coloration in the rodent's fecal matter suggests there were high concentrations of anthocyanins in the gut. High concentrations of anthocyanins may possess gastrointestinal benefits, especially in the colon. Rats consuming anthocyanin-rich diets grew fewer and smaller aberrant crypt foci (aberrant crypt foci are early biomarkers of colon cancer) compared to the controls (He et al 2005).

Overall, the inconsistencies in absorption studies may indicate anthocyanin's ability to be absorbed through multiple routes. Also berries and other anthocyanin-rich foods typically contain multiple anthocyanins, making it difficult to trace all the metabolites in the plasma and urine in vivo (Kay et al 2004; Crozier et al 2009). However it appears anthocyanins can be

absorbed intact, remain largely in the gut, and have health effects despite their low bioavailability (Bornsek et al 2012).

Diets containing anthocyanins have inhibited carcinogenesis in multiple *in vivo* studies. In our laboratory, rodents supplemented with a diet containing 1% blueberry flavonoids had decreased DNA damage in the liver compared to controls (Dulebohn et al 2008). Kang et al. (2003) found that APC^{Min} mice fed 2.4 and 0.6 mg anthocyanins/animal/d of cyanidin or 600 mg of tart cherries/animal/d developed a reduced number of cecal adenomas compared to the control animals, or animals receiving the non-steroidal anti-inflammatory drug sulindac. Cooke et al (2005) observed that mice fed an anthocyanin-rich blueberry extract or pure cyanidin-3-glucoside at 0.1% (w/w) had fewer small intestinal adenomas compared to the controls. Treatments with anthocyanins prior to carcinogen administration have also inhibited carcinogenesis. Rats receiving 35 mg/animal/d anthocyanin-rich extracts one week prior to an administered carcinogen developed less aberrant crypt foci compared to the controls (Magnuson et al 2003). Esophageal tumors in rats were prevented by a diet containing 5% black raspberry anthocyanins (Wang et al 2009). There are a limited number of human trials, however a German study showed participants who had high intakes of anthocyanin/polyphenolic-rich fruit juice had a decrease in oxidative DNA damage and increase in reduced glutathione (Weisel et al 2006). A reduction in cancer cell proliferation rates and increase apoptosis was seen in biopsied tumors of 25 colon cancer patients, without any previous treatments, who consumed 20 g/3x/d of black raspberry powder for 2-4 weeks. Healthy tissues were also biopsied before and after the treatment and were unaffected by the anthocyanin supplement (Wang and Stoner 2008).

Anthocyanins may be useful chemopreventative agents because of their antioxidant potential and ability to inhibit cell viability, inflammation and angiogenesis, and increase

apoptosis. Yi et al (2005) found anthocyanins decreased colon cancer cell proliferation to a greater extent compared to flavonol and tannin fractions. Similar to other flavonoids, anthocyanins have a biphasic effect within cancer cells and are less toxic to healthy tissue. Concentrations of 10 umol/L or less have shown to increase cell populations and act as antioxidants by enhancing antioxidant enzyme activity such as glutathione S- transferase and/or glutathione reductase (Turner 2009; Tokarev 2010; Galambos 2012), while higher concentrations, 25 umol and above have shown to inhibit cell proliferation and induce apoptosis (Yi et al 2005; Brown et al 2012; Shih et al 2007).

In vitro studies have investigated the effect of anthocyanidins on proliferation and apoptosis in various cancer cell lines (**Table 2.2**). Berry anthocyanin fractions in high concentrations (ranging from 50-200 umol/L) have significantly reduced cell viability by 50-90%, increased DNA fragmentation, and/or induced apoptosis (Yi et al 2005; Brown et al 2012). Isolated anthocyanidins at approximately 25-50 umol also can increase the beneficial apoptotic process and reduce cell populations of colon cancer cell lines. Anthocyanins potentially interact with multiple steps in the carcinogenesis process including 1) inhibition in cell proliferation through MAPK pathway and activator protein I factor (AP-1), 2) decreasing inflammation through the inhibition of COX-2 gene and NF-kB pathway, and 3) by inducing apoptosis (Hou et al 2004). Anthocyanins have shown to induce apoptosis by increasing caspase-3 activity; changing mitochondrial membrane potential and causing morphological changes like nuclear condensation, and damaged DNA (Lazze et al 2004). In multiple cancer cell lines anthocyanidins, at varying doses, have induced apoptosis by increasing p53 and Bax, and by reducing the expression of anti-apoptotic proteins like Bcl-2. Huang et al (2011) also reported a mulberry anthocyanin-rich extract initiated the extrinsic apoptosis pathway in gastrointestinal

adenocarcinoma cells (AGS) through p38/c-jun signaling pathways which activated Fas/Fas ligand and ultimately stimulated caspase-8. Intrinsic apoptosis occurred through enhancement of p38/p53. However few studies have looked if anthocyanins induce apoptosis through the intrinsic or extrinsic pathway to the same extent.

In higher concentrations anthocyanins have acted as pro-oxidants. Delphinidin and cyanidin were found to be cytotoxic to only colon cancer cells, Lovo and LoVo/ADR cells, by increasing ROS production and decreasing activity of the antioxidant enzymes GR and GSHT. However in a primary tumor cell line Caco-2, the same treatments with delphinidin and cyanidin acted as antioxidants. The different reactions in the cell lines could stem from the different neoplastic changes between the colon cancer cells (Cvorovic et al 2010). There are conflicting results about the ability of anthocyanins to increase ROS, however their pro-oxidant activity has been associated with their interference with the glutathione antioxidant system and involvement with peroxide scavenging (Feng et al 2007). Despite delphinidin and cyanidin decreasing GR activity in Lovo/ADR cells, GSSG was unable to be detected, possibly because the cells over-expressed MRP proteins, which have a higher affinity for GSSG. Without the necessary reduced glutathione pool, oxygen species can increase, ATP may become depleted, and cell death can occur (Cvorovic et al 2010). Anthocyanin treatments have also shown to damage DNA, resulting in cell death. Concentrations of >50 umol/L malvidin induced DNA stand breaks in HT-29 cells, and resulted in the buildup of cells in the G2 phase of the cell cycle (Fritz et al 2006). In a contradicting study, berry extracts, at concentrations > 50 ug/mL, inhibited topoisomerase 1 activity in HT-29 cells, and prevent DNA breakage (Esselen et al 2011). Other studies have found anthocyanin-rich extracts slow cells down in the G1 phase and G2/M in the cell cycle (Flis et al 2012; Fritz et al 2006). However in a few studies anthocyanidins inhibited

cell proliferation but did not induce apoptosis (Brown et al 2012). Interestingly berry extracts were able to induce apoptosis in cyclooxygenase-2 expressing cell lines, HT-29, but not in other lines, HCT-116, which lacked the enzyme (Wu et al 2007; Katsube et al 2003).

Some anthocyanidins, when combined, appear to have a synergistic effect on enzyme activity, while others combinations seem to have antagonistic effects. A combination of cyanidin, malvidin, peonidin, petunidin, and delphinidin induced cell-cycle arrest, apoptosis, and inhibited NSCLC cell invasion more than delphinidin alone. *In vivo,* the anthocyanidin mixture and delphinidin inhibited H1299 xenograph cell growth in mice, however the combination anthocyanidins required a lower dose to reduce cell growth (Kauser et al 2012). However, combined anthocyanidins do not always have a synergistic effect. In HepG2 cells a combined treatment of delphinidin and malvidin had on antagonistic effect compared to the effect of single anthocyanidins on GR activity (Galambos 2012).

Among the anthocyanidins, malvidin and delphinidin have exhibited the most significant antiproliferative potential, however results tend to be conflicting on which one is more potent (Fritz et al 2006). Delphinidin inhibited the epidermal growth factor receptor (EGFR), JNK phosphorylation, and interfered with topoisomerases, but malvidin had no influence on these targets (Hou et al 2004; Fritz et al 2006). In contrast, treatments with malvidin prevented 3',5'-cyclic adenosine monophosphate (cAMP)-specific phosphodieterases (PDE), which are generally overexpressed in cancerous tumors. If PDE is inhibited then intracellular levels of cAMP are increased, and protein kinase A (PKA) is activated. The substrate of PKA, serine/threonine kinase Raf-1, is subsequently phosphorylated which then suppresses activation of the MAPK cascade. However malvidin only marginally affected the G1-phase, except at higher concentrations of 100 umol/L, but it did interfere with G2/M-progression (Fritz et al 2006).

Delphinidin, cyanidin, and petunidin (anthocyanidins with an ortho-dihydroxyl structure) inhibited AP-1 transcription in a mouse epidermal cell line infected with tumor promoters (Hou et al 2004).

The potency of each individual anthocyanidin appears to be cell-specific. For example, in gastric adenocarcinoma cells (AGS), malvidin inhibited cell proliferation to the greatest extinct compared to the other anthocyanidins. AGS cells treated with malvidin had an increase in capase-3 activity, disrupted mitochondria membrane potential, increased p38 kinase expression and inhibited ERK activity. Also malvidin's effect on caspase-3 activity was blocked when ERK and p38 inhibitors were used, clarifying its antiproliferative effects and cytotoxicity on AGS cells were because of the induction of apoptosis instead of necrosis (Shih et al 2005).

It is questioned if single compounds, like anthocyanins have the same beneficial effects as whole fruits and vegetables. The properties of an anthocyanin in a berry may be different from when it is extracted (Yi et al 2005). However a few *in vivo* studies have found anthocyanins had a greater effect than the ingestion of whole berries. Diet-induced obese mice were fed whole blueberries or strawberries, or purified anthocyanin extracts from blueberries or strawberries. The mice fed pure anthocyanins had a reduction in body weight, improved insulin sensitivity, and normalized serum lipid and leptin levels, but the mice fed the diets containing the whole berries did not have any improvements (Prior et al 2008; Prior et al 2009).

