ABSTRACT

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                     5-FLUROURACIL CHEMOTHERAPY

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Anthocyanins are natural colorants, with potential chemopreventive activity.
5-Flurouracil (5-Fu) is a widely used cancer chemotherapeutic drug. However, it has
serious side effects. **Objective**: To determine whether anthocyanin-rich-extracts
(ARE) can increase the effectiveness of 5-Fu. **Methods:** Colon cancer cells were
treated with ARE in combination with 5-Fu to assess *in vitro* whether ARE would
enhance 5-Fu cytotoxicity. *In vivo*, rats were given diets with or without ARE for 14
days. On day 11, rats were injected with either 5-Fu or its vehicle. Total blood cells
counts and pathological analyses were conducted. **Results:** ARE enhanced the
growth inhibition by 5-Fu in colon cancer cell lines, but almost had no effect on
normal colon cells. 5-Fu had serious side effects on the rats. Dietary ARE partially
reduced the gastrointestinal damage by 5-Fu. Further studies with advanced tumor
models are needed to assess effect of ARE on 5-Fu tumor inhibition *in vivo*.

EFFECT OF ANTHOCYANIN-RICH EXTRACTS ON

5-FLUROURACIL CHEMOTHERAPY

By

Ye Su

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Chapter 1. Literature Review:

1.1. Anthocyanins and their anticancer effects

1.1.1. Abstract: Anthocyanins are natural colorants and have many bioactivities, including antioxidant, anti-inflammatory, and antitumor activity, and potentially the ability to decrease the risk of heart disease. More and more researchers are interested in anthocyanins and have conducted considerable work on them. This paper reviews the structures, stability, bioavailability and the possible mechanisms of anticancer function of anthocyanins.

1.1.3. Introduction

Anthocyanins are natural colorants and impart attractive colors to vegetables and fruits. In recent years, many epidemiological studies showed that high consumption of fruits and vegetables have health-promoting benefits, such as protecting against oxidant damage, reducing the risk of cardiovascular diseases, improving eyesight, as well as being antiinflammatory, antibacterial, antimutagenic, and anticarcingoenic [1-5, 12, 15, 17]. These functions partially belong to anthocyanins in the fruits and vegetables. Thus, anthocyanins are very promising functional food materials and additives.

There are many fruits and vegetables rich in anthocyanins such as berries, purple corns, purple cabbages, red soybeans, and purple potatoes [1-3]. Right now some kinds of anthocyanin extracts are commercially available like chokeberry, bilberry,

elderberry and grape anthocyanin extracts. Some of them already are used in the food industry as natural colorants. This article reviews the recent development about the structures, stability, bioavailability, anticancer function and possible mechanisms of anthocyanins or anthocyanidin rich extracts from multiple materials.

1.1.4. The structures of anthocyanins

Anthocyanins belong to the flavonoids family. In nature, the anthocyanins are present as glycosylated forms [4, 5]. The nonglycosylated form is called anthocyanidin (Fig. 1). There are more than 400 anthocyanins and 17 known naturally occurring anthocyanidins (Table 1). Only six anthocyanidins are common in higher plants, which are listed in Figure 1. Among the six anthocyanidins, cyanidin is most abundant in nature and represents almost 50% of all anthocyanidins. The rank of the distribution of the six anthocyanidins is cyanindin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) [4]. Four classes of anthocyanins are common in the nature. They are 3-monosides, 3-biosides, 3, 5-diglycosides and 3, 7-diglycosides. Cyanidin 3-glycoside is the most widespread one [4, 6].

The structures of anthocyanins are related to their function. Regarding the antioxidative activity, the B-ring structure has a marked effect on antioxidant activity with *ortho*-hydroxylation and methoxylation substantially increasing antioxidant activity [7].
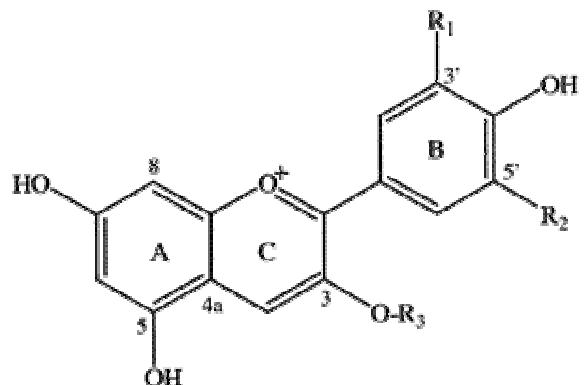
Anthocyanidins & Anthocyanins	R_1	R_2	R_3
Pelargonidin	H	H	H
Cyanidin	OH	H	H
Delphinidin	OH	OH	H
Peonidin	OMe	OH	H
Malvidin	OMe	OMe	H
Pelargonidin-3-galactoside	H	H	galactose
Cyanidin-3-galactoside	OH	H	galactose
Cyanidin-3-rutinoside	OH	OH	rutinose
Cyanidin-3-glucosylrutinoside	OH	OH	glucose-rutinose
Delphinidin-3-galactoside	OH	OH	galactose

Figure 1 Structures of 6 common anthocyanidins and their anthocyanins [7]

Table 1 Naturally occurring anthocyanidins [4]

Name	Abbreviation	Substitution							Color
		3	5	6	7	3'	4'	5'	
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	Orange
Aurantinidin	Au	OH	OH	OH	OH	H	OH	H	Orange
Capesninidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	Bluish-red
Cyanindin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Bluish-red
Europinidin	Eu	OH	OMe	H	OH	OMe	OH	OH	Bluish-red
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OH	OMe	Bluish-red
6-Hydroxycyanidin	6OHCy	OH	OH	OH	OH	OH	OH	H	Red
Luteolinidin	Lt	H	OH	H	OH	OH	OH	H	Orange
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
5-Methylcyanidin	5-MCy	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H	Orange
Peonidin	Pu	OH	OH	H	OH	OMe	OH	OH	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Bluish-red
Pulchellidin	Pl	OH	OMe	H	OH	OH	OH	OH	Bluish-red
Rosinidin	Rs	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidin	Tr	H	OH	H	OH	OH	OH	OH	Red

1.1.5. Stability of anthocyanins

Stability is important for bioactive functions and colors of anthocyanins, and especially important for the colorants used for foods. Anthocyanins are not stable pigments. The hydrolysis of glucose from the anthocyanin molecules is responsible for the pigment degradation during processing [8]. The stability of anthocyanin pigments is dependent on several factors, including structure, concentration of the pigment, pH, temperature, light intensity and quality, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation products and sulfur dioxide [1, 2, 9].

The chemical structure of anthocyanins is a main factor affecting the stability of the anthocyanins. Increases in glycosidic substitution, acylation and methoxylation improve the stability of anthocyanins [1, 6, 8]. Acylation with hydroxylated aromatic organic acids increases the stability of anthocyanins, but with some exceptions. Acylation of cyanidin 3-glycosides and mavidin 3-glucoside distinctly increased their antioxidant activity [6]. Methoxylation also increases the stability of anthocyanins [1]. Malvidin glycosides are more stable than most other anthocyanins because of the dimethoxylation of the molecule.

The pH has a marked effect on the structures of anthocyanins. Anthocyanins exist in a variety of protonated, deprotonated, hydrated, and isomeric forms, and the relative proportion of these molecules is strongly dependent on pH [10]. Consequently, their colors also undergo dynamic changes with the structure change. In acidic media, four anthocyanin structures exist in equilibrium: flavylium cation, quinonoidal base, carbinol pseudobase and chalcone. The relative amounts of these structures at

equilibrium vary with pH and anthocyanins structure [1]. When pH is 1-3, the color of anthocyanins ranges from orange to blue-red, and exists predominantly as a flavylium cation [10, 11]. As the pH is raised to 4-5, hydration reactions generate the colorless carbinol pseudo-base, which can further undergo ring opening to the light yellow chalcones, most rapidly at pH 2.5-5 [10, 11]. At pH between 6 and 7, the quinonoidal bases through proton transfer from flavylium cation can be further converted to blue-purple quinonoid anions [9-12].
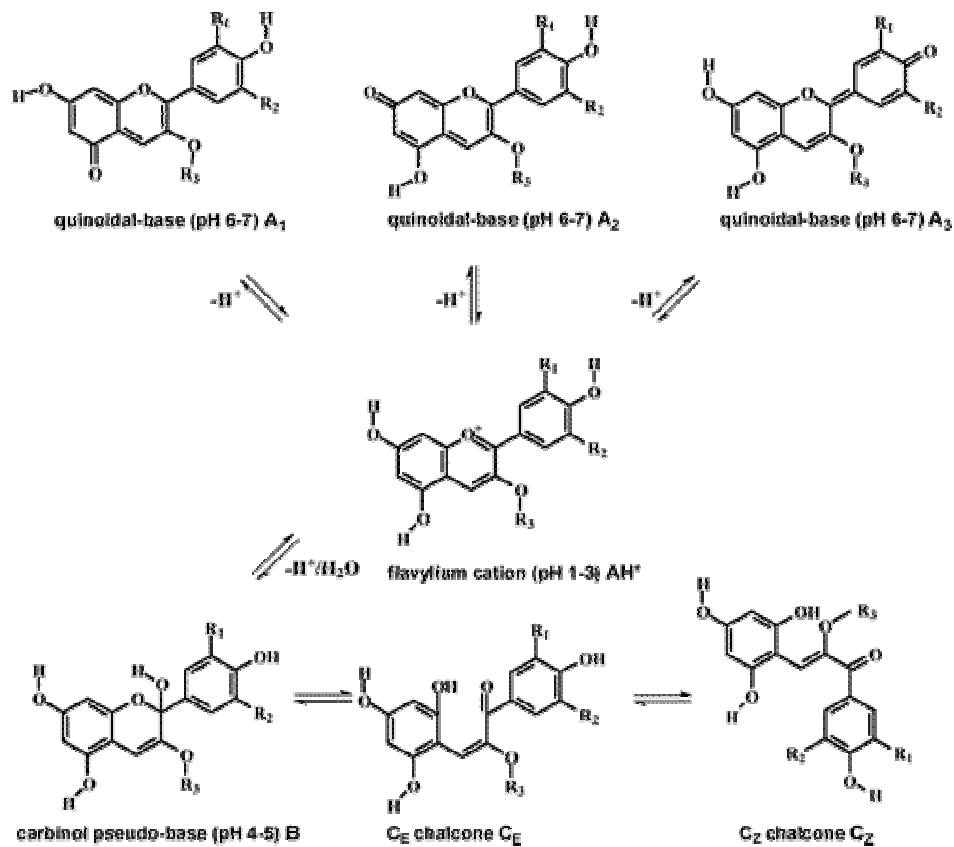
Figure 2 Structural changes of anthocyanins in aqueous solutions, based on transformations of red malvidin-3-O-β-glucoside flavylium cation into possible purple quinone methides (A1, A2, and A3), colorless carbinol pseudobase (B), and yellow chalcone forms (CE and CZ), at different pH (R1, R2 = OMe, R3 = glucosyl) [12]

Formation of copigments with other compounds in the mixture or solution could increase the stability of anthocyanins. Molecular complexation of anthocyanins with other phenols-pigments is the main color-stabilizing mechanism in plants. Copigmentation consists of the stacking of the copigment molecule on the planar polarizable nuclei of the anthocyanin-colored forms [9]. Acylated anthocyanins have high stability due to the intramolecular copigmentation. Nonacylated anthocyanins could increase their stability by forming intermolecular copigment [9]. Copigmentation can increase the light and temperature resistance of anthocyanins, increasing their stability.