Curcumin, a proapoptotic polyphenol

Curcumin, the curcuminoid in the spice turmeric, is an example of a polyphenol with pharmacological, and in particular, anticancer properties. Curcumin was included in this study as an agent with a known effect upon apoptosis and to compare its effects with those of the anthocyanidins. Curcumin induces intrinsic apoptosis through the mitochondrial-mediated

pathway in HT-29 cells and extrinsic apoptosis through the Fas-associated death domain/ caspase-8 pathway (Wang et al 2009; Reuter et al 2008). It also inhibits 12-O-tetracenoyl-phorbol-13-acetate (TPA)-induced inflammation, hyperplasia, proliferation, ornithine decarboxylase (ODC), ROS production, COX, and lipoxygenase in mice (Gullet et al 2010). Curcumin is able to down regulate the NF-κB pathway, which is found in nearly all animal cell types. The NF-κB pathway regulates infection and inflammation and is activated by > 150 stimuli such as cytokines, viruses, toll-like receptors (TLRS), and antigen receptors. The activation of NF-κB has been associated with the transformation of inflammation to cancer. Once activated, NF-κB induces over > 200 pro-inflammatory, pro-angiogenic and anti-apoptotic genes (Guo et al 2009). Curcumin's ability to inactivate NF-κB results in a down regulation of NF-κB gene products and a decrease in cell proliferation. Colorectal cancer cell lines are especially susceptible to curcumin; growth inhibition was observed in human colorectal cancer cell lines; SW480, HT-29, SW1116, WiDr and KM12, as well as one murine colon26 cell line (Chen et al 2011). Cell viability was inhibited in a dose and time-dependent manner. Wang et al (2009) reported 40 umol/L curcumin decreased HT-29 cell viability by 6% after 8 hours and by 39% after 24 hours. At a higher concentration of 80 umol/L curcumin decreased cell viability by 11% after 8 hours and by 89% after 24 hours. Intrinsic apoptosis was also affected in a dose-dependent manner; the release of cytochrome c and pro-apoptotic proteins (Bad, Bax, and caspase-3) increased in HT-29 cells incubated with 20-80 umol/L curcumin.

Rationale

A remaining question is whether the anthocyanidin concentrations used in in vitro studies are feasible in vivo, and particularly whether they can be reached in a diet that is high in fruits and vegetables (Brown et al 2012). Anthocyanidins induce apoptosis in some cancer cell lines,

but studies describing the dose-dependent effects of anthocyanidins on cell populations are limited or are only tested at high concentrations. At lower concentrations, ≤ 10 umol/L, anthocyanidins have shown to enhance cell proliferation, while higher concentrations have the opposite effect. There are also limited studies showing which apoptotic-pathway anthocyanidins effect more; intrinsic or extrinsic.

Objectives

To investigate the dose-dependent response of malvidin and delphinidin on cell viability and apoptosis in HT-29 cells.

To compare the effects of these two structurally different anthocyanidins to those of curcumin.

Hypotheses

Concentrations of 25-100 umol/L will decrease cell viability and induce apoptosis. The effect of malvidin and delphinidin on cell proliferation and apoptosis will differ. Delphinidin will have a greater impact due to the presence of three hydroxyl groups on the peripheral phenol ring. Curcumin will have similar antiproliferative and pro-apoptotic effects compared to the anthocyanidins.

Table 2.1. Anthocyanin content in selected common foods[1]

Food	Anthocyanin/100 g [2]
Cultivated blueberry	387 mg
Red cabbage	322 mg
Blackberry	245 mg
Cranberry	140 mg
Sweet cherry	122 mg
Concord grape	120 mg
Red radish	100 mg
Raspberry	92 mg
Eggplant	86 mg
Red onion	49 mg
Black beans	45 mg
Red grape	27 mg
Strawberry	21 mg
Red delicious apple	12 mg

[1]Data from adapted from Wu et al, 2006 by Tokarev, 2010
[2]100 g of fresh weight or consumed form

Table 2.2. Effect of anthocyanins on cell viability and apoptosis in *in vitro* and *in vivo* models

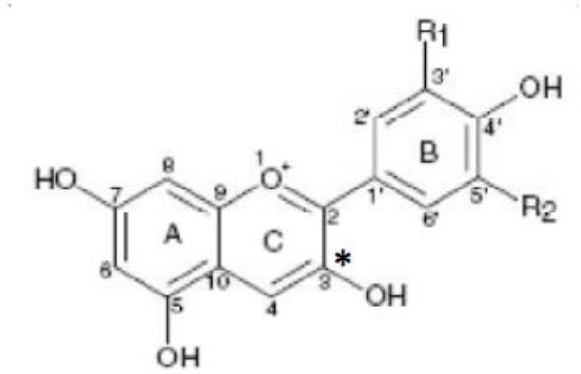
Reference	Model	Compound	Concentration	Length	Results
Aqil et al 2012	Human non-small cell lung carcinoma A549	Anthocyanin-rich extract (ARE) from Indian Blackberry pulp	12.5–200 ug/ml	72 h	• Hydrolyzed pulp had IC_{50} value of 59 ± 4 µg/ml but unhydrolyzed pulp was very weak
Cvorovic et al 2010	Colorectal cancer cell lines: Caco-2, LoVo, and LoVo/ADR	Dp and cy	25-100 umol/L	68 h	• Dp and cy were only cytotoxic in LoVo and LoVo/ADR (metastatic cell lines) • In LoVo/ADR ↓ GR and GSH
Hsu et al 2012	COLO 320DM, normal colon cells HT-29, colon carcinoma cells	Anthocyanidin-rich purple-shoot tea extract	0,25,50,100,200, or 400 ug/mL	24 and 48 h	• ↓ cell growth in dose-dependent manner, with COLO 320DM (IC50 = 64.9 ug/mL) and HT-29 (IC_{50} = 55.2 ug/mL)
Hwang et al 2012	ARPE-19 human retinal pigment epithelium cells	Anthocyanin oligomer	0.1, 0.5, and 1 mg/mL	18 h preincubation then stressed in 0.5 umol/L H_2O_2 for 24 h	• Anthocyanins ↑cell proliferation and maintained 60% of cell viability in in 0.5 umol/L H_2O_2
Hyun et al 2004	U936 human monocytic leukemia cells	Cy and mv extracted from oryza sativa, an anthocyanin-rich pigmented rich	20, 40, 60, 80 ug/mL	96 h	• ↓ cell viability in a dose-dependent pattern, IC_{50} for cy and mv were 60 um/mL and 40 ug/mL • Apoptosis was induced with 60 ug/mL cy and 40 ug/mL mv
Johnson et al 2011	HT-29 colon carcinoma cells	Freeze dried black raspberries	0.6 and 1.2 mg of extract/mL	48 h	• ↓ proliferation in a dose-dependent manner • Extent of cell proliferation was affected by cultivar, production site, and stage of maturity

Kim et al 2008	HT-29 colon carcinoma cells	Cy, pg, dp	1,10, and 50 umol/L	48 and 72 h	• Cy and dp ↓ growth at 1umol/L over 48-72 h, and pg at 50 μM over 72 h
Marko et al 2004	HT-29 colon carcinoma cels	Cy, mv, dp, pg, pn	0-300 umol/L	72 h	• Mv and dp ↓cell viability the most, with treatments of 25 umol/L viability was < 80% of the control.
Paixao et al 2011	Bovine aortic endothelial cells	Cy-, dp-, and pg-3-glucoside	25 umol/L	14 h	• Prevented peroxynitrite induced apoptosis • ↓caspase-9,3 activity, Bax levels, and mitochondrial membrane potential loss
Paixao et al 2012	Bovine arterial endothelial cells	Mv-3-glucoside	25 umol/L	14 h	• ↑ eNOS mRNA, eNOS activity, and NO production • ↓ pro-inflammatory mediators
Patterson, 2008	HT-29 colon carcinoma cells	Mv, pn	1-10, 50 ug/mL	2 h	• 50 ug/mL mv ↑ H2O2 induced DNA damage • ↓1ug/mL H2O2 induced DNA damage • 5-10 ug/mL ↑H2O2 apoptosis • Mv was more effective at inducing apoptosis compared to pn
Renis et al 2008	Caco-2 cells	Cy, cyn-3-O-B-pyranoside	5,10,25,50,100, and 200 umol/L	48 h	• 5-10 μM did not inhibit proliferation, 25-100 μM ↓cell proliferation • ($p < 0.001$)
Shih et al 2005	AGS gastric adenocarcinoma cells	Cy, dp, mv, pg, pn glycosylated cy, mv, pn and pg	200 umol/L	12-48 h	• Mv, dp and mv-3-glucoside ↓ viability to 63, 67 and 69% of the control • Mv induced apoptosis in a dose and time-dependent manner (started to significantly ↑ at 100

					umol/L)
Song et al 2012	JB6 P+ mouse epidermal (JB6 P+) cells	Cy and cy-3-glucoside	0–40 umol/L	1 h	• Dose-dependent response in radical scavenging abilities • Cy inhibited EGF-induced Akt/p70S6K phosphorlytaion and PI3K
Srivastava et al 2007	HT-29 cells colon carcinoma cells	Frozen blueberries anthocyanin fractions	50-150 ug/mL	6 h	• ↑ Caspase-3 activity • ↓ antioxidant enzymes of GST family
Tokarev et al 2010	HT-29 colon carcinoma cells	Dp	1,5,10,25 umol/L	4 h then 100 μM H_2O_2 for 2 h	• 1-10 umol/L dp ↑cell proliferation in H_2O_2 treated cells vs H_2O_2 alone
Tsuyuki et al 2012	HeLa cervical carcinoma cells	Pg, cy, mv, dp	25, 50, 100 umol/L	24, 48, and 72 h	• Mv ↓ cell proliferation at 100 umol/L and dp ↓ cell proliferation at 25 umol/L • Anthocyanidins did not influence caspase-3,7 activity
Wang et al 2009	Male F344 rats, 4 to 5 wk old	Freeze dried black raspberries (BRB), anthocyanin-rich fractions	Diets containing: a)5% BRB powder, b)anthocyanin-rich faction, c)organic	Fed the various diets for 2 wk before treatment with	• Anthocyanin treatments suppressed N-nitrosomethylbenzylamine-(NMBA) induced tumors in the rat esophagus
Yi et al 2005	HT-29 and Caco-0 colon cancer cells	blueberry anthocyanin fractions	1-100 ug/mL	48 h	• 1 ug/mL ↓ viability in both cell lines, 50% inhibition in Caco-2/HT-29 by 15-50 ug/mL • Anthocyanins ↑ DNA fragmentation by 3-7X, with greatest effect at ~40 ug/mL in HT-29 cells and 80 ug/mL in Caco-2 cells

Yun et al 2009	HCT-116 colon cancer cells	Dp	30, 60, 120, 180 and 240 umol/L	48 h	• In a dose-dependent response del ↓cell proliferation from 60-240 umol/L
Zhang et al 2005	Human cancer cells: AGS (gastric) HCT-116 (colon), MCF-F (breast), NCI H460 (lung) and SF-268 (central nervous system)	Dp, cy, mv, pn, pet and 4 glycosylated anthocyanins	12.5, 25, 50, 100 and 200 ug/mL	48 h	• Glycosylated anthocyanins had no effects at 200 ug/mL. Cy and dp at 100-200 ug/mL ↓ only MCF-7, pet only ↓ MCF-7 and AGS at 200 ug/mL. Pn and mv ↓ all lines at 200 and 100 ug/mL respectively
Zhao et al 2004	HT-29 colon carcinoma cells and NCM460 nontumorigenic colon cells	Commercially prepared anthocyanin-rich extracts (ARE) grape, bilberry, and chokeberry	10-75ug/mL	Up to 72 h	• All ARE inhibited cell growth, chokeberry was the most potent • ~50% HT-29 cell growth was inhibited after treatment of 25 ug/mL chokeberry • NCM460 cells were only affected by 75 ug/mL grape ARE after 72 h incubation

Abbreviations: cy (cyaniding), dp (delphinidin), mv (malvidin), pet (petunidin), pg (pelargonidin), pn (peonidin)



Anthocyanin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Pelargonidin	H	H

*Sugar moieties in glycosylated anthocyanins are usually attached at this position

Figure 2.1. Structure of common anthocyanidin

CHAPTER III

MALVIDIN AND DELPHINIDIN EXHIBIT A DOSE-DEPENDENT EFFECT ON CELL V IABILITY AND APOPTOSIS IN HT-29 CELLS[1]

[1] Krauss, A.J., Fischer, J.G. To be submitted for publication in *Nutrition Research*.

Abstract

Anthocyanidins induce apoptosis in some cancer cell lines, but studies describing the dose-dependent effects on cell populations are limited. My objective was to evaluate the dose-dependent response of malvidin and delphinidin, anthocyanidins with different chemical structures, on cell viability and apoptosis in HT-29 cells compared to curcumin, a known inducer of apoptosis. Lower concentrations of anthocyanidins increased cell viability by 12-16% compared to control. Higher concentrations of ≥80 umol/L of curcumin, malvidin, and delphinidin decreased cell viability by 16-44%. Apoptosis was assessed by measuring caspase-3 and caspase-8 activities, as well as mitochondrial permeability. Only high concentrations (80 umol/L) of anthocyanidins and curcumin increased caspase-3 and caspase-8 activity. Concentrations of 25, 50 and 80 umol/L malvidin and delphinidin significantly disrupted mitochondrial permeability, showing anthocyanidins may be more effective at inducing apoptosis intrinsically. There was a significant interaction between anthocyanidin concentration and anthocyanidin type on cell viability, caspase-8 activity, and mitochondria permeability.

Introduction

In 2012 cancer accounted for every one in four deaths in the US (American Cancer Society 2012). Epidemiological studies have shown a diet rich in fruits and vegetables reduces the risk of some cancers, especially those of the upper digestive tract (WCRF/AICR 2007). The health benefits from consuming high intakes of fruits and vegetables may be linked, in part, to their non-nutritive compounds. Anthocyanins, a subgroup of flavonoids, are responsible for the bright reds, blues, and purples that are found in a variety fruits and vegetables (Prior and Wu 2006). The average intake of anthocyanins in the US is estimated to be 11.7 mg/d (Murphy et al

2012). Anthocyanin bioavailability is low and it is believed that a high percentage reach the lower intestinal tract (He et al 2005; Kahle et al 2006).

Anthocyanins are formed from aglycone bases called anthocyanidins. In vitro studies have investigated the effect of anthocyanidin treatments on proliferation and apoptosis in various cancer cell lines. Anthocyanidins in high concentrations (ranging from 50-200 umol/L) have significantly reduced cell viability by 50-90% (Brown et al 2012). Several studies have shown anthocyanidins to induce apoptosis through both intrinsic and extrinsic mechanisms. However, in some studies anthocyanidins inhibited cell proliferation but did not induce apoptosis (Brown et al 2012). Interestingly, berry extracts were able to induce apoptosis in cyclooxygenase-2 expressing cell lines such as HT-29 cells, but not in other lines, such as HCT-116, which lacked the enzyme (Wu et al 2007; Katsube et al 2003). A remaining question is whether the anthocyanidin concentrations that have a significant impact on cell proliferation and apoptosis are feasible in vivo, and particularly whether they can be reached with a diet that is high in fruits and vegetables (Brown et al 2012).

The differences in chemical structure between individual anthocyanidins may explain their varying anticancer properties (Prior and Wu 2006). Jing et al (2008) reported an increase in free hydroxyl groups enhances antioxidant potential. A free hydroxyl group at the 3' position in anthocyanidins was also shown to more significantly influence cell proliferation and inhibitory activity, either increase or decrease depending on concentration of treatment (Zhang et al 2005). The two anthocyanidins of interest in this study are delphinidin and malvidin. Delphinidin contains three free hydroxyl groups on its peripheral phenol ring, while malvidin contains one hydroxyl group and two methoxyl groups.

Other dietary compounds such as curcumin, the primary curcuminoid in the spice turmeric, have been investigated for anticancer potential. Curcumin induces intrinsic apoptosis through the mitochondrial-mediated pathway in HT-29 cells and extrinsic apoptosis through Fas-associated death domain/ caspase-8 initiation (Wang et al 2009; Reuter et al 2008). It is unclear whether anthocyanidins are able to induce apoptosis to the same extent.

The objectives of this study were to investigate the dose-dependent response of malvidin and delphinidin on cell viability and apoptosis in HT-29 cells. The second objective was to compare the effects of these two structurally different anthocyanidins to curcumin. It was hypothesized that concentrations of 25-100 umol/L would decrease cell viability and induce apoptosis. The effect of malvidin and delphinidin on cell proliferation and apoptosis would differ with delphinidin having a greater impact due to the presence of three hydroxyl groups on the peripheral phenol ring. Curcumin would have similar antiproliferative and pro-apoptotic effects compared to the anthocyanidins.

Methods

Cell culture and media

HT-29 cells were purchased from ATCC (Manassas, Virginia) and cultured in 75 cm^2 flasks using ATCC's McCoy's 5a medium with 10% fetal bovine serum. The cells were incubated in 37 ° C in a humidified atmosphere of 95% air, 5% CO_2. The media was changed every 2-3 d; subculturing was done at 80% confluence and cells were passed at a ratio of 1:4.

Cells were treated with 0, 5, 10, 25, 50, 80, or 100 umol/L malvidin or delphinidin or 40 or 80 umol/L curcumin for 48 h prior to an assay. Compounds were purchased from Chromadex (Irvine, CA).

Cell viability

Cell viability was determined using a MTT cell proliferation assay kit (ATCC, Manassas, VA). In metabolically viable cells the yellow MTT reagent (3-)4,5-dimethylthizolyl-2)-2,5-diphenyltretrazolium bromide) is reduced by dehydrogenase enzymes to produce NADH and NADPH, forming a purple formazan. The absorbance of the purple formazan was measured by spectrophotometry. 100 uL of cells were seeded in 96 well plates at 10^5 cells/mL. The untreated cells were incubated for 24 h, old media was removed and designated wells were treated with 0, 5, 10, 25, 50, 80, or 100 malvidin or delphindin or 40 or 80 umol/L curcumin (n=3 per group). After 48 h, media was removed from all wells and 100 uL of fresh media was added with 10 uL of MTT reagent. This was incubated at 37 °C for 3 h until a purple precipitate was visible under a microscope. 100 uL MTT detergent reagent was added to all the wells, the plate was then covered with foil and left in a dark room at room temperature for 2-4 h. Cell viability was measured at 570-655 nm with a microtiter plate reader (Turner 2010; Patterson 2009; Galambos 2012).

Apoptosis

The caspase-3 activity assay, caspase-8 activity assay, and mitochondrial membrane potential were used to assess apoptosis. There are multiple ways to assess apoptosis, however caspase-3 activity is commonly used as a general biomarker of apoptosis. The protease caspase-3 is involved early in the process that commits the cell to apoptosis (Kim et al 2006). Caspase-8 is an initiator protein within the extrinsic apoptosis pathway, its activation by DISC triggers caspase-3, stimulating a cascade of proteolytic activity (Jain et al 2013). Mitochondrial membrane potential will also be measured because it represents apoptosis occurring intrinsically (Jain et al 2013). The mitochondrial membrane potential will increase if it is disrupted, which will result in proapoptoic compounds being released that signal the cell to undergo apoptosis. It

has been reported that substances that induce intrinsic apoptosis have a better ability to disrupt cancer pathogenesis (Hanahan and Weinberg 2011).

Caspase-3 Activity

A colorimetric Caspase-3 Assay Kit was purchased from EMD Millipore (Billerica, MA). Caspase-3 is an important marker of apoptosis because the activation of caspase-3 commits the cell to apoptosis (Jain et al 2013). If present, caspase-3 can cleave a substrate containing the DEVD sequence (Asparte-Glutamate-Valine-Aspartate). Caspase-3 activity was determined by measuring the amount of free chromophore *p*-nitroaniline (*p*NA) that was cleaved from DEVD-*p*NA (Millipore).