Heat and light treatment have negative effects on the stability of anthocyanins. They induce the degradation of anthocyanins, decrease the color intensity, and increase the polymerization of monomeric anthocyanins [1].

1.1.6. Bioavailability

Bioavailability is the foundation of biological effects of anthocyanins. The bioavailability includes the absorption, distribution, metabolism and excretion of anthocyanins. For a long time, people thought anthocyanins only can be absorbed in their aglycon forms [3, 5, 13]. Some hypotheses came from the studies of absorption of flavonoids, especially quercetin [5, 13, 14], because both of them belong to the same flavonoid family and share a similar basic structure. Studies suggested that quercetin could be absorbed in their glycosylated form [5]. Two possible mechanisms for the transport of quercetin glycosides by enterocytes were transport of quercetin glycosides by sodium-dependent glucose transporter (SGLT)1, and extracellular hydrolysis by lactase phlorizin hydrolase (LPH), a brush border ß-glucosidase,

followed by passive diffusion of the aglycone [5, 15, 16]. Similar hypotheses were suggested for cyanidins too [5].

As discussed above, the stability of anthocyanins is affected by the pH, so the pH change throughout the gastrointestinal tract may influence the form of anthocyanins and may further influence the absorption of anthocyanins. The absorptive sites of anthocyanins seem to be stomach, and small intestine [17-19]. Up to now, most studies on the bioavailability of anthocyanins have been done with cyanidins and their glycosides. However, the opinions regarding the absorption of anthocyanins are still contradictory and need to be further confirmed.

Recent studies have shown that the glycosylated cyanidins and other dietary anthocyanins (most from berries) could be absorbed in their intact glycosylated forms [3, 14, 17, 20-25]. The intact anthocyanins were detected in the plasma and urine of human subjects [3, 13, 14, 20, 22, 26] and rats [3, 14, 24-26]. There was a significant positive correlation between the serum anthocyanin content and postprandial antioxidant status [22]. After oral ingestion, the anthocyanins could be absorbed rapidly and had a relatively short half-life [24, 25]. The elimination of plasma anthocyanins appeared to follow first-order kinetics and most anthocyanin compounds were excreted in urine within four hours after feeding [3, 20, 23]. The mechanism of the absorption of anthocyanins as glycosides is still not clearly understood. One study suggested that glucose transport receptors in the mucosal epithelium may be involved in the process based on the results from quercetin studies [5, 21].

Some other studies showed that anthocyanins were metabolized and absorbed as their degraded products [3, 20, 27] and these products are present in urine and other tissues

as liver and kidney. *In vivo*, Wu *et al.* [20] demonstrated that the cyanidins were methylated to peonidin and glucuronide conjugate formation after people consumed the berry anthocyanins. Felgines *et al.* [24] found that anthocyanin 3-glucosides were partly hydrolyzed by intestinal β-glucosidase releasing aglycons. The aglycons were rapidly transformed to other unknown metabolites. Some of the absorbed anthocyanins were likely metabolized to some noncolored forms that can not be detected [24]. A more recent study [28] showed that human subjects had the capacity to metabolize the cyanidin 3-glycosides. The metabolites were glucuronide conjugates, methylated and oxidized derivatives of cyanidin 3 - galactoside and cyanidin glucuronide. These metabolites may contribute to the bioactive effects of anthocyanins. The proposed metabolic pathway is shown in Figure 3 [28].

Overall, the bioavailability of anthocyanins is very low, lower than 1% [3, 20, 24, 29]. Most anthocyanins are nonbioavailable and enter into the colon. *In vitro* studies showed that in colon, the gut microflora could utilize both glycosides and aglycons of anthocyanins and degrade them into small molecules [12, 29, 30]. The glycosidic bonds within anthocyanins were hydrolyzed by gut microbes. The unstable aglycon degraded spontaneously into phenolic acids and aldehydes at neutral pH [29, 30]. The metabolites of phenolic acids or/and other yet unidentified compounds may also contribute the antioxidant activity of anthocyanins.
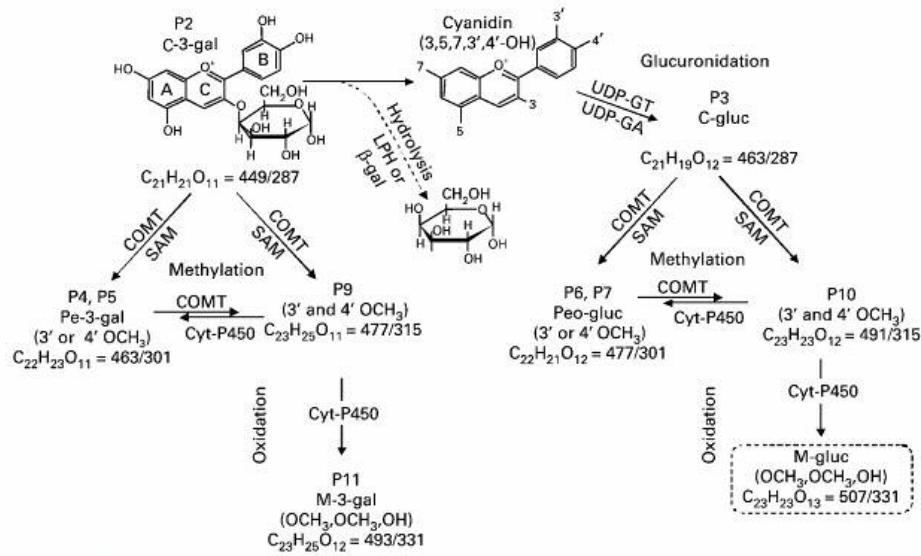
Figure 3 Pathways for the formation of anthocyanin metabolites in human urine as proposed by [28]

1.1.7. Antitumor effects and mechanisms

Tumor initiation is a very complicated process, which includes damage to DNA, accumulation of gene mutations, up- or down-regulation of key enzymes, shutting off of signaling transduction pathways. Although different kinds of cancer have different mechanisms and characteristics, one of the main features of cancer cells is uncontrolled cell proliferation. Tumor cells typically acquire damages to genes that directly regulate the cell cycle. Therefore inhibition of the growth of tumor cells, regulation of some pathways of cell growth, control of the activities of some critical enzymes, and protection of DNA from damage, are effective ways to prevent and/or treat cancers [31]. The basic concept to cure cancer is to control the growth of tumor cells. Following are some mechanisms of anticancer action of anthocyanins.

1.1.7.1. Inhibition of the tumor cells growth

Anthocyanins can effectively inhibit growth of some cancer cell lines, including breast cancer MCF7, lung cancer NCI-H460, colon cancer HT29, HCT115 and HCT116 [32-34]. There are several different mechanisms to inhibit the cell growth, including inhibition of the kinases of cell cycle, inhibition of the endothelial growth factor receptor, or alteration of the cyclins of the cell cycle. These will be discussed below.

Kang *et al.* [32] conducted an experiment with APC^{Min} mice[1] treated with tart cherry extract (rich in anthocyanins), purified tart cherry anthocyanins, and their respective

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[1]Apc^{Min} mice are a mutant mouse lineage predisposed to multiple intestinal neoplasia (Min) due to a mutation in the murine homolog of the adenomatous polyposis coli (APC) gene. APC gene is considered a gatekeeper in the carcinogenic process. The primary phenotype of Apc^{Min} mice is the development of multiple intestinal adenomas [32].

cyanidins. The results showed that the anthocyanins and cyanidins significantly reduced the cecal adenomas, comparing with the mice consuming the control diet, but colonic tumor numbers and volume were not significantly influenced by the treatment. The cherry anthocyanins and cyanidin also reduced cell growth of human colon cancer cell lines HT29 and HCT116 [32]. These results suggested that tart cherry anthocyanins and cyanidin could reduce the risk of colon cancer [32].

Similar reports were published by Kamei and research group [35], who tested the antitumor effect of methanol extracts from red and white wines and determined that the anthocyanins are the active fraction of this extract. Their findings indicated that the anthocyanins inhibited the growth of HCT15 cells derived from human colon cancer, and AGS cells derived from human gastric cancer. Cells treated with anthocyanins were blocked in the S, G2, and M phases of the cell cycle.

In addition, Malik *et al.* investigated the effect of semipurified anthocyanin-rich extract (ARE) from fruits of *Aronia meloncarpa* E on normal colon and colon cancer cell lines [11, 34]. They found 50 μg monomeric anthocyanin/mL could inhibit up to 60% growth of human HT-29 colon cancer cells. Culturing cells in the presence of the extract resulted in the HT29 cells arresting at G1/G0 and G2/M phases of the cell cycle. In contrast, almost no effect on the growth of normal colon cells was observed when the concentration of ARE was less than 50 μg/mL.

Zhao *et al.* [11] investigated cancer cell growth inhibition effects of three commercially available anthocyanin-rich extracts (ARE) from grape (*Vitis vinifera*), bilberry (*Vaccinium myrtillus* L.), and chokeberry (*Aronia meloncarpa* E.) based on monomeric anthocyanin concentration ranging from 10 μg to 75 μg. They found that all three extracts had inhibitory effects on HT29 colon cancer cells, but chokeberry ARE was the most effective one. As low as 10 μg /mL, chokeberry ARE could significantly inhibit the proliferation of HT29 cells after 24 hour treatment, but not the normal nontransformed colon cell line, NCM460. The chokeberry ARE only inhibited the NCM460 growth when monomeric anthocyanin concentration was greater than 50 μg/mL after 48 hour exposure. This suggested that chokeberry ARE is a potential chemopreventive agent for colon cancer, but has little effect on normal colon cells.

1.1.7.2. Antioxidation

Oxidative reactions can produce a large amount of reactive oxygen radicals, peroxyl and other free radicals. In normal physiological conditions, the body keeps the balance between oxidation and oxidant scavenging. However, when the free radicals exceed the scavenging ability of the body, they can induce extensive damage to the lipid, protein, DNA, membranes, and enzymes of the cells. Damage to DNA may produce mutations that cause permanent alterations in the genetic messages and increase the risk of the carcinogenesis [36]. The oxidative damage is widely accepted as one of major causes of diseases including cancer [37]. Therefore, protection of the DNA from oxidative damage can prevent gene mutation, decrease neoplastic development, and is considered as an effective method to prevent tumor initiation.