For the assay, cells were grown to confluence in 75 cm^2 flasks. Apoptosis was induced by incubating flasks with 0, 25, 50, or 80 malvidin or delphinidin or 80 curcumin for 48 h or 250 tert-butyl hydroperoxide (t-BOOH) for 2 h at 37 °C. The old media was removed from all flasks and 10 mL of fresh media was added and incubated for ~5-15 min. Cells were then harvested by trypsinization, cells were counted and 1 mL of 1.5 X 10^6cells/mL were obtained for each group. Cells were centrifuged at 125 x g for 10 minutes in a microcentrifuge. The supernatant was removed and the cells were suspended in 300 uL 1X cell lysis buffer, incubated on ice for 10 min, then centrifuged for 5 min at 10,000 x g. In a 96 well plate, each test well contained 20 uL of 5X assay buffer, 10 uL of caspase-3 substrate, and 70 uL of supernatant from the designated sample. Samples from the untreated cells acted as the controls. A buffer blank was created by adding 20 uL 5X assay buffer and 80 uL of distilled water and a substrate blank was created by adding 20 uL 5X assay buffer, 70 uL of distilled water, and 10 uL caspase-3 substrate. The 96-well plate was incubated for 1-2 h at 37 °C. Samples were read at 405 nm by a microtiter plate

reader. Fold-increases were calculated by using the equation (Sample-Substrate Blank)/(Control-Substrate Blank) (Tokarev 2010).

Caspase-8 Activity

A colorimetric caspase-8 assay kit was purchased from EMD Millipore (Billerica, MA). The caspase-8 activity assay had a similar protocol to caspase-3 but the samples were treated with the caspase-8 substrate, Acetyl-Isoleucine-Glutamine- Threonine-Aspartate p-nitroaniline (Ac-IETD-pNA). Caspase-8 activity was determined by measuring the amount of cleaved pNA on a spectrophotometer at 405 nm (Nicholson et al 1995). Apoptosis-induced samples were compared to the un-induced controls to find the fold increases in caspase-8 activity.

To induce apoptosis, cells were treated with 50 or 80 umol/L of malvidin or delphindin or 80 umol/L curcumin for 48 h or 250 t-BOOH for 2 h at 37 °C. Cells were counted and collected in 1 mL aliquots of 1.5 $X10^6$cells/mL in Eppendorf tubes. A cell pellet was formed using a microcentrifuge at 125xg for 10 min. The media supernatant was discarded and cells were resuspended with 300 umol/L 1X Cell Lysis Buffer and incubated on ice for 10 minutes, then centrifuged for 5 min at 10000 x g. In a 96 well plate, each test well contained; 20 uL of 5X assay buffer, 10 uL of defrosted caspase-8 substrate, and 70 uL of supernatant from the designated sample. Samples from the untreated cells acted as the controls. A buffer blank was created by adding 20 uL 5X assay buffer and 80 uL of distilled water and a substrate blank was created by adding 20 uL 5X assay buffer, 70 uL of distilled water, and 10 uL caspase-8 substrate. The 96-well plate was incubated for 1-2 h at 37 °C. Samples were read at 405 nm by a microtiter plate reader. Fold-increases were calculated by using the equation (Sample-Substrate Blank)/(Control-Substrate Blank)(Wang et al 2008).

Mitochondrial membrane potential

Mitochondrial membrane potential was assessed with a MitoPT JC-1 kit (ImmunoChemistry Technologies LLC., Bloomington, MN), which uses 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine I- / Cl- salt, referred to as JC-1, to emit fluorescence. A change in mitochondria permeability transition (PT) can be used to detect early indicators of apoptosis. PT is the disruption of the electrochemical gradient across the mitochondrial membrane, and is measured by mitochondrial membrane potential. The change in membrane potential results in the release of proapototic proteins, BID, BAK, BAD, or BAX, the proteins create pores allowing cytochrome-c to be released into the cytoplasm (Jain et al 2013).

JC-1 detects the loss of mitochondrial membrane potential. In healthy cells JC-1 redistributes itself within mitochondrial organelles, emitting fluoresces of orange-red at 488 nm excitation and 590-600 nm emission for red fluorescence. If the mitochondrial membrane collapses because of stressed and apoptotic conditions JC-1 will be disperse throughout the cell in a monomeric form. This will cause JC-1 to emit a green fluorescence upon excitation at 488 nm and have a lower red fluorescence absorbance.

Apoptosis was induced by treating the cells with 25, 50, or 80 umol/L malvidin or delphinidin or 40 or 80 umol/L curcumin for 48 h, or 250 t-BOOH for 2 h at 37 °C. An additional flask was treated with 50 mM carbonylcyanide m-chlorophenylhydrazone (CCCP) as a positive control, CCCP was diluted (1:10) into 10 ml of media and incubated for 1 h at 37 °C. Another flask was treated with the same volume of DMSO-media, to represent a negative control, and incubated for 1 h at 37 °C.

The HT-29 cells were cultured to ~$1.5x10^6$ cells/mL, and pelleted by centrifugation at 125x g for 7 min at room temperature. The media supernatant was discarded and the cells were re-suspended with 1 mL of 1X Mito PT JC-1 solution, and incubated for 10-15 min in CO^2 at 37

°C. Then 2 mL of warmed 1X assay buffer was added to the JC-1 cell solution and mixed. The cells were centrifuged at 125 x g for 7 min at room temperature. Once again the supernatant was removed and 1 mL of 1X assay buffer was added to the cell pellets, and mixed well. A small aliquot from each sample was taken to determine a cell count. The cells were then centrifuged at 400 x g for 5 min at room temperature. The supernatant was removed and replaced with adequate amounts of 1X assay buffer to yield a cell population of $1.5x10^6$ cells/mL for each sample. 100 uL of each sample was dispensed into 4 wells in a black 96-well plate. The plate was read at an excitation wavelength of 488-490 nm and red fluorescence emission wavelengths at 590-600 nm. The red fluorescence (RFU) readings were compared between the samples for change in the mitochondria membrane (Desagher et al 1999).

Statistical Analysis

Differences among treatment group means were determined with analysis of variance (ANOVA) and Fisher's least significant difference test (Statistical Analysis Systems, Version 9.3, Cary NC). Differences were considered significant at $p< 0.05$.

Results

Cell Viability: The effects of the various treatments on cell viability in HT-29 cells are shown in **Table 3.2** and **Figure 3.1**. Overall the treatments exhibited a significant dose-dependent response on cell viability ($p<0.0001$). Lower concentrations of delphinidin (5 and 10 umol/L) and malvidin (5 umol/L) increased cell viability by 12-16% compared to control. Higher concentrations of curcumin, (80 umol/L), malvidin (80 umol/L and 100 umol/L), and delphinidin (80 umol/L and 100 umol/L) decreased cell viability by 16-44%. The greatest inhibition of cell viability was found with 100 umol/L delphinidin, which was significantly greater than the other treatments. A separate ANOVA examined the effect of malvidin and delphinidin on cell

viability compared to control. There was a significant effect of concentration ($P<0.001$) and a significant concentration times anthocyanin interaction ($p<0.02$) due to the different effects of of malvidin and delphinidin at concentrations of 50 and 100 umol/L. The results suggested that concentrations of anthocyanidins (5 and/or 10 umol/L) increase cell viability and concentrations of 80 umol/L or higher are required to decrease cell proliferation.

Apoptosis: Since the anthocyanidins did not affect cell viability at concentrations at 5 and 10 umol/L further assays were conducted at only anthocyanidin concentrations of 25 umol/L or higher.

Caspase-3 activity: The results for caspase-3 activity are shown in **Table 3.3** and **Figure 3.2**. An increase in caspase-3 activity indicates apoptosis is occurring because its initiation commits the cell to death. Treatment with t-BOOH, curcumin, malvidin and delphinidin impacted caspase-3 activity ($p<0.0001$). Post-hoc tests showed that a significant increase in caspase-3 activity, of about 1.6 fold, occurred in cells treated with 80 umol/L of malvidin, delphinidin, and curcumin. There was no increase in caspase-3 activity with 25umol/L malvidin and delphinidin over control, and a slight, but significant increase with 50 umol/L malvidin... A separate analysis of only anthocyanidin effects on caspase-3 activity confirmed there was a significant dose-response effect of both anthocyanidins on caspase-3 activity ($p<0.001$). Preliminary results (not shown) found only treatments of 80 umol/L of anthocyanidins or curcumin significantly increased caspase-3 activity. These results indicate concentrations of malvidin and delphinidin that are greater than 25 umol/L may be needed to induce apoptosis in HT-29 cells.

Caspase-8 activity: The results of caspase-8 activity are shown in **Table 3.4** and **Figure 3.3**. An increase in caspase-8 activity suggests apoptosis was stimulated through the extrinsic pathway. Through the extrinsic pathway an apoptosis-inducing agent binds to Fas- receptor

stimulating activation of procaspase-8 to caspase-8, and forms the Fas-Associated protein with Death Domain (FADD). The impact of malvidin and delphinidin on caspase 8 activity was only examined at concentrations of 50 and 80 umol/L since caspase 3 activity and cell viability were not impacted by lower concentrations of these anthocyanidins. There was a dose-dependent response for both anthocyanidins on caspase-8 activity ($p<0.002$), however, only 80 umol/L malvidin, with a fold increase of 1.47, was significantly different compared to the control ($p<0.05$). This was also demonstrated with a significant a concentration times anthocyanin type interaction ($p<0.02$).