The chemical structures of anthocyanins cause them to be very potent antioxidants. Anthocyanins are electrophilic and can accept the unpaired electrons so that they have active antioxidant function. There are many studies reporting that the anthocyanins act as antioxidants *in vitro* and *in vivo* [10, 38-42]. To date, there are several antioxidant mechanisms reported, including hydrogen donation, metal chelation, and protein binding [4, 5]. Anthocyanins can effectively clear the reactive oxygen radicals and prevent lipid and liver microsome peroxidation. There are also some reports that anthocyanins can prevent the oxidation of low-density lipoproteins (LDL) [43].

1.1.7.3. Protection DNA from damage

Studies [4, 5] show that anthocyanins can protect the DNA damage via protecting DNA from oxidation. One of possible mechanisms is that the anthocyanin forms the copigmentation with DNA, and further protects the DNA from the damage [4].

Duthie *et al.* [36] and Ramirez-Tortosa *et al.* [43] found that consumption of anthocyanins could significantly improve the low antioxidative capacity of rats due to the vitamin E deficiency. The level of 8-Oxo-deoxyguanosine (a biomarker of DNA damage), which was increased due to the depletion of vitamin E in rats, was decreased in rats fed anthocyanins diet compared with the rats consuming the control diet. Duthie *et al.* [36] also demonstrated that diet containing 100 mg/kg cyanidin-3-glycoside for 12 weeks could protect the DNA single strand break induced by oxidative damage in vitamin E deficient rats.

1.1.7.4. Inhibition of epidermal growth factor receptor

Meiers *et al.* [44] studied the inhibitory effects of different kinds of anthocyanins and anthocyanidins on the epidermal growth factor receptor (EGFR). They tested the

activity of the tyrosine kinase activity of EGFR isolated from A431 cells and measured the phosphorylation of the transcription factor Elk-1. They also compared the effects of the different structures of anthocyanins and anthocyanidins on the inhibition of EGFR. The results showed that the aglycon forms were more effective than the glycosides in inhibition of the EGFR activity, and the cyanidin and delphinidin were the most effective compounds among them. For the inhibition of the tyrosine kinase activity, the free hydroxyl groups were necessary, especially in the 3 position. When the hydroxyl group in position 3 was replaced by sugar or methoxy group at the B ring, the anthocyanin would lose the ability to inhibit the enzyme activity. EGFR is a crucial signaling event in the regulation of the cell growth. Thus the results suggest that it could be one of possible pathways for anthocyanidins to inhibit growth of cancer cells.

1.1.7.5. Induction of apoptosis of tumor cells

Apoptosis, namely programmed cell death, is a mechanism that the body uses to control cell numbers and responds to internal or external damages by induction of the death of extra cells and damaged cells [45]. Apoptosis is a complex procedure, involving proapoptotic and apoptotic suppression proteins like the Bax and Bcl families. Recently, induction of apoptosis is a novel target used in cancer prevention and therapy [45].

Katsube *et al.* [46] tested the apoptosis-inducing effects of ethanol extracts from bilberry, which were rich in anthocyanins, in two cell lines, HL60 human leukemia and HCT116 human colon carcinoma cells *in vitro*. At the 4 mg/mL concentration based on the dry material, the extract can induce apoptosis significantly in the HL60

cells, but not in HCT116 cells. Katsuzaki *et al.* [47] studied the apoptotic effect of the anthocyanins and cyanidins isolated from the skin of red grapes and skin of black bean seed coats in human lymphoid leukemia Molt 4B cells. Apoptosis was evaluated by observing the induction of DNA ladder fragments in this study. They found that the anthocyanins can induce apoptosis in the Molt 4B cells dose-dependently.

1.1.7.6. Anti-angiogenesis

Angiogenesis is defined as the formation of new blood vessels from pre-existing ones and is active in physiological conditions such as embryonic development, menstrual cycle and wound healing [48]. In the normal state, it is an important process for the body. But it is also very important for the growth of tumors. Through the angiogenesis, the tumor cells gain the nutrition for growth and metastasis. Therefore, inhibition of the new blood vessel formation seems to be an effective way to inhibit the growth of tumor cells. Anti-angiogenic approach to prevent and treat the cancer is a new priority [37].

Favot *et al.* [48] reported the inhibitory effect of delphinidin on angiogenesis *in vivo* in the chorioallantoic membrane model in developing embryo. Their findings showed that delphinidin inhibited angiogenesis *in vivo* in a dose-dependent manner ranging from 10-50 μg/embryo. This effect was associated with inhibition of two major steps of angiogenesis: endothelial cell migration and proliferation. Delphinidin induced a down-regulation of cyclin A and D1 expression and an upregulation of p27 [kip1] expression, an inhibitor of cyclin-dependent kinase (CDK). The authors proposed that, *in vivo*, delphinidin played an important role as an anti-angiogenic compound by altering key protein expression in cell migration and cell proliferation. Bagchi *et al.* [37] evaluated the effects of a formulated berry extract, from the combination of six

berry extracts including wild blueberry, bilberry, cranberry, elderberry, raspberry seed, and strawberry rich in anthocyanins. They reported 50 µg/mL formulated extract effectively inhibited the expression of the vascular endothelial growth factor (VEGF) in HaCaT cells induced by H_2O_2 and TNF (tumor necrosis factor). VEGF is critical for the tumor blood vessel formation. .

1.1.8. Summary

Anthocyanins are beneficial for the health of people and have several different bioactivities. Up to now, there are no adverse side effects reported from consumption of anthocyanins, so they have the potential to be used for the functional food material or combination with chemotherapeutic drug.

1.2. Effects of diet or diet components on the chemotherapy of 5-fluorouracil

----Improvement of Sensitivity or Reduction of Adverse Effects

1.2.1. Introduction

Chemotherapy uses one or several drugs to kill dividing cancer cells, or to inhibit the proliferation of cancer cells. 5-Fluorouracil (5-Fu) is an antimetabolic chemotherapy drug, a fluorinated uracil base, used for a variety of solid cancers [49-51], liver carcinomas, breast cancer, and especially for colorectal cancer [52-55]. It was first synthesized in 1957 based on the observation that tumor cells utilized the uracil much faster than normal cells [56]. So it is possible that tumor cells also use 5-Fu faster because the similar structure of 5-Fu to uracil. Even though 5-Fu has been widely used for cancer chemotherapy for more than 50 years, it is still the first line for colorectal cancer. However, the response of cancer cells to 5-Fu is only 10-30% depending on the sensitivity of the cancer cells [49, 57, 58]. So, clinically, 5-Fu is routinely used in combination with methotrexate (MTX), an thymidylate synthase inhibitor, leucovorin (LV) and other antitumor drugs to increase its effectiveness and response [52, 57].

Many studies showed that dietary components, antioxidants, proteins, polysaccharide, unsaturated fatty acids, have chemopreventive potential and/or modulate the chemotherapy effectiveness of 5-Fu [57, 59-62]. The dietary intake of these nutrients either can increase the sensitivity of the cancer cells to drugs, or increase the tolerance of the host to the toxicity of chemotherapy drugs and reduce side effects induced by chemotherapeutic agents beyond the traditional nutritional value.

This review focuses on dietary components, especially antioxidants, which have been most studied in combination with 5-Fu chemotherapy for cancer treatment, or side effect reduction.

1.2.2. 5-Fu Introduction

1.2.2.1 The structure of 5-Fu

5-Fu is a derivative of uracil. It is fluorinated at position 5 on the ring of uracil. This structure is very similar to uracil (Figure 5). Because it is similar to this base, it was expected to have the same metabolic pathway of uracil. However, it is incorporated into DNA and inhibits the cancer cell DNA synthesis and cell division through different pathways. The structures of 5-Fu and base analogues are shown in Figure 4.
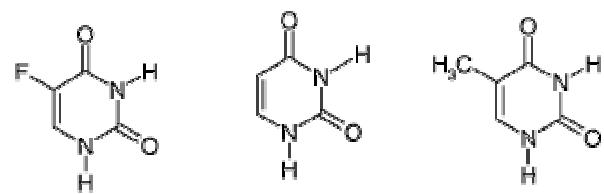
Figure 4. Structures of 5-Fu and its base analogues [47]

1.2.2.2 Metabolic pathways of 5-Fu

5-Fu is not active. It is a prodrug, which needs to be converted into its active metabolites by intracellular reactions through anabolic pathways [56, 63, 64]. The metabolic pathways of 5-Fu are cell-line dependent. The three key cytotoxic metabolites are 5-fluro-2'-deoxyuridin-5'-monophosphate (5-FdUMP) (inhibits TMP synthetase and can convert to FdUTP), FUTP (incorporated into RNA synthesis), and FdUTP (incorporated into DNA) [65]. So, the cytotoxicity of 5-Fu is associated with the activities of anabolic enzymes [56]. The anabolic pathway of 5-Fu is shown in Figure 6.