Mitochondrial permeability transition: The results of the effects of curcumin, malvidin and delphinidin on mitochondrial permeability transition are shown in **Table 3.5** and **Figure 3.4**. Disruption of mitochondrial permeability can trigger the apoptosis intrinsic pathway. Higher values of red fluorescence signify healthy cells with intact mitochondria, while lower values indicate the mitochondria membrane has been damaged. Cells treated with DMSO, the recommended negative control, did not differ from the untreated control group. Cells treated with t-BOOH induced intrinsic apoptosis by 17% compared to the control. Cells treated with CCCP, the provided positive control, induced apoptosis by 28% compared to the control group. Concentrations of ≥25 umol/L malvidin and, delphinidin, and >40 umol/L curcumin significantly induced intrinsic apoptosis by disrupting mitochondrial membrane permeability. In a separate analysis of the effects of only the anthocyanidins, there was a significant dose-dependent effect for both anthocyanidins on intrinsic apoptosis ($p<0.0001$). Also, malvidin and delphinidin disrupted mitochondrial permeability differently ($p<0.0001$) with malvidin having a greater effect. Both anthocyanidins at 25 umol/L increased intrinsic apoptosis by 18-21%, comparable to treatment of t-BOOH. A concentration times anthocyanin effect was observed, due to a

greater effect of malvidin, compared to delphinidin at 50 umol/L ($p<0.0001$). Cells treated with 50 umol/L malvidin and 40 umol/L curcumin significantly increased intrinsic apoptosis by about 47%. Treatment with of 80 umol/L malvidin, delphinidin, and curcumin stimulated intrinsic apoptosis to the greatest extent, by 56-62%. These results may suggest anthocyanidins are able to target the intrinsic apoptosis pathway more effectively compared to the extrinsic pathway.

Discussion

Healthy cell populations are controlled by a balance of cell proliferation and cell death/ apoptosis. Carcinogenesis disrupts the homeostasis by suppressing cell death and increasing cell renewal (Hanahan and Weinberg 2011). Cancer treatments aim to stop the progression of carcinogenesis and eliminate the malignant cells. Anthocyanidins may have the ability to reestablish cellular homeostasis and inhibit cancer progression (Wang and Stoner 2008). Several *in vitro* studies have shown anthocyanidins act as chemopreventative agents by performing as antioxidants, or by inhibiting proliferation, inducing apoptosis, and halting the progression of angiogenesis. Anthocyanidins have been described as having antioxidant as well as pro-oxidant effects (Ramos 2008; Tokarev 2010).

The purpose of this study was to examine the dose dependent response of delphinidin and malvidin on cell proliferation and apoptosis of HT-29 cells, and compare this with curcumin. The differences in chemical structure between individual anthocyanidin may explain their varying anticancer properties (Prior and Wu 2006). Jing et al (2008) reported an increase in free hydroxyl groups enhances antioxidant potential. A free hydroxyl group at 3' position in anthocyanidin also contributes to cell proliferation and inhibition (Zhang et al 2005). Delphinidin contains three free hydroxyl groups on its peripheral phenol ring, while malvidin contains one hydroxyl group and two methoxyl groups. Delphinidin has exhibited more

antioxidant potential compared to other anthocyanidins, mostly like because of its three free hydroxyl groups (Rahman et al 2006). It has been suggested anthocyanidins with more hydroxyl groups on the B-ring (delphinidin and cyanidin) inhibit topoisomerase I and II, resulting in the disruption on G2/M-passage in the cell cycle. Malvidin also interferes with G2/M-progression, not through inhibition of topoisomerases but potentially because of its ability to damage DNA (Fritz et al 2006).

A broad range of concentrations, 5-100 umol/L, was initially tested to examine the anthocyanin's potential to increase as well as inhibit effects that control cell populations. Concentrations of ≤25 umol/L of delphinidin and malvidin have been previously tested in our lab under oxidative-stressed conditions using H_20_2 or t-BOOH. Concentrations ≤ 10 umol/L of delphinidin have shown the greatest antioxidant ability (Turner 2010; Patterson 2009; Galambos 2012). Multiple in vivo studies have tested higher concentrations of anthocyanidins (≥50 ug/mL or 100-250 umol/L), which decreased cell viability by 50-100% and induced apoptosis in a dose-dependent manner (Brown et al 2012). However, higher concentrations are less feasible in vivo and are more likely to cause cytotoxic affects in healthy tissues, so a lower range of 5-100 umol/L was selected for the current study. The phenolic compound curcumin was used because it has a well documented effect on cell proliferation and apoptosis. Curcumin has shown to reduce cell proliferation by inhibiting NF-κB and inducing apoptosis, mainly through the intrinsic pathway by mitochondrial disruption, but also by binding to Fas-associated death domain (Wang et al 2009; Reuter et al 2009).

First, the dose-dependent response of the anthocyanidins and curcumin on cell viability was evaluated. It was hypothesized that concentrations of 5 and 10 umol/L of the anthocyanidins would increase cell viability. The results showed the lowest concentrations of 5 and 10 umol/L

delphinidin and 5 umol/L malvidin, increased cell proliferation compared to the untreated controls. These results suggest anthocyanidins may have a protective effect at lower concentrations. Previous studies have reported similar findings of anthocyanidins being protective (against oxidative-induced stress) and increasing cell proliferation (Tokarev 2010; Galambos 2012; Rahman et al 2006). The greatest increase in cell viability was with the lowest concentration of 5 umol/L. Previous studies in our lab reported similar findings in which concentrations around 5 umol/L exhibited the greatest increases in cell viability (Patterson 2009; Tokarev 2010). Others have found concentrations of anthocyanidins >10 umol/L to be the most protective (Esselen et al 2011; Afaq et al 2005).

I hypothesized that concentrations of 25-100 umol/L anthocyanidins would decrease cell proliferation, but found under the conditions of this study that concentrations of 80 umol/L curcumin, malvidin, and delphinidin were necessary to significantly decrease cell viability. It was predicted delphinidin would affect cell viability more than malvidin, and at 100 umol/L delphinidin decreased cell viability to a greater extent compared to the control. The antiproliferative effects of 80 umol/L were comparable to other studies (Yi et al 2005; Brown et al 2012). Surprisingly 50 umol/L delphinidin increased cell proliferation compared to the control. Anthocyanidins at higher concentrations may increase the amount of reactive oxygen species within the cell and simultaneously deplete ATP, which would result in a decrease in viability and cell death (Cvorovic et al 2010). Higher concentrations of anthocyanin treatments have also been shown to damage DNA integrity and cause a buildup of cells in the G2 phase of the cell cycle (Fritz et al 2006). Curcumin did exhibit similar effects compared to malvidin and delphinidin. However, its effect on cell viability was lower compared to previous studies using equivalent concentrations. Wang et al (2009) reported 40 umol/L curcumin caused reduction of

38% in cell viability in HT-29 cells and 80 umol/L curcumin had an 89% reduction in cell viability compared to only a 30% reduction in the current study. In Wang's study the cells were only incubated for only 24 h and used a different MTT assay to assess cell viability, which could potentially explain the varying results.

The effectiveness of malvidin and delphinidin at decreasing cell proliferation has differed among cell lines. Zhang et al (2005) compared the effects of anthocyanidins on various cancer cell lines; AGS (gastric), HCT-116 (colon), MCF-F (breast), NCI-H460 (lung), and SF-268 (CNS). Delphinidin, at 100-200 umol/L, only reduced cell proliferation in MCF-F cells but malvidin affected proliferation of all the cell lines at 100-200 umol/L. In contrast, a study using HCT-116 cells showed delphinidin had a dose-dependent response at 60-240 umol/L (Yun et al 2009). Also, in HeLa cervical cancer cells, delphinidin reduced cell proliferation at a lower concentration of 25 umol/L compared to malvidin at 100 umol/L (Tsuyuki et al 2012). However, malvidin decreased cell viability and induced apoptosis to the greatest extent compared to all the anthocyanidins in AGS cells (Shih et al 2005). Thus, studies at this time suggest the effects of malvidin and delphinidin on cell viability are cell and dose-dependent.

Caspase-3 activity, caspase-8 activity, and mitochondrial membrane permeability were used to assess the impact of anthocyanins on apoptosis. Since the lower concentrations of anthocyanidins, 5 and 10 umol/L, did not affect cell viability, they were not assessed in the apoptosis assays. The maximum concentration tested was 80 umol/L malvidin and delphinidin, because the study focus was on the lowest concentration required to induce apoptosis. It was initially hypothesized 25-80 umol/L anthocyanidins would increase caspase-3 activity. Caspase-3 is an important effector enzyme; when activated, through either intrinsic or extrinsic pathways, it commits the cell to apoptoticdeath (Huai et al 2010). In our study, caspase-3 activity only

increased at the concentration of 80 umol/L for malvidin, delphinidin, and curcumin, This corresponds to the concentration needed to decrease cell viability. Other studies found similar results, where similar concentrations of 100 umol/L delphindin decreased proliferation and increased caspase-3 activity (Cvorovic et al 2010).

Caspase-8 activity is a measure of the activation of apoptosis through the extrinsic pathway. There is a limited amount of research about the ability of anthocyanidins to trigger the extrinsic pathway directly. In our study caspase-8 only increased with 80 umol/L malvidin. Curcumin was expected to significantly increase caspase-8 activity; although there was a slight increase in activity, it did not differ from the control. One explanation is the extrinsic pathway may require a higher concentration of some polyphenolics to trigger a response. Another reason could be because anthocyanidins and curcumin increase ROS production, and may be more tailored to target the intrinsic pathway versus the extrinsic pathway (Fimognari et al 2012). However, since malvidin at 80 umol/L increased activity, while delphinidin did not, it may suggest structural composition is an important determinant of this effect.

The intrinsic pathway of apoptosis is commonly targeted more by clinical treatments such as irradiation and chemotherapy (Jain et al 2013). Disruption of the mitochondrial membrane, stemming from DNA damage, defective cell cycle, hypoxia, and other types of stress, can lead to apoptosis. A change in the membrane permeability can cause an increase in pro-apoptotic proteins such as Bax, Bad, Bid, and ultimately result in the release of cytochrome c (Kim et al 2006).

Anthocyanidins and curcumin target several proteins within the intrinsic pathway to stimulate apoptosis; up-regulating pro-apoptotic proteins of the Bcl-2 family like BAX, BIM and BAK, and down regulating the anti-apoptotic proteins Bcl-2 and Bcl-xL. Both types of phenolic

compounds also can generate ROS within the malignant cell (Hyun et al 2004; Wang et al 2009; Reuter et al 2008). Several studies have showed curcumin-inducing apoptosis through the Fas-receptor/ caspase-8 pathway independently of p53, but like anthocyanidins, curcumin has shown to induce p53 dependent apoptosis (Reuter et al 2008). I hypothesized that concentrations of 25-80 umol/L anthocyanidins would disrupt mitochondria permeability and stimulate intrinsic apoptosis. All tested concentrations, 25-80 umol/L of malvidin, delphinidin significantly altered mitochondrial membrane permeability compared to the controls. Malvidin had a clear dose-dependent response with concentrations of 25, 50 and80 umol/L increasing mitochondrial damage in incremental amounts. Malvidin had a greater inhibitory effect at a concentration of 50 umol/L compared to delphinidin, but the effects were generally comparable at 80 umol/L. Curcumin had a comparable response to malvidin at similar concentrations. Since the cells treated with anthocyanidins were not subjected to any oxidative stress agents the results suggest the anthocyanidins generated ROS, causing the induction of intrinsic apoptosis. While the activation of apoptosis is not exclusive to one pathway or another, it does appear anthocyanidins and curcumin have a more significant impact on the intrinsic rather than extrinsic pathway.

It is difficult to quantify *in vitro* concentration to the equivalent treatment doses *in vivo* populations. The average intake of anthocyanins in the U.S has been estimated to be around 11.7 mg/d (Murphy et al 2012). Anthocyanin intake could be easily be increased by consuming more anthocyanin-rich foods. For example, cultivated blueberries contain 387 mg/100 g of anthocyanins (Wu et al 2006). While the bioavailability of anthocyanins is low, they are found in the gastrointestinal lumen where they could have a significant impact (Esselsen et al. 2011). In an in vivo study 32 rats received chokeberry, bilberry, grape-enriched (3.85 g monomeric anthocyanin per kg diet), or control diet for 14 weeks. Fecal contents were examined and

anthocyanin concentrations ranged from 0.7-2 g/kg, with the rats fed the grape diet having the highest anthocyanin-fecal content. The high anthocyanin concentrations within the rat feces could indicate the potential for a high amount of anthocyanin absorption into colon epithelial cells (He et al 2005). The bioavailability of anthocyanins could be underestimated because of undetected anthocyanin metabolites or because the compounds are found largely within the gastrointestinal track instead of plasma (Yi et al 2005). There is evidence, though, that anthocyanins do reach distant tissues. In another animal study, pigs were fed diets containing 1, 2, or 4% w/w blueberries for 4 weeks. No anthocyanins were found in plasma or urine samples, but intact anthocyanins were detected in all of the collected tissues (liver, eye, and brain) (Kalt et al 2008). There are several limitations to working with anthocyanidins in an *in vitro* model. While the aglycone forms exhibit greater antiproliferative effects, anthocyanins are typically consumed in a glycosylated form (Prior 2003). For example, the aglycones- cyanidin and delphinidin were more potent inhibitors against human vulva carcinoma cell growth compared to cyanidin-3-beta-D-galactoside or malvidin-3-beta-D-glucoside (Meiers et al 2001). Also *in vitro* models are exposed more to oxygen, which produces more reactive oxygen species and could alter the prooxidant bioactivity of anthocyanidins (Flis et al 2012). The complexity of *in vivo* models could differ in anthocyanidin disposition, resulting in different overall effects.

The findings of this study showed low concentrations of anthocyanidins increase cell proliferation, which is consistent with previous studies (Turner 2010; Galambos 2012). Higher concentrations of malvidin and delphinidin are needed to reduce cell viability and induce apoptosis. While a dose-response effect was evident, the anthocyanidins significantly reduced cell viability and increased caspase-3 and -8 activities at only 80 umol/L anthocyanidin. There were significant interactions between anthocyanidin concentration and anthocyanidin type on

cell viability, caspase-8 activity, and change in mitochondrial membrane permeability. Overall high concentrations of anthocyanidin, ≥80 umol/L, are needed to induce apoptosis; however concentrations of ≥ 25 umol/L significantly altered mitochondrial membrane permeability. This may suggest anthocyanidin are able to target the intrinsic apoptosis pathway more effectively. The apoptosis intrinsic pathway has been associated with oxidative stress, which corresponds to anthocyanidin's ability to act as pro-oxidants at high concentrations (Brown et al 2012; Ramos 2008). Also high concentrations of malvidin and delphinidin reduced cell viability and induced apoptosis to the same extent as the equivalent dose of curcumin. These results may indicate anthocyanidin have similar anticancer potential as curcumin. Additional studies are needed to understand the effects of a full range of concentrations of anthocyanins on cell populations to decrease any adverse effects and increase potential benefits.

Table 3.1
Treatment concentration conversions

Treatment	umol/L	ug/mL
Curcumin[1]		
	40	14.7
	80	29.5
Malvidin[2]		
	5	1.83
	10	3.67
	25	9.17
	50	18.34
	80	29.34
	100	36.68
Delphinidin[3]		
	5	1.69
	10	3.39
	25	8.47
	50	16.94
	80	27.10
	100	33.87

[1] Molecular weight of curcumin is 368.38 g/mol.
[2] Molecular weight of malvidin is 366.75 g/mol.
[3] Molecular weight of delphinidin is 338.70 g/mol.

Table 3.2

Cell viability of HT-29 human adenocarcinoma cells treated with curcumin, malvidin and delphinidin for 48 hours[1]

Treatment (umol/L)		% Viability[2]
Control[3]		100[c,d]
Curcumin		
	40	92 ± 4[d,e]
	80	74 ± 2[f]
Malvidin		
	5	116 ± 1[a]
	10	103± 7[a]
	25	95 ± 7[c,d,e]
	50	101 ± 3[c ,d]
	80	85 ± 4[e,f]
	100	74 ± 3[f]
Delphinidin		
	5	116 ± 5[a]
	10	113 ± 3[a,b]
	25	103 ± 3[b,c,d]
	50	116 ± 4[a]
	80	84 ± 5[e,f]
	100	56 ± 2[g]

[1] Mean ± SEM (n=3/group). Means with different letters are significantly different ($p<0.05$).
[2] % viability was calculated by comparing the treatments to the control cell population.
[3] Controls were not pretreated with anthocyanidins or curcumin.

Table 3.3
Caspase-3 activity in HT-29 human adenocarcinoma cells treated with curcumin, malvidin, or delphinidin for 48 hours[1]

Treatment (umol/L)		Fold Increase[2]
Control[3]		1.00 ± 0.13[c,d]
t-BOOH[4]		1.51 ± 0.07[b]
Curcumin		
	80	1.76 ± 0.06[a]
Malvidin		
	25	0.90 ± 0.08[d]
	50	1.12 ± 0.04[c]
	80	1.63 ± 0.02[a,b]
Delphinidin		
	25	0.88 ± 0.05[d]
	50	1.02 ± 0.04[c,d]
	80	1.56 ± 0.10[a,b]

[1] Mean ± SEM (n=4/group). Results with different letters are significantly different ($p<0.05$).
[2] Fold increase compared to control was calculated using the equation: (treatment-background control)/(control-background control).
[3] Control was not pretreated with anthocyanidins, curcumin, or t-butyl hydroperoxide (t-BOOH).
[4] t-BOOH group was treated with 200 umol/L t-BOOH 2 h prior to assay.

Table 3.4

Caspase-8 activity in HT-29 human adenocarcinoma cells treated with curcumin, malvidin, and delphinidin for 48 hours[1]

Treatment (umol/L)		Fold Increase[2]
Control[3]		$1.00 \pm 0.003^{c,b}$
Curcumin		
	80	$1.23 \pm 0.11^{a,b}$
Malvidin		
	50	0.95 ± 0.04^{c}
	80	1.47 ± 0.04^{a}
Delphinidin		
	50	$1.12 \pm 0.15^{c,b}$
	80	1.21 ± 0.02^{b}

[1] Mean ± SEM (n=3/group). Results with different letters are significantly different ($p<0.05$).

[2] Fold Increase compared to control was calculated using the equation: (treatment-background control)/(control-background control).

[3] Control was not pretreated with anthocyanidins or curcumin.

Table 3.5

Mitochondrial permeability transition in HT-29 human adenocarcinoma cells treated with curcumin, malvidin, and delphinidin for 48 hours[1]

Treatment (umol/L)		RFU[2] at 590 nm
Control[3]		277 ± 1[a]
DMSO[5]		269 ± 4[a]
t-BOOH[4]		232 ± 22[b]
CCCP[5]		199 ± 8[c]
Curcumin		
	40	142 ± 10[e,d]
	80	107 ± 3[f]
Malvidin		
	25	229 ± 4[b]
	50	147 ± 7[d]
	80	110± 2[f]
Delphinidin		
	25	220 ± 12[b,c]
	50	212 ± 2[b,c]
	80	122± 3[e,f]

[1] Mean ± SEM (n=4/group). Results with different letters are significantly different ($p<0.05$).
[2]RFU= red fluorescence
[3] Controls were not pretreated with anthocyanidins, curcumin, or t-BOOH.
[4] t-BOOH group was treated with 200 umol/L t-BOOH 2 h prior to assay.
[5] CCCP and DMSO groups were treated with 50 mM CCCP or DMSO for 1 h.