In addition, 5-Fu also undergoes catabolic metabolism using the same pathway as uracil and thymine [56]. The initial reduction of 5-Fu to dihydro 5-Fu is catalyzed by dihypyrimidine dehydrogenase (DPD) [64], the key rate-limiting enzyme in 5-Fu degradation. More than 80% of administrated 5-Fu was eliminated by metabolism through DPD [64]. DPD activity is high in human liver and variable in tumor tissues. The higher the DPD enzyme activity, the lower the cytotoxicity of 5-Fu [64]. In order to increase the antiproliferative effect of 5-Fu, various modulators were developed to inhibit the activity of DPD, to inhibit the degradation of 5-Fu and increase 5-Fu content in tissues.
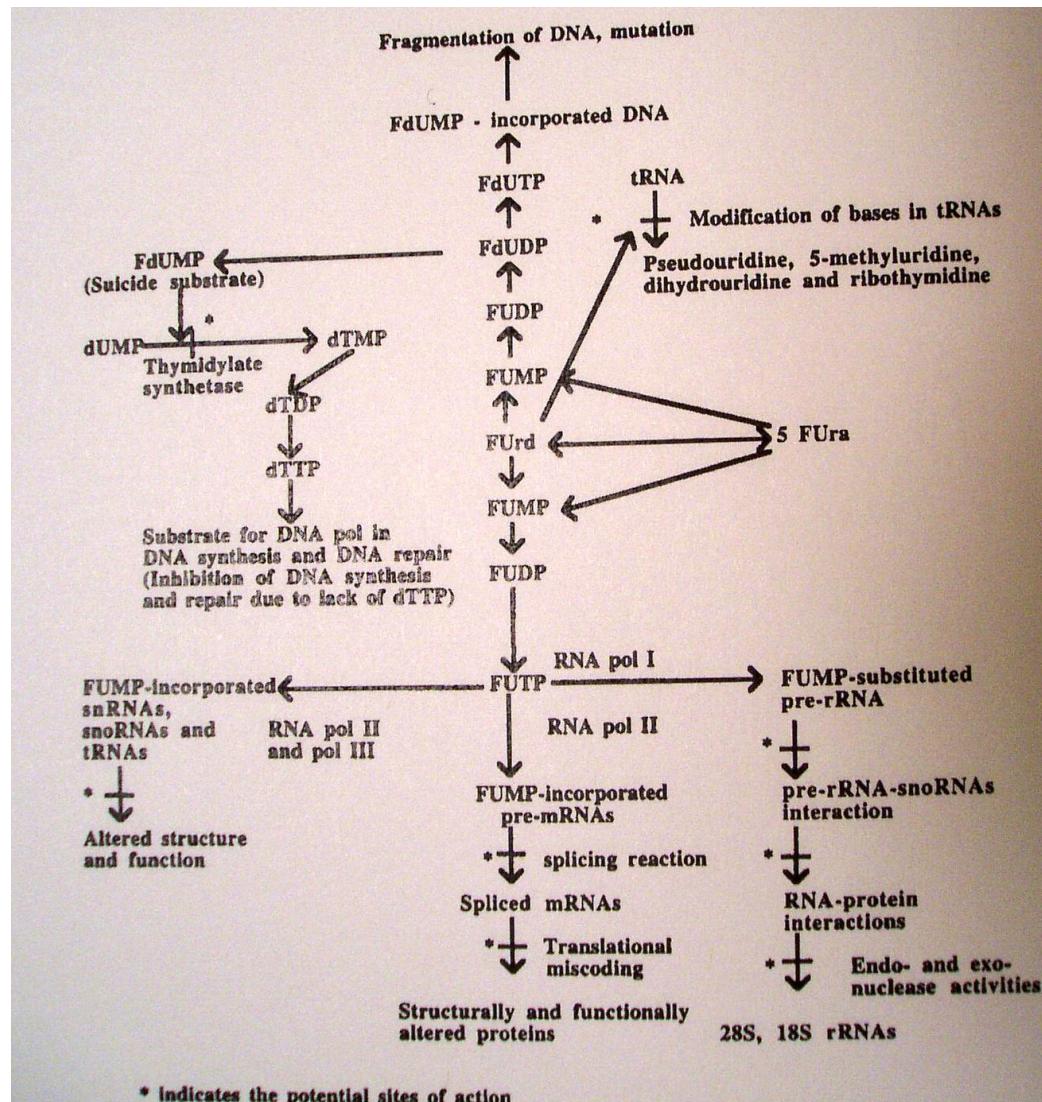
Figure 5. Metabolic pathway of 5-Fu [55]

1.2.2.3 Mechanisms of 5-Fu effect

So far, there are three main mechanisms by which 5-Fu is thought to exert its cytotoxic effects on cancer cells [52]. These are: inhibition of the activity of thymidylate synthase, DNA-directed cytotoxicity, and RNA-directed cytotoxicity. Each mechanism will be discussed below.

- Inhibition of the activity of thymidylate synthase (TS). One metabolite of 5-Fu, 5FdUMP, inhibits the activity of TS, a key enzyme of deoxythymidine triphosphate (dTTP) synthesis, by formation the stable complex with TS and 5, 10 methylenetetrahydrofolate (CH2-THF)[51]. TS is the key enzyme that catalyzes the *de novo* synthesis of thymidylic acid which will be converted to dTTP, a substrate of DNA. Through this pathway, 5-Fu finally inhibits the synthesis of DNA and depletes the DNA for cancer cells division. This effect is expressed as the cell cycle block and/or induction of apoptosis [49].

- DNA-directed cytotoxicity. The metabolite FdUTP of 5-Fu incorporates into genomic DNA and results in DNA fragmentation due to the formation of streak breaks, gene mutations and base mismatches [55]. After the 5-Fu-induced damage of DNA occurs, the tumor suppressor protein p53 can active proapoptotic proteins to initiate apoptosis and inhibit cell growth [52].

- RNA-directed cytotoxicity. One of metabolites of 5-Fu, FUTP, incorporates into RNA and leads to changes of mRNA expression, inhibition of mRNA splicing, and interference with tRNA modification [55, 65].

1.2.2.3 The limitation of 5-Fu chemotherapy

Because 5-Fu is an analogue of uracil, its metabolites can incorporate into RNA and DNA, leading to malfunction of these macromolecules [52, 58]. In the end, 5-Fu affects all rapid growing cells, normal and abnormal. As a result, the drug not only kills tumor cells, but also kills rapidly growing normal cells, including gastrointestinal (GI) cells and bone marrow cells [58]. This lack of target differentiation limits the use of the drug because the toxicity it creates in the host. Toxicity is expressed as vomiting, diarrhea, structural alteration of mucosal cells, decreased nutrient absorption, white blood cell (WBC) depression, and decreased platelet cells [58]. The most serious toxicities are gastrointestinal toxicity, bone marrow inhibition, and immunotoxicity [62]

Gastrointestinal toxicity and bone marrow inhibition are always the dose limiting factors and hamper the use of higher and possibly more effective doses of 5-Fu [66]. The gastrointestinal damage includes stomatistis, diarrhea, denudation of villi, destruction of the integrity of mucosa, an increase of the permeability of intestine, and decrease of activities of intestinal enzymes such as thymidine-kinase (the marker of the activity of dividing crypt cells), alkaline-phosphatase, sucrase and maltase (the markers of digestive function of differentiated enterocytes) [1].

Intestinal permeability is an index of intestinal barrier function and is used for screening and diagnosis of many intestinal diseases [67]. Noninvasive *in vivo* test methods for intestinal permeability were discovered in last decades by measuring urinary excretion of orally administrated substances [67]. Disaccharides and monosacchrides probes are usually used together for the intestinal permeability test.

The urinary excretion ratio of disaccharide/monosacchride is used as an index of intestinal permeability because it is relatively stable in normal condition and less affected by other factors [67]. In a disease condition, these small, hydrophilic sugars penetrate damaged intestinal epithelium far more rapidly than normal, healthy mucosa. Thus, the small molecule markers are orally ingested and urine is collected to test the content of markers. More damaged intestine results in excretion of more probes in urine. Lactulose/L-rhamnose and lactulose/mannitol are the most commonly used combination. The ratio of disaccharide/monosacchride above 0.04 is considered normal [68]. The use of sucrose as a novel permeability marker for gastroduodenal damage was initiated by Meddings [69]. Compared with disaccharide/monosacchride markers, the sucrose marker test is simpler, more convenient, and practical for large samples screen [69]. Healthy gastrointestinal mucosa is relatively impermeable to sucrose. Sucrose is rapidly degraded in the small intestine by sucrase. Increased amount of sucrose in urine means the sucrase activity is damaged and the permeability of the small intestine is increased [69].

Bone marrow is another target of 5-Fu toxicity because of rapid turnover of bone marrow cells. As a result, 5-Fu induces serious myelotoxicity, such as leucopenia, anemia, peripheral thrombocytopenia, and medullary erythrocytopenia [62]. In order to solve this problem, a variety of myelopoietic growth factors were used in combination with 5-Fu clinically to recover or ameliorate myelopeiteic function. These factors are granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL) -3, IL-6, IL-11 and stem cell factor [62, 70].

1.2.2.4 Current or developing strategies for improving the limitations of 5-Fu

Because the overall low response and serious adverse side effects, people have been looking for approaches to enhance 5-Fu therapeutic index and reduce side effects [55, 56]. The antitumor and toxic side effects of 5-Fu can be modified by changing the dose schedules, route of administration and by using combinations with biochemical modifiers such as methotrexate, leucovorin, uridine and dietary compounds [66].

One approach to solve this problem is to increase the toxicity of 5-Fu by combination 5-Fu with other drugs, that is to increase the tumor cells response rate or sensitivity to 5-Fu. Leucovorin, (LX), also known as folinic acid, or formyltetrahydrofolate (formylTHF), a precursor of 5-10 methylenetetrahydrofolate, is one of the most common modulators. By transformation to 5-10 methylTHF, it stabilizes the complex of 5-10 methylTHF, 5-Fu, and TS. The complex inhibits the activity of TS and further increases the cytotoxity of 5-Fu [56, 71]. Methotrexate (MTX), an antifolate agent, one of mechanisms to exert its function by inhibiting activity of TS [52], is another anticancer drug routinely used in combination with 5-Fu clinically to increase the cytotoxicity of 5-Fu.

The other approach is to reduce side effects of 5-Fu on normal tissues. Selective protection of normal host tissues from the toxicity of 5-Fu would allow the use of higher, more effective doses of 5-Fu [66]. Dietary modification is one of the effective approaches being explored. One of reason that dietary compound can improve the effectiveness of 5-Fu is they could increase the host tolerance to 5-Fu. Besides, dietary antioxidant compounds could protect the host from oxidative stress resulted from cancer and chemotherapy [59, 72].

1.2.3. Effects of dietary compounds on 5-Fu chemotherapy

1.2.3.1 Dietary antioxidants

Free radical and other reactive oxygen specials (ROS) are necessary for maintaining the normal physiological process of organisms in cell signaling, mitochondrial respiration, and phagocyte of bacteria [59]. In normal condition, there is a balance between ROS and antioxidants system. Cancer and chemotherapy drugs both induce excessive free radicals and ROS. Excessive free radicals and ROS will lead oxidative stress to organisms, and can involve in cancer initiation and progression [59]. Oxidative stress could induce lipid peroxidation and DNA damage. Lipid peroxidation prolongs the cell cycle and reduces the chemotherapy effectiveness of anticancer drug, because most anticancer drugs affect rapid dividing cells. Antioxidants have the potential to scavenge the oxidants produced by cancer and chemotherapy drug, and protect lipid from peroxidation, so that they can increase the chemotherapy effect and reduce side effects due to oxidative stress [59].

1.2.3.1.1 Vitamin E

Vitamin E is a group of naturally lipid-soluble antioxidants including eight structurally related tocopherols and tocotrienols [73]. Vitamin E can scavenge ROS and protect polyunsaturated lipid from peroxidation [74]. Study findings showed that vitamin E also can inhibit cancer cells growth, prevent cancer chronic diseases, and enhance cytotoxicity of anticancer drugs [60, 73]. As antioxidants, vitamin E is widely used in diet supplements, and used with chemotherapy drug for cancer therapy.