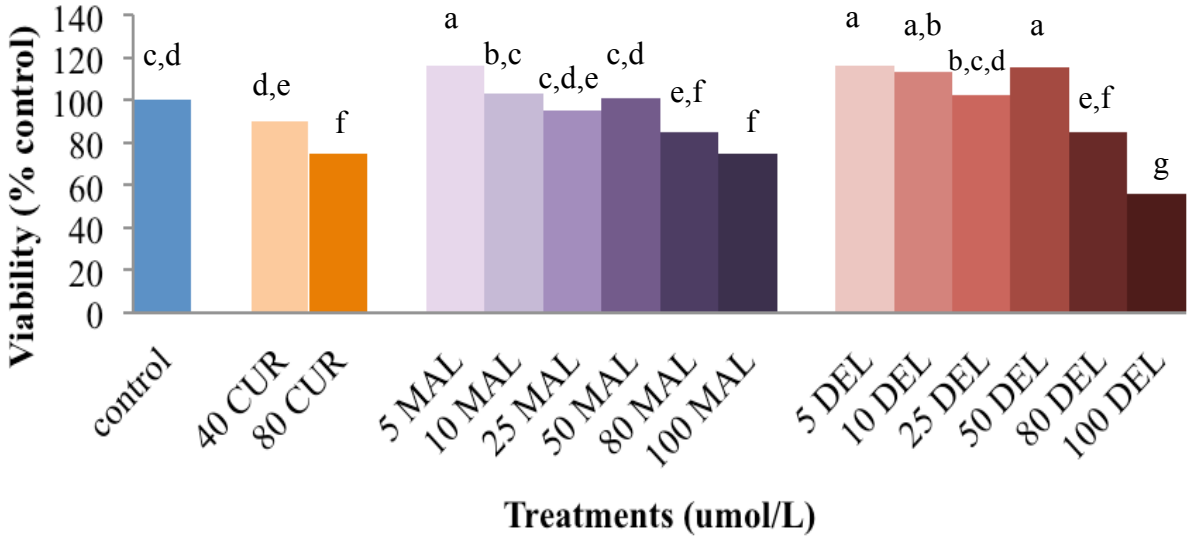


Figure 3.1: Cell viability in HT-29 cells treated with curcumin, malvidin, and delphinidin for 48 hours[1,2,3,4]

[1] Mean ± SEM (n=3/group). Means with different letters are significantly different ($p<0.05$).
[2] % viability was calculated by comparing the treatments to the control cell population.
[3] Controls were not pretreated with anthocyanidins or curcumin.
[4] Remaining cells were pretreated with curcumin, malvidin, or delphinidin for 48 h.

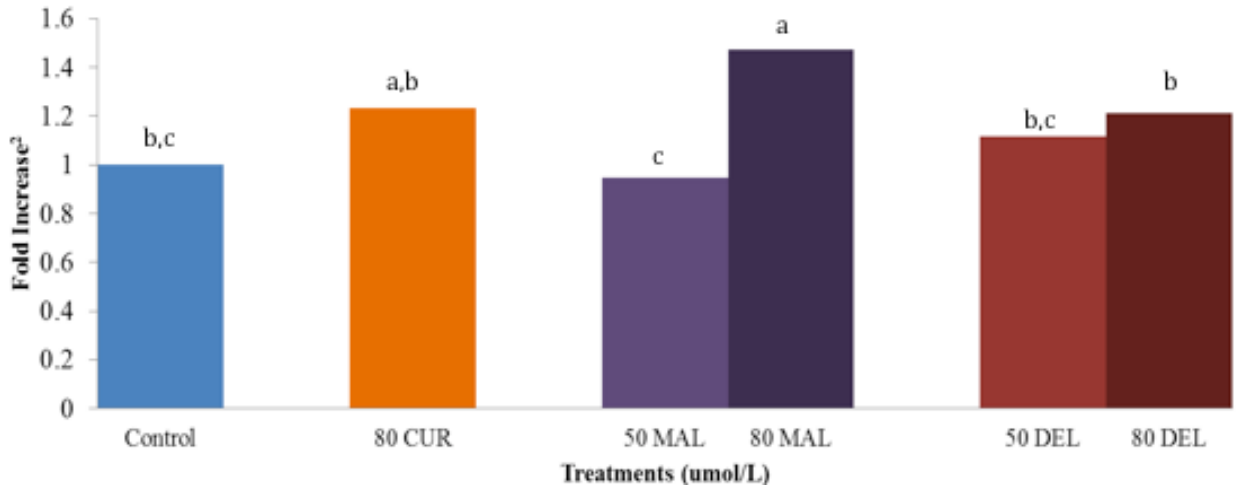


Figure 3.2: Caspase-3 fold increases in HT-29 cells treated with curcumin, malvidin, and delphinidin[1,2,3,4,5]

[1] Mean ± SEM (n=4/group). Results with different letters are significantly different (P<0.05).
[2] Fold Increase compared to control was calculated using the equation: (treatment-background control)/(control-background control).
[3] Controls were not pretreated with anthocyanidins, curcumin, or t-BOOH.
[4] t-BOOH group was treated with 200 umol/L t-BOOH 2 h prior to assay.
[5] Remaining groups were pretreated with curcumin, malvidin, or delphinidin for 48 h.

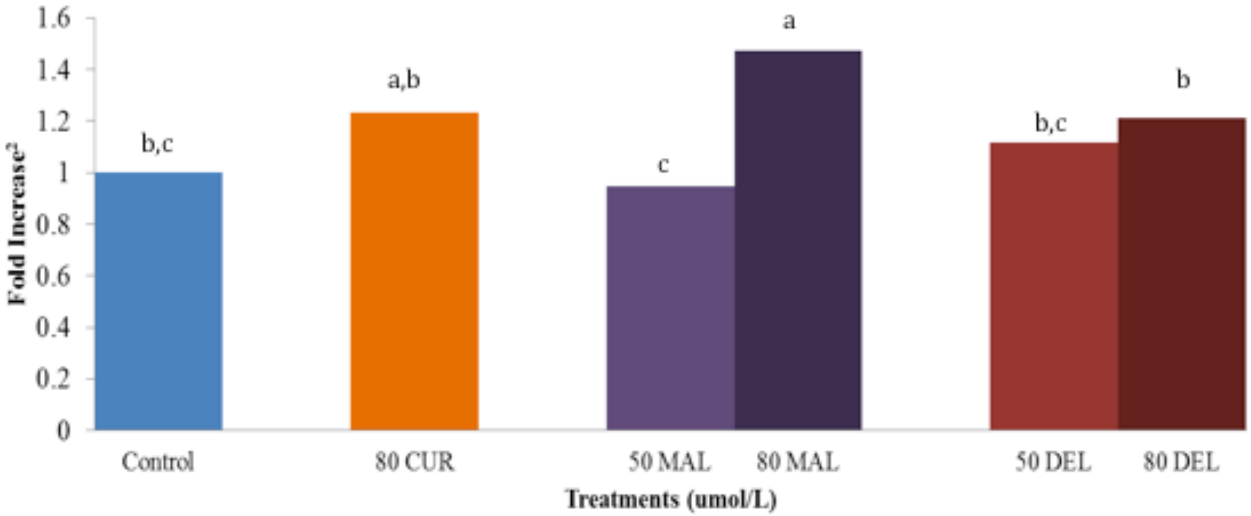


Figure 3.3: Caspase-8 activity in HT-29 cells treated with curcumin, malvidin, and delphinidin[1,23,4]

[1] Mean ± SEM (n=3/group). Results with different letters are significantly different (P<0.05)
[2] Fold Increase compared to control was calculated using the equation: (treatment-background control)/(control-background control).
[3] Controls were not pretreated with anthocyanidins, curcumin, or t-BOOH.
[4] Remaining groups were pretreated with curcumin, malvidin, or delphinidin for 48 h.

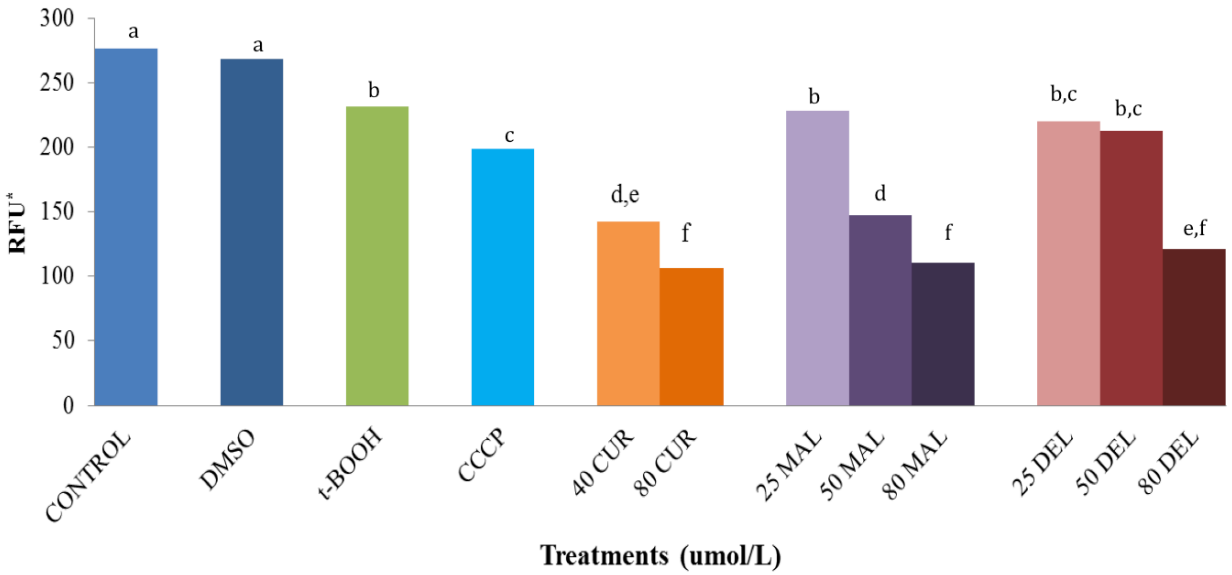


Figure 3.4: Mitochondria permeability transition in HT-29 cells treated with curcumin, malvidin, and delphinidin for 48 hours

*RFU= red fluorescence
[1] Mean ± SEM (n=4/group). Results with different letters are significantly different (P<0.05)
[2] Control was not pretreated with anthocyanidins, curcumin, or t-BOOH.
[3] t-BOOH group was treated with 200 umol/L t-BOOH 2 h prior to assay.
[4] CCCP and DMSO groups were treated with 50 mM CCCP or DMSO for 1 hr.
[5] Remaining groups were pretreated with curcumin, malvidin, or delphinidin for 48 h.