Chancery and associates [57] found that vitamin E antioxidant enhanced the antitumor effect of 5-Fu *in vitro* and *in vivo*. Vitamin E could increase the sensitivity of HCT116 and HCT15 cells to 5-Fu. Both cell lines grew in the presence or absence with the approximate IC50 concentration (3 mM) vitamin E together with different concentrations of 5-Fu. Addition of vitamin E in the media significantly decreased the IC50 of 5-Fu from 3.80 μM to 1.70 μM. They also conducted an *in vivo* test of the combination effect of 5-Fu and vitamin E. Athymic mice bearing HCT116 or HCT15 tumor were treated with 750 mg/kg vitamin E and / or 5-Fu, or saline negative control by introperitoneal (ip) injection. The combination of 5-Fu and vitamin E significantly increased the cytotoxic effect of 5-Fu and resulted in the growth cessation in three weeks. In addition, apoptotic index tested by TUNNEL method was also significantly increased by vitamin E addition in the two 5-Fu treated cell lines. Mechanistic studies showed that one of the possible mechanisms that vitamin E increased the sensitivity of cancer cells to 5-Fu was to induce a single transcription factor (C/EBPβ), which can further elevate the expression of $p21^{WAF1/CIPI}$, a cyclin dependent kinase inhibitor of cell cycle, and cell death. This process was independent of p53 protein.

1.2.3.1.2 Vitamin C

Vitamin C, a water-soluble antioxidant, prevalent in fruits and vegetables, is widely used in functional foods, dietary supplements, and cosmetics [75]. Not only can vitamin C protect an organism from oxidative stress, but it also can have chemopreventive activity by inhibiting tumor promotion [75]. As discussed below, it also can improve the chemotherapy effect of anticancer drugs.

In order to evaluate the synergistic effect of vitamin C and 5-Fu, Nagy and co-workers [76] determined the growth inhibition effect of simultaneous exposure the 5-Fu and vitamin C in three cell lines, mouse lymphoma, a chemosensitive one, HEp-2, a chemoresistant one and a human lung fibroblast cell line. The results showed that vitamin C itself did not have antiproliferative effect on fibroblasts, but increased the growth inhibition effect of 5-Fu with different concentrations on mouse lymphoma with dose-dependent manner once the concentration of vitamin C was greater than 5 μg/mL. When the concentration of vitamin C was over 50 μg/mL, vitamin C also enhanced the growth inhibitory effect of 5-Fu on HEp-2 chemoresistant cell line. One of mechanisms by which vitamin C increased the antiproliferation of 5-Fu was enhancement of apoptotic cell death. At higher concentrations of vitamin C, apoptosis increased.

DeLoecker *et al.* [77] investigated the synergistic effect of vitamin C and vitamin K3 combined with chemotherapeutic agents. Fifty percent confluent AN3CA (human endometrial adenocarcinama) cells treated with 5-Fu 0.15 μg/mL together with 200 μM vitamin C and 2 μM of vitamin K3. The growth inhibition increased from 25% 5-Fu alone to 71% combined with both vitamins. They found that the concentration of the 5-Fu and vitamins should be adjusted to proportional to the density of the treated cells to exert their best effects.

1.2.3.2 Folic acid

Folic acid is a necessary vitamin for people and necessary for healthy neural system development. Dietary folic acid deficiency has been observed in cancer patients due to the combination of inadequate dietary content, poor dietary intake and absorption,

and increased metabolic needs of tumors [78]. Folic acid is one of substrates of TS. So, it is possible that folic acid has the ability to modulate the effect of 5-Fu. However, study findings regarding the effects of folic acid on 5-Fu chemotherapy were complicated.

Branda *et al.* [78] found that folate deficiency for six weeks (no folic acid in diet) retarded the growth of transplanted MADB106 mammary tumor in Fisher 344 rats compared with folate replete diet (2 mg/kg). They also found that folate deficiency rats were more sensitive to side effects of chemotherapy. In another experiment, they treated rats with three folic acid concentrations diets, folate deficiency, (0 folate), replete, (2 mg/kg), and high folate, (2 mg/kg plus 50 mg/kg ip injection) for 7 weeks. Then the rats were transplanted with mammary tumor cells and treated with 50 mg/kg 5-Fu chemotherapy 3 times, once every 4 days. Later, they did another experiment with the same treatment as above but with 75 mg/kg 5-Fu. The findings were that high folate diet increased the host resistance to 5-Fu toxicity, including higher survival rate and less weight loss. These results indicated that diet rich in folic acid has the potential to reduce the toxic side effects of 5-Fu and that dietary deficiency of folic acid increases the toxicity of 5-Fu.

1.2.3.3 Glutathione

Glutathione (GSH), a tripeptide thiol of glutamate, cysteine, and glycine (GluCysGly), is a strong antioxidant found in almost all cells [54]. Freshly prepared meat, boiled ham, and hamburger are rich in GSH, and fruits and vegetables are moderately high [54]. GSH contains a thiol group, which is strongly nucleophilic, and forms a stable covalent compound with electrophilic compounds. This character makes glutathione

able to form the complex with some antineoplastic agents to lower their activity and also lower their toxicity. Some alkylating anticancer drugs are also electrophilic agents, which can compete with GSH to form the complex with DNA. So GSH has the potential to lower the anticancer effect when in combination with this kind of agent [59]. Cozzaglio *et al.* [79] studied the modulator effect of GSH in combination with cisplatin and 5-Fu in metastatic colorectal cancer. First, patients were treated with 5-Fu dose of 750 mg/m^2 for five days with infusion. After that, reduced GSH was administrated via intravenous treatment. One hour later, cisplatin dose of 40 mg/m^2 was administrated in a one-hour infusion for three days. They found that the GSH reduced the toxicity of nausea, vomiting and nephrotoxicity induced by cisplatin or 5-Fu and cisplatin. This study demonstrated that GSH interfered with the cytotoxicity of cisplatin and may have the ability to protect the renal tissues from the damage of cisplatin, but GSH had no effect on 5-Fu treatment.

Chen and co-workers [54] found that GSH level in HT29 cells treated with 5 μM 5-Fu increased dramatically compared with nontreated control cells, but when the cells were treated with a GSH inhibitor and then 5-Fu, the GSH level decreased compared with the control cells. GSH itself did not stimulate the growth of HT29 cells. They found that 34% increase of GSH level in the cells did not have any effect on the cytotoxicity of 5-Fu, but 50% decrease of GSH in the cells had significant increasing effect on the cytotocity of 5 and 10 μM 5-Fu.

1.2.3.4 Docosahexaenoic Acid

Docosahexaenoic Acid (DHA) is a polyunsaturated fatty acid rich in marine fish. Epidemiological studies have shown that diet rich in DHA can lower the risk of heart

diseases and cancer [80]. Recent findings suggested that DHA itself can inhibit cancer cells growth, decrease side effects of cancer chemotherapy and increase sensitivity of cancer cells to anticancer drugs [61, 80, 81].

Calviello *et al.* [80] found that DHA can increase cancer growth inhibition effect of low dose 5-Fu in four colon cancer cell lines. DHA potentiated the 5-Fu effect by downregulating the expression of the apoptotic-suppressing proteins, Bcl-2 and Bcl-X_L. In their study, two p53 wild type colon cancer cell lines, LS174 and Colo320, and two p53 mutant cell lines, HT29 and Colo205, were used. IC20 DHA concentrations ranging from 2.5 to 10.1 μM were used in four cell lines in combination with increasing concentrations of 5-Fu from 0 to 2.5 μM. The reason they used IC20 DHA was that high concentrations of DHA may cause peroxidation of lipid membranes. Results showed that IC20 DHA significantly increased the cytotoxic effect of 5-Fu on all four cell lines independent of p53 status. DHA and 5-Fu had a synergistic effect rather than additive effect. Further study showed that combination of 5-Fu and DHA both at their IC20 concentrations could markedly induce apoptosis by cell cycle arrest at G_2/M phase and by inhibition of expression of antiapoptotic protein Bcl-X_L and Bcl-2 in LS174 cells.

Intestinal toxicity is one of severe side effects of antitumor therapy. Gomez de Segura *et al.* [61] examined the intestinal protection effect of diets rich in DHA (1%), protein, and DHA + protein in 5-Fu-treated male Wistar rats. Rats were given their assigned diets for six days (8 rats/group). At day 3, 900 mg/kg 5-Fu was injected by ip in a single dose. Results showed that in the rats with DHA rich diet, the intestinal mucosal height was not significantly different from control diet group. Diet supplied with

DHA and protein significantly increased mucosa height and recovered it to normal level. Diet with DHA and protein also reduced apoptosis of crypt cells in small intestine and increased the crypt cells proliferation. These findings suggested that diet supplemented with DHA gave moderate protection to the intestine from 5-Fu toxicity, and additional supplementation with protein fortified the protection of DHA.

1.2.3.5 Other compounds

Liao *et al.* [62] measured the bone marrow protection effect of a novel high molecular polysaccharide isolated from black soybean (PSBS) by liquid chromatography and gel filtration in 5-Fu treated mice. Male ICR mice (6 each group) were ip injected 150 mg/kg 5-Fu single time. Six hours later, 400 mg/kg PSBS was orally administrated in drinking water twice a day for five days. The experiment lasted 11 days. PSBS treatment could reconstitute the suppressed bone marrow function by increasing leukocyte counts, shortening the recovery time of leukocyte counts, increasing colony-forming unit-granulocyte/macrophage (CFU-GM) due to toxicity of 5-Fu. So, PSBS is a potential mylopoietic agent.

Horie *et al.* [72] found that aged garlic extract could protect the small intestine of Wistar rats from 5-Fu damage. Rats were treated with 5-Fu 30 mg/kg orally, and provided 2% aged garlic extract. The extract reduced the intestinal permeability tested with fluorescein iosthiocyanate-labeled dextran (FD-4) by *in vitro* everted intestine technique. This indicated that aged garlic extract may reduce intestinal toxicity of 5-Fu therapy.

Takano *et al.* [70] found that (+)-catechin isolated from *Actinidea arguta* Planch can promote bone marrow cells proliferation, recovery of white blood cells counts and

platelets counts damaged by 5-Fu. Female C57B1/6 mice were single injected with 150 mg/kg 5-Fu at day one. (+)-Catechin was ip injected once a day for 9 days after 5-Fu treatment. The experiment lasted for 16 days totally. The effects of (+)-catechin on 5-Fu toxicity were evaluated at day 9 and day 16. One mg/kg and 10 mg/kg (+)-catechin significantly increased the numbers of white blood cells and platelets at day 9. At day 16, (+)-catechin enhanced the numbers of myelocytes and splenocytes remarkedly compared with 5-Fu alone. These findings suggested that (+)-catechin may promote the recovery of myleotoxicity induced by 5-Fu.

Protein intake also may alleviate the side effects of 5-Fu treatment. Protein is a necessary nutrient for organism growth. In cancer status, absorption of nutrients lowers, because of intestinal toxicity of chemotherapy, and possible lead to malnutrition of host. Torosian *et al.* [82] investigated the effect of protein on 5-Fu toxicity in a tumor model. Wistar rats with implanted mammary tumor cells were treated with 25 mg/kg 5-Fu for 5 days. 5-Fu induced severe leucopenia. Diet supplemented with 22% protein significantly increased white blood cell number of the rats. In addition, a diet rich in protein reduced diarrhea due to intestinal toxicity of 5-Fu.

1.2.4. Summary

Dietary supplements may modulate chemotherapeutic effect of 5-Fu, either increase response rate of cancer cells to 5-Fu, or reduce side effects of 5-Fu and increase host tolerance. However, there are no uniform methods and evaluation standards for these studies. Mechanisms of their effects are still under determination and most of them are still in preclinical study. Thereby, further investigation on effect mechanisms and clinical studies are needed.

Chapter 2: Enhancement of growth inhibition of 5-flurouracil on colon cancer cells by Anthocyanin-Rich Extracts

2.1. Introduction

Epidemiological studies have shown that diets rich in fruits and vegetables are related to reduced risk of chronic diseases cancers and cardiovascular diseases [83]. There are emerging researches that anthocyanins contribute to protective effects of fruits and vegetables on chronic diseases. Anthocyanins are water-soluble plant secondary metabolites responsible for blue, purple, and red colors of fruits and vegetables [84]. In nature, most anthocyanins are glycosylated. The position and number of hydroxyl group and types of sugar attachments determine the type and activity of anthocyanins. Anthocyanins in berries have the activities of antioxidation, growth inhibition of cancer cells, anti-angiogenicity, protection of genomic DNA integrity, and reduction of age-related diseases [37]. *In vivo* and *in vitro* studies showed anthocyanins are very good nutraceutical ingredients. Therefore, fruits and vegetables containing anthocyanins are considered to be promising functional compounds for cancer prevention and other chronic diseases. Our group's studies have demonstrated that Anthocyanin-Rich-Extract (ARE) from chokeberry had chemopreventive effect on HT29 cells [11, 34].

The daily intake of anthocyanins is estimated to be 200 mg in the United States [44, 85]. Among them, the anthocyanins from berries contribute a very large part, 100-300 mg in a single portion. Black Chokeberry, native in North America and Russia, contains high levels of total phenolics [27]. The berries contain 725–800 mg anthocyanins/100 g fresh weight [86]. The anthocyanins in black chokeberry consist mainly of cyanidin-3-galactoside and cyanidin-3-arabinoside, which are reported to

constitute 64% and 29%, respectively and are found in the peel as well as throughout the fruit flesh [87].

5-Flurouracil (5-Fu), an antimetabolic anticancer drug, has been widely used for cancer chemotherapy more than 50 years, especially in the advanced stages of colon, breast, and prostate cancers [49]. It inhibits the cancer cell growth by inducing apoptosis [58], inhibiting the activity of thymidylate synthase (TS) and further inhibiting the synthesis of DNA and RNA [58, 65]. 5-Fu is a cytotoxic agent, affecting rapid growing cells including cancer cells and some rapid growing normal cells including intestinal cells and bone marrow cells. Because of nonselective target of 5-Fu, it has certain side effects and those side effects limit its usage and dosage. The most serious adverse side effects of 5-Fu are gastrointestinal toxicity, expressed as diarrhea, decreased absorption of nutrients, vomiting, bone marrow suppression, and immunotoxicity.

The response to 5-Fu of colorectal carcinoma is approximately 20%, relatively low [49, 58]. Therefore, people have been trying to find some new regimen or modifications of administration schedules to increase the sensitivity of cancer cells to 5-Fu. Combination with other anticancer drugs or agents is one of these approaches. Modulators include cisplatin, N-(phosphoneacetyl)-L-aspartic acid (PALA), thymidine, methotrexate, leucovorin (LV) [17]. There were also findings that the antioxidants enhanced the cytotoxicity of the chemotherapeutic reagents [57, 58]. The antioxidants, vitamin E and vitamin C, sensitized cancer cells to 5-Fu [76]. Based on this rationale, we investigated if the chokeberry extract rich in anthocyanin, a strong antioxidant, could enhance the sensitivity of colon cancer cells to 5-Fu. Previously,

Malik *et al*. [34] reported that chokeberry extract inhibited growth of colon cancer cells to a greater degree than normal cells. Therefore, this experiment also investigated the effect of combined 5-Fu and chokeberry extract on normal colon cells. The overall goal is to find a combinational treatment that: (1) enhances the toxicity of 5-Fu to cancer cells, without increasing toxicity to normal cells to increase the effectiveness of the drug, and/or (2) reduces the toxicity of 5-Fu to normal cells to reduce side effects.

2.2 Materials and methods

2.2.1 Chemicals

5-Fu powder, purchased from Sigma Chemical Company (St. Louis, MO), was dissolved in dimethyl sulfoxide (DMSO) and diluted with cell culture media to different concentrations. The final concentration of DMSO was 0.125%. Food grade chokeberry (*Aronia meloncarpa* E.*)* extract powder (10% Anthocyanin) was supplied by Artemis International, Inc. (Fort Wayne, IN).

2.2.2 Cell culture

Human colon cell lines, HT29 and SW620, were purchased from American Type Culture Collection (Rockville, MD). Both the HT29 cell line and SW620 cell line were maintained in flasks in McCoy's 5A medium (Biowhitaker Inc., Walkersville, MD) containing 10% fetal bovine serum (FBS) purchased from Hyclone (Logan, UT). The HT29 cell line was isolated from a primary tumor. The SW620 was isolated from metastatic site of lymph node originated from colon tumor. The tumor stage of this cell line is Dukes' type C. NCM460, a cell line derived from a normal human colon (InCell Corp. San Antonio, TX), grew in M3 Base media (InCell Corp. San Antonio,

TX) supplemented with 10% FBS. All these three cell lines were cultured at 37° C in a 5% CO_2 humidified atmosphere and grew exponentially as monolayers during all the courses of all experiments. Five thousand cells were seeded to each well of 96-well plates. After 24 hours incubation, when the cells grew to the log phase, combined 5-Fu and ARE solutions and media control were randomly added to the wells. After another 48 hours for HT29, NCM460 or 72 hours for SW620 cell lines, the cell growth was assessed. Every treatment was assigned to six wells, and the whole experiments were replicated at least three times.

2.2.3 Cell growth inhibition assay

Cell growth inhibition was assessed using sulforhodamine B (SRB) method based on the *in vitro* toxicology assay kit sulforhodamine B (Sigma) according to the manufacturer's instruction. The key component of this kit is the dye, sulforhodamine B (Acid Red 52). The dye binds to cellular protein and is then solubilized in base. At the end of the experiment, cells are briefly washed, fixed and stained with the dye. The incorporated dye is then liberated from the cells in a tris base solution. An increase or decrease in the number of cells (total biomass) results in a concomitant change in the amount of dye incorporated by the cells in the culture. This indicates the degree of growth inhibition caused by the test material. Growth inhibition was calculated by $\dfrac{ODcontrol - ODtreatment}{ODcontrol} \times 100\%$.

2.2.4 Fifty percent inhibition concentration test

Fifty percent inhibition concentration (IC50) is a parameter of sensitivity of targets to drugs. It is the concentration that the test material can inhibit 50% cell growth. Five thousand colon cells were seeded to 96-well plates growing for 24 hours. Then at

least five different concentrations of 5-Fu were added to the plates and incubated for another 48 hours for HT29, NCM460 and 72 hours for SW620. The cell inhibition was measured by SRB method. The experiments were done three times independently. Then the dose-effect curve was drawn according to the average of the inhibition percentage of three experiments. The log transformation of the 5-Fu concentration was the X axis, and the inhibition percentage was the Y axis. An equation and correlation coefficient were obtained by Excel software. With the equation, 50% inhibition concentration was calculated [58, 88].

2.2.5 Chokeberry Anthocyanin-Rich Extract (ARE) preparation

One gram chokeberry extract powder was dissolved in 10 mL 0.01% HCl acidified deionized water, and then the supernatant was semi-purified with C18 cartridge (as below). The eluted liquid was concentrated with rotary evaporator and redissolved in 10 mL acidified water. The ARE was stored in -80 °C until use.

2.2.6 Solid phase (C18) semi-purification of anthocyanins

After conditioning the five g C18 cartridge (Waters Corp, Milford) with methanol, the cartridge was washed with acidified deionized distilled water to remove the methanol. Twenty milliliters supernatant was forced through the cartridge, and then the cartridge was washed with two columns acidified water to remove sugar and other polar compounds in the extract, which were not adsorbed to the cartridge. Anthocyanins and other phenolics were eluted with two columns acidified ethanol (0.01% HCl) and collected in a 150 mL boiling flask. The ethanol was totally removed in a rotary evaporator at 40 °C under vacuum. The concentrated extract was redissolved in acidified water. The monomeric anthocyanins content was tested. The extract was stored in aliquots at -80 °C for future use and analysis. Usually, the ARE was

prepared just the day before use to insure the anthocyanins are intact and did not degrade.

2.2.7 Determination of monomeric anthocyanin concentration

The monomeric anthocyanin content of extracts was determined using the pH differential method [14]. The absorption value was determined at 510 and 700 nm at pH 1.0 and pH 4.5. Anthocyanin content was calculated as cyanidin-3-glucoside, because cyanidin is the most abundant anthocyanin, using an extinction coefficient (ε) of 26900L cm^{-1} mol^{-1} and molecular weight (MW) of 449.2 g mol^{-1}.

2.2.8 Statistical analysis

The results were expressed as means±SE. The normality of the data was tested using SAS software (version 9.1, SAS Institute Inc, Cary, NC). One-way ANOVA was used for all the data analyses with SAS software to determine if there were significant differences between different treatment concentrations. Pairwise comparisons adjusted with Tukeys were done once the difference was significant. $P<0.05$ was considered as statistical significant difference

2.2.9 Combinational 5-Fu and anthocyanin extracts

Colon cells were exposed to the IC50 concentrations of 5-Fu with and without chokeberry ARE at different concentrations of monomeric anthocyanins per mL growth media. After 48 or 72 hours treatment, cell growth was assessed using SRB assay and the cell growth inhibition percentage was calculated.

2.3 Results

2.3.1 IC50 of 5-Fu on HT29 and SW620 cell lines

The effects of 5-Fu on the growth of HT29 and SW620 cell lines were dose dependent (Figure 6 and Figure 7). The 50-percent growth-inhibitory concentration (IC50) for any cell line is simply an index of cytotoxicity or cytostasis. It is a parameter often used to compare the effectiveness of compounds *in vitro*. The lower the IC50, the more efficient is the compound in inhibiting the growth of the testing cells. The IC50 values were calculated with the graphic method, which is an old but most common method for IC50 estimation [21].