CHAPTER IV

SUMMARY AND CONCLUSION

Summary

In the U.S. cancer accounts for every one in four deaths (American Cancer Society 2013). Epidemiological studies have shown a diet rich in fruits and vegetables reduces the risk of some cancers, especially those of the upper digestive tract (WCRF/AICR 2007). The health benefits from consuming high intakes of fruits and vegetables may be linked, in part, to their non-nutritive compounds. Anthocyanins, a subgroup of flavonoids, are responsible for the bright reds, blues, and purples that are found in a variety fruits and vegetables (Prior and Wu 2006). Anthocyanins are formed from aglycone bases called anthocyanidins. In vitro studies have investigated the effect of anthocyanidin treatments on proliferation and apoptosis in various cancer cell lines. Anthocyanidins in high concentrations (ranging from 50-200 umol/L) have significantly reduced cell viability by 50-90% (Brown et al 2012). Several studies have shown anthocyanidins induce apoptosis through both intrinsic and extrinsic mechanisms. Yet, in some studies anthocyanidins inhibited cell proliferation but did not induce apoptosis (Brown et al 2012). The differences in chemical structure between individual anthocyanidins may explain their varying anticancer properties (Prior and Wu 2006). At low concentrations delphinidin has shown to have the greatest antioxidant potential due to the three free hydroxyl groups on its B-ring (Rahman et al 2006). However studies are inconclusive on its inhibitory effects compared to other anthocyanidins, like malvidin, which has only one hydroxyl group and two methoxyl groups. It is still unknown if the anthocyanidin concentrations that impact cell proliferation and

apoptosis are feasible in vivo, and particularly whether they are attainable in a diet high in fruits and vegetables (Brown et al 2012).

The objective of this study was to investigate the dose-dependent response of malvidin and delphinidin on cell viability and apoptosis in HT-29 cells. A second objective was to compare the effects of these two structurally different anthocyanidins to another phenolic compound, curcumin. Anthocyanidins had a significant impact on cell viability ($p<0.0001$). Low concentrations of anthocyanidins, ≤ 10 umol/L, increased cell proliferation, which is consistent with previous studies in our laboratory. Higher concentrations of the anthocyanidins and curcumin, ≥80 umol/L, significantly reduced cell proliferation. There was a significant dose-response of both anthocyanidins on caspase-3 activity. Significant increases above control of caspase-3 activity occurred at 80 umol/L of anthocyanidins and curcumin. Only 80 umol/L malvidin significantly increased caspase-8 activity, a marker of extrinsic apoptosis. Concentrations of ≥ 25 umol/L anthocyanidin and curcumin induced intrinsic apoptosis as assessed by disruption of mitochondrial membrane permeability, and malvidin had a significantly greater effect than delphinidin. Further, there were significant interactions between concentration and anthocyanidin type for effects on cell viability, caspase-8 activity and mitochondrial membrance permeability, suggesting that anthocyanin effects on cell viability and apoptosis may be dependent on structure. Both anthocyanidins at 80 umol/L had a similar effect on cell viability and apoptosis compared to 80 umol/L curcumin. Despite a dose-dependent response it appears anthocyanidins generally require a concentration of ≥80 umol/L to exhibit antiproliferative effects and induce apoptosis.

Limitations

There are several limitations to working with anthocyanidins in an *in vitro* model. Cell cultures are exposed to more oxygen, creating excess ROS that might not occur in an *in vivo* environment (Fimognari et al 2012). The increase in ROS species could increase the anthocyanidin's prooxidant abilities (Flis et al 2012). Another limitation could be the use of anthocyanidins, instead of anthocyanins. Unlike other flavonoids, which are hydrolyzed in the small intestine, some anthocyanins have shown to be absorbed intact (Prior and Wu 2006). Anthocyanins, with their attached sugar moieties, are the compounds found in food (Prior and Wu 2006). However anthocyanidins were used for this study because they have been shown to be more effective at inhibiting cell proliferation and inducing apoptosis (Prior 2003; Cooke et al 2005). Despite anthocyanidins being more potent inhibitors, they may be less relevant to the effect of anthocyanin-rich foods on cancer cells. In addition the incubation time of 48 hours was based on protocols of previous cell proliferation studies (Yi et al 2005; Renis et al 2008; Kim et al 2008) and results may differ depending on incubation time. The maximum antiproliferative effect of anthocyanidins found *in vitro* was seen after 72 hours incubation (Brown et al 2012). Overall the complexity of *in vivo* models could change anthocyanidin effects.

Future Studies

The purpose of the study was to evaluate how the anthocyanidins effect cell viability and apoptosis in HT-29 cells at the various concentrations of 5-100 umol/L. A higher concentration of 80 umol/L malvidin, delphinidin, and curcumin inhibited cell proliferation and induced apoptosis. Future studies could test comparable doses *in vivo* using rodent models, to find out if anthocyanidins effect the development or progression of cancer. Also future studies are needed to understand the preventative effect of consuming anthocyanins prior to tumor induction *in vivo*.

This study did not investigate the synergistic effect of the anthocyanidins. It is possible a lower concentration of combined anthocyanidins could result in inhibition of cell viability and induction of apoptosis. It would be interesting to observe how anthocyanidins react with an anticancer drug. Anthocyanidins may have a synergistic or antagonistic effect with a conventional treatment, like a chemotherapy drug. Future studies could also look at the effect of anthocyanidins, such as malvidin and delphinidin, in combination with other bioactive phytochemicals, like curcumin.

Application

These findings suggest a concentration ≥80 umol/L anthocyanidins reduces cell proliferation and induce apoptosis more effectively through the intrinsic pathway. At this time these findings cannot be extrapolated into nutrition recommendations, but they can be used as concentration selections for further *in vitro* studies. Potentially in the future the effect of anthocyanidins can be tested *in vivo* models to advance our understanding about how these compounds can be used for chemoprevention.

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APPENDIX A

Caspase-8 Activity Assay Kit

Treatment

*Apoptosis was induced in the cells through the following treatments:

1. Five 75 cm^2 flasks were treated with 10 mL of 50 or 80 umol/L of malvidin or delphinidin or 80 umol/L curcumin for 48 hours.
2. A designated 75 cm^2 control flask was treated with 10 mL fresh media.
3. Treatment media was removed from all the flasks (n=6) and replaced with fresh media and incubated for about 20 minutes.

Cell Harvesting

4. The media was removed from all the flasks.
5. Cells were detached by trypsinization, 9 mL fresh media was added to each flask to deactivate trypsin.
6. Cells were counted and diluted to 1.5 $X10^6$ cells/mL, and 1 mL aliquots of cell solution were added 1.5 mL Eppendorf tubes.

Caspase-8 Assay

6. A cell pellet was formed using a microcentrifuge at 125xg for 10 min.
7. The media supernatant was discarded and cells were resuspended with 300 umol/L 1X Cell Lysis Buffer and incubated on ice for 10 minutes, then centrifuged for 5 min at 10000 xg.
8. In a 96-well clear plate, each test well contained;

Sample	Assay Mixture			Caspase-8 Substrate	Total Volume
	5X Assay Buffer	Caspase-8 Sample	D_I H2O		
Buffer Blank	20 uL	0 uL	80 uL	0 uL	100 uL
Substrate Blank	20 uL	0 uL	70 uL	10 uL	100 uL
Test Sample	20 uL	70 uL	0 uL	10 uL	100 uL

9. The 96-well plate was incubated for 1-2 h at 37 °C.
10. Samples were read at 405 nm by a microtiter plate reader. Fold-increases were calculated by using the equation (Sample-Substrate Blank)/(Control-Substrate Blank).

APPENDEX B

Mitochondrial Permeability Transition Detection

Treatment

*Apoptosis was induced in the cells through the following treatments:

1. Cells were grown to yield a concentration of 1.5-2 x 10^6 cells/mL.
2. Eight 75 cm^2 flasks were treated with 10 mL of 25, 50 or 80 umol/L of malvidin or delphinidin or 40 or 80 umol/L curcumin for 48 hours.
3. 10 mL fresh media was added to four 75 cm^2 that were designated as the controls; t-BOOH, CCCP, DMSO, and the untreated control.
4. Media was removed from the t-BOOH flask after 46 hours, and replaced with 10 mL of 250 umol/L t-BOOH for 2 hours.
5. Media was removed from the CCCP and DMSO flasks after 47 hours and replaced with 10 mL of 50 umol/L CCCP and DMSO, respectively.
6. After 48 hours the treatment media was removed from all the flasks (n=12) and replaced with fresh media and incubated for about 20 minutes.

Cell Harvesting

7. The media was removed from all the flasks.
8. Cells were detached by trypsinization, 9 mL fresh media was added to each flask to deactivate trypsin.
9. 1.5 mL aliquots of cell solution were added to15 mL centrifuge tubes (n=12 centrifuge tubes).

Mitochondrial PT™ JC-1 Assay

10. Cells were pelleted by centrifugation at 125 xg for 7 minutes at room temperature.
11. The supernatants were carefully removed and discarded. The cells were resuspended in 1 mL 1X working strength MitoPT™ JC-1 solution.
12. Cells were incubated for 10-15 minutes in 37 °C in a CO_2 incubator and the 1X assay buffer was warmed to 37 °C.
13. 2 mL of 1X assay buffer was added to each sample and mixed.
14. Cell pellets were reformed by centrifugation at 125 xg for 7 minutes at room temperature.
15. Supernatants were carefully removed and discarded and the cells were resuspended with 1 mL 1X assay buffer.
16. Cells counts were performed using a small aliquot of 10 uL from each sample.
17. The remaining cells were centrifuged at 400 xg for 5 minutes at room temperature.
18. Supernatant was removed from all the samples and .5-1 mL of 1X assay buffer was added back to each sample to yield a cell count of 1.5 X 10^6 cells/mL.
19. For each sample 100 uL was dispensed in to 4 wells in a black flat bottom 96-well microtiter plate.

96-well fluorescence plate reader set up

20. The plate reader was set to an endpoint read.
21. Excitation wavelength was set to 488-490 nm.
22. Emission wavelength was set to the red fluorescence setting of 590-600 nm.
23. Samples were red fluorescence output (RFU).