The IC50s of 5-Fu for SW620 cells were 89.2 μg/mL and 12.9 μg/mL at 48 hour and 72 hour, respectively. The IC50 of 5-Fu for HT29 cells at 48 hour was 5.5 μg /mL. The IC50 of 5-Fu on HT29 at 72 hour was not determined, because 48 hour was long enough to inhibit 50% cell growth. From the IC50 values, it is clear that HT29 cells were more sensitive to 5-Fu than the SW620 cells. For future combinational studies, the IC50 of HT29 cells at 48 hour and of the SW620 cells at 72 hour were used. The SW620 cell line was less sensitive to 5-Fu and needed more exposure time than the HT29 cell line. The effect of 5-Fu on both cell lines was time dependent. We exposed the cancer cells to IC50 5-Fu concentrations of these two cell lines. IC50 is the equitoxic concentration for these two cell lines, so that we could compare the effects of the ARE among the two cell lines.

$$y = 27.385x + 31.668$$
$$R^2 = 0.982$$
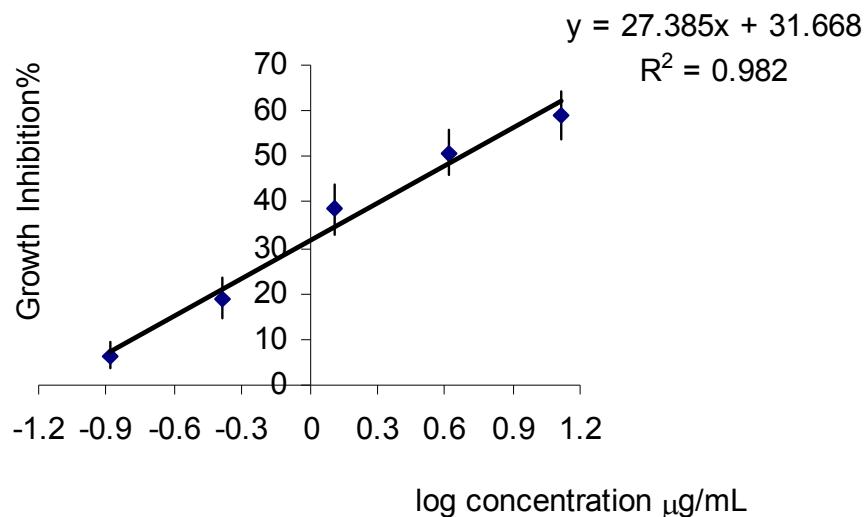
Figure 6 Dose-effect curve of 5-Fu on HT29 cells at 48 hour

Five different concentrations (0.13, 0.41, 1.30, 4.11, 13.00 μg/mL) of 5-Fu were added to HT29 cells. After 48 hours, cell growth inhibition was assayed with SRB method. Values are expressed as mean±SE. Results were from three independent experiments. Log 5-Fu concentration is X axis. Growth inhibition percentage is Y axis. The linear equation was obtained through Excel. IC50 was calculated by y=50%.
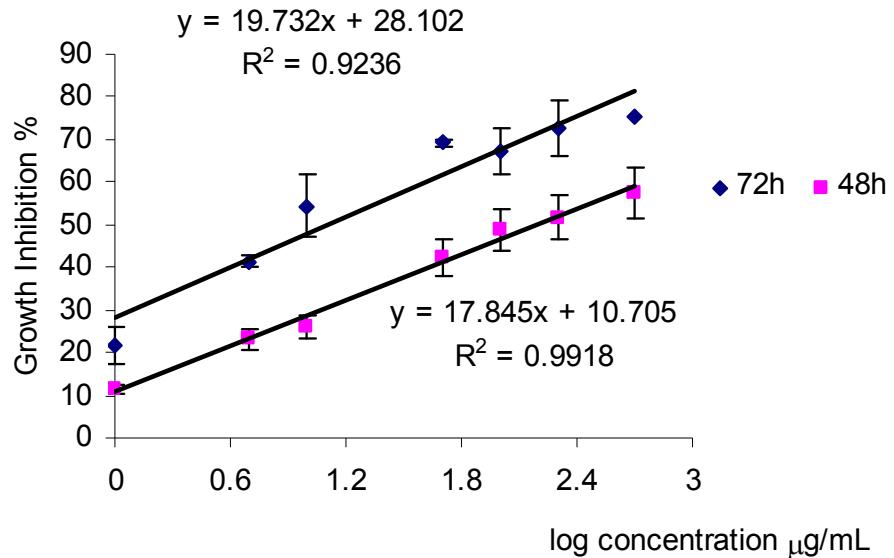
Figure 7 Dose-effect curves of 5-Fu on SW620 cells at 48 and 72 h

Seven different concentrations of 5-Fu (1.0, 5.0, 10.0, 50.0, 100.0, 200.0, 500.0 μg/mL) were added to SW620 cells. After 48 hours and 72 hours treatment, cell growth inhibition was assayed with SRB method. Values are expressed as mean±SE. Results were from three independent experiments. Log 5-Fu concentration is X axis. Growth inhibition percentage is Y axis. The linear equation was obtained through Excel. IC50 was calculated by y=50%.

2.3.2 The combined effect of ARE and 5-Fu on HT29 cell line

In the HT29 cell line, addition of ARE increased growth inhibition by 5-Fu (Figure 8).

Using an IC50 concentration of 5.5 µg/mL 5-Fu, low concentrations of ARE had little

to no effect on HT29 cells, and the cell growth inhibition was very similar to cells

treated with only 5-Fu. However, growth inhibition was significant ($p<0.05$) when the

concentration of ARE was increased to 50 µg/mL. Compared to inhibition with 5-Fu

alone (64%), 5-Fu plus 50 µg/mL ARE resulted in over 90% inhibition. Because

addition of 50 µg/mL ARE resulted in more than 90% growth inhibition, we did not

test higher concentration ARE in this study.
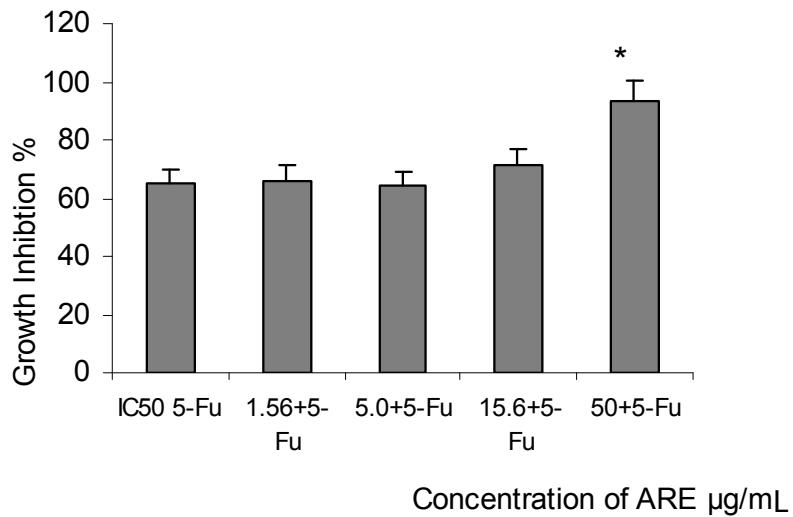
Figure 8 Growth inhibition on HT29 colon cancer cells by IC50 5-Fu and different concentrations ARE

Five thousand HT29 cells were seeded into 96-well plates. After incubation for 24 hours, 100 μL mixture of ARE with different concentrations (1.56 to 50 μg/mL) and IC50 5-Fu were added to the plates. After another 48 hours, the cell growth was assay with SRB method. The results came from three independent experiments. Data are mean ± SE. Values are expressed in percentage and referred to the control cells. The inhibition percentage for control cells was 0.

2.2.3 Combined effect of ARE and 5-Fu on SW620 cell line

In SW620 colon cancer cells, addition of ARE increased growth inhibition by 5-Fu at high ARE concentration (Figure 9). Compared with IC50 5-Fu alone, the growth inhibition percentage was increased by combination of different concentrations of AREs with IC50 5-Fu, and the inhibition percentage was dependent on the dose of monomeric anthocyanins. IC50 5-Fu inhibited cell growth by almost 42%. Fifty μg/mL monomeric anthocyanins ARE increased the inhibition rate up to 68%, which was a significant increase of the growth inhibition percentage compared with only 5-Fu.
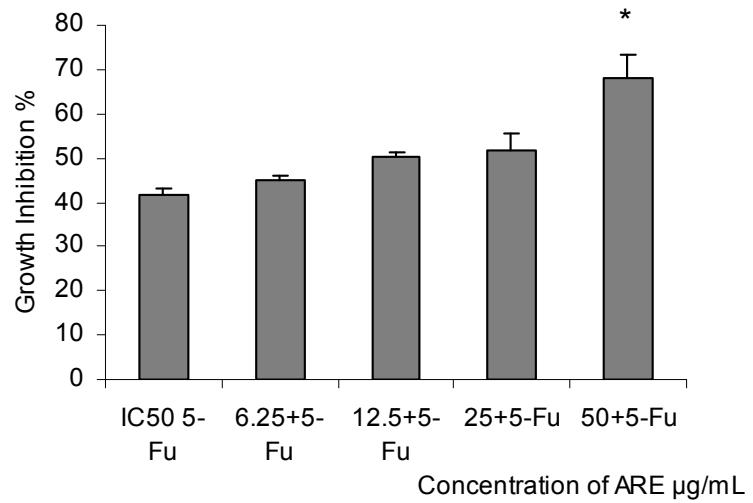
Figure 9 Growth inhibition on SW620 colon cancer cells by IC50 5-Fu and different concentrations ARE

Five thousand SW620 cells were seeded into 96-well plates. After incubation for 24 hours, 100 μL mixture of ARE with different concentrations (6.25 to 50 μg/mL) and IC50 5-Fu were added to the plates. After another 72 hours, the cell growth was assay with SRB method. The results came from three independent experiments. Data are mean ± SE. Value are expressed in percentage and referred to the control cells. The inhibition percentage for control cells was 0.

2.2.4 The combined effect of ARE and 5-Fu on NCM460 normal cell line

In order to test if ARE also increased the damage by 5Fu to the normal cells, we tested growth inhibition effect by combination of the IC50 5-Fu and the different concentrations ARE on NCM460 normal colon cell line (Figure 8). The ARE almost had no effects on the growth inhibition of 5-Fu even with the highest concentration 50 µg/mL ARE (60% inhibition), compared with 5-Fu alone (53%). There was no significant inhibition difference between the 5-Fu alone and 50 µg/mL ARE+5-Fu. Therefore, ARE had no influence on the sensitivity of the normal cells to 5-Fu.
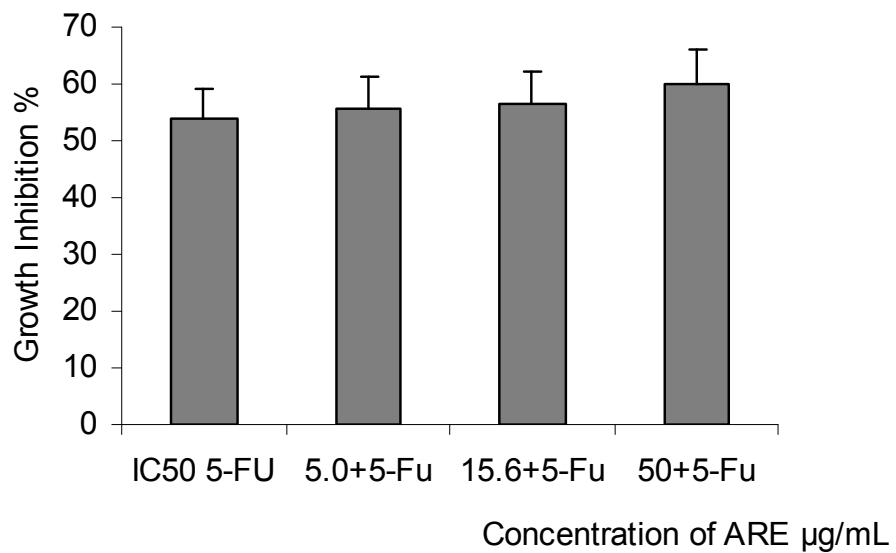
Figure 10 Growth inhibition in NCM460 normal colon cells by IC50 5-Fu and different concentrations ARE

Five thousand NCM460 cells were seeded into 96-well plates. After incubation for 24 hours, 100 μL mixture of ARE with different concentrations and IC50 5-Fu were added to the plates. After another 48 hours, the cell growth was assay with SRB method. The results came from three independent experiments. Data are mean ± SE. Value are expressed in percentage and referred to the control cells. The inhibition percentage for control cells was 0.

2.4 Discussion

2.4.1 ARE enhanced growth inhibition effect of 5-Fu on colon cancer cell lines, but had little or no effect on normal colon cell line

Even though 5-Fu is a widely used colon cancer chemotherapeutic agent, the response rate of tumor patients is only 15%-20%. One of the methods to increase the response rate is to increase the sensitivity of cancer cells to 5-Fu [23]. In clinical therapy, the 5-Fu is often combined with folonic acid (LV), cisplatin (CDDP), cyclophosphamide, methotrexate or other chemotherapeutic drugs [66]. There were reports that the CDDP increased the sensitivity of tumor cells to 5-Fu both *in vitro* and *in vivo*. The response rate of gastric cancer cells to 5-Fu was increased 43% to 45% by CDDP [24], and the response rate could be improved by LV approximately 20% [18]. Some other reports found that the antioxidants vitamin E, vitamin C and N-acetylcysteine could enhance the susceptibility of colon cells to the 5-Fu by induction of apoptosis [58, 89]. Nagy and co-workers [76] tested the antiproliferative effect and apoptosis-inducing action of 5-Fu in combination with vitamin C in a mouse lymphoma cell line, HEp-2, and a human lung fibroblast cell line [76]. They found vitamin C itself had no effect on the proliferation of cancer cells, but it could increase the anticancer effect of 5-Fu dose dependently once the concentration of vitamin C was greater than 5 μg/mL. When the concentration of vitamin C was over 50 μg/mL, vitamin C also enhanced the growth inhibitory effect of 5-Fu on HEp-2, a chemoresistant cell line. The results suggested that vitamin C could serve as a potential sensitizer of 5-Fu for cancer chemotherapy.

In the current study, ARE in combination with 5-Fu increased the antiproliferative effect of 5-Fu on colon cancer cell lines, HT29 and SW620. The results showed that

ARE can enhance the chemosensitivity of HT29 and SW620 cells to 5-Fu. For both cell lines, ARE increased the growth inhibition by more than 30% when the anthocyanins concentration was 50 μg/mL. At low dose anthocyanins, the enhancement effect was very little and not significant, but when dose increased to 50 μg/mL, the enhanced effect was very significant. This finding suggested that ARE had the potential to be a sensitizer for 5-Fu in colon cancer therapy. The difference between Nagy's study on vitamin C and this study was that Nagy also used different 5-Fu concentrations from 100 μg/mL to 300 μg/mL. In the current study the IC50 5-Fu was used. Vitamin C was more effective than ARE in combination with 5-Fu. Vitamin C increased 5-Fu effectiveness at concentration as low as 5 μg/mL. ARE was effective at concentration of 50.0 μg/mL. This difference may be because the sensitivity of different cell lines used in these two studies.

The interesting thing was that ARE increased the sensitivity of colon cancer cells to 5-Fu, but had no effect on the sensitivity of normal colon cell NCM460 to 5-Fu. This could be a useful aspect for ARE in combination with 5-Fu. 5-Fu is an antimetabolism chemotherapeutic drug. It has serious side effects because it not only damages the cancer cells, but also damages the normal cells, especially rapid growing cells like epithelial cells in intestines. The side effects are gastrointestinal damage, light to serious diarrhea, and apoptosis of normal rapid growing cells [58]. However, ARE increased the sensitivity of HT29 and SW620 colon cancer cells to 5-Fu, not the normal cells. This indicated that ARE could be a source for side effect reduction of 5-Fu in clinical use by lowering the dose of 5-Fu administration, because it can increase the effectiveness of 5-Fu. Kimura *et al.* [90] reported that chitosan, a dietary fiber, can prevent gastrointestinal toxicity and immunotoxicity induced by 5-Fu in

mice, and without loss of antitumor activity. This is similar to what was found in this *in vitro* study. So further animal study of combination of 5-Fu and anthocyanin rich extract is necessary to investigate effectiveness of ARE on 5-Fu chemotherapy.

2.4.2 IC50 test of HT29 cell line and SW620 cell line

IC50 is a parameter of sensitivity of the targets to drugs. The IC50 of HT29 at 48 hour (5.5 μg/mL) was much lower than SW620 (89.2 μg/mL), so the HT29 is much more sensitive to the 5-Fu than SW620. The IC50 difference of these two cell lines was as we expected. The HT29 was isolated from primary colon cancer, and SW620 was isolated from lymph node metastasized from colon. The SW620 cells were from more advanced stage of colon cancer than HT29 cells, so the SW620 cells would be expected to be more resistant to anticancer drugs than HT29 cells. We tested the IC50 for the further combined study of 5-Fu with ARE. With IC50 5-Fu and 50μg/mL ARE, the growth inhibition percentage was more than 20% higher than with only 5-Fu treatment in both cell lines. Therefore, if we use the dose of 5-Fu lower than IC50 with 50 μg/mL ARE, it is possible to get 50% growth inhibition percentage, because ARE increased the growth inhibition effect of 5-Fu. With the low concentration of 5-Fu, in the consequence, side effects induced by 5-Fu also will be reduced, because the side effects are dose dependent too. This is a good aspect of ARE to be used in later chemotherapy studies.

2.4.3 Mechanism of growth inhibition enhancement by anthocyanins

5-Fu inhibits the cell growth by several mechanisms, including cell cycle arrest, apoptosis induction, and COX enzyme inhibition [91, 92]. Anthocyanins can induce cancer cell apoptosis [46]. By induction of apoptosis, inhibition of the enzyme

activity or induction of cell cycle arrest, ARE in combination with 5-Fu may increase the sensitivity of cancer cells to 5-Fu.

Adeyemo and co-workers [58] found that combining 5-Fu with 5mM vitamin E or 25mM *N*-acetylcystein augmented pro-apoptotic Bax protein expression 2.5-3 fold compared with 5-Fu alone in the colon cancer cell lines Colo201 and Colo205. One of possible mechanisms that vitamin E and *N*-acetylcystein enhanced the susceptibility of colon cells to 5-Fu is to increase apoptosis. Bax protein is a member of the pro-apoptotic family, promoting apoptosis by triggering the release of cytochrome C from mitochondria [58]. As anthocyanins are also strong antioxidants, similar to vitamin E, the induction of apoptosis may be one of the possible mechanisms for anthocyanins augmenting the sensitivity of colon cancer cells to 5-Fu.

The anthocyanidins of cyanidin and delphinidin, are potent inhibitors of the epidermal growth factor receptor, shutting off the downstream signaling cascades [44]. Therefore, an effect of ARE on the pathway also could be one of mechanisms of increasing the sensitivity. However, up to now, there is no study regarding this aspect.

5-Fu moderates the cell cycle stages of growing cells. It blocks all the stages of the cell cycles depending on the dose and exposure time [49, 91]. It is possible that ARE and 5-Fu may have some synergistic effect when both of them block the same stage of cell cycle. However, the real mechanisms of the enhancement effect of ARE on the cytotoxicity of 5-Fu to cancer cells are remained to be determined.

2.4.4 Schedule of addition of ARE

When 5-Fu is administrated to patients combined with other reagents, the regimen of drug administration is also a very important factor and will affect the therapeutic effects. Cho *et al.* [93] investigated the timing effect of combination of 5-Fu and cisplatin (CDDP) on the gastric cancer cell lines. They found that the cytotoxicity of a combination of 5-Fu and CDDP against human gastric cancer cells is both cell line and schedule dependent and is especially affected by the timing of the CDDP treatment. The MKN-1, MKN-28, MKN-45, and MKN-74 cell lines were exposed to IC50 5-Fu and CDDP for 72 hours and 8 hours, respectively. Three treatment schedules were used for application of CDDP: before, simultaneously with, and after the treatment with 5-Fu. The treatment effect was cell line dependent. For MKN-28, MKN-45, and MKN-74, the regimen of addition of CDDP after 5-Fu treatment was best. Against MKN-1, the addition of CDDP before 5-Fu treatment was best. Therefore, in the future study, different treatment schedules of 5-Fu and ARE should be done. In this study, only simultaneous treatment of 5-Fu and ARE was used. We should try pretreatment of colon cancer cells with ARE for a certain time then followed by 5-Fu treatment or the reverse. In addition, the sensitivity to 5-Fu also cell line dependent, so different colon cancer cell lines with different gene types or stages should be done in future study with ARE and 5-Fu combinations.

Chapter 3: Reduction of side effects of 5-flurouracil by Anthocyanin-Rich Extracts in rats

3.1. Introduction

Even though 5-Flurouracil (5-Fu) is one of most useful antitumor drugs [44], serious side effects including gastrointestinal toxicity, myelotoxicity and immunosuppression [44, 83] can affect and reduce the response rate and therapeutic index. Serious side effects like nausea, vomiting, diarrhea, and stomatitis result in the patients losing their appetites, further effects on malnutrition and weakness such that they have to stop the therapy. These side effects limit the acceptable dose, therapeutic time and the therapeutic efficacy. Therefore, scientists are trying to find some drugs or natural agents, which can improve the sensitivity or/and reduce the side effects of 5-Fu when combined with 5-Fu chemotherapy. In this way, physicians can either lengthen the therapy treatment time or increase the therapeutic doses, when needed, to achieve improved response rates.

Dietary modulation is one of these approaches that have been explored and positive impacts on the side effects of chemotherapy have been reported [66]. By reducing the toxicity of chemotherapeutic drugs, the normal tissues could tolerate higher doses of drugs and enhance their anticancer action. There were reports that dietary fiber chitosan, soy protein, and docosahexaenoic acid (DHA) increased the anticancer action and prevent the side effects of 5-Fu [50, 61, 83]. Horie *et al.* also found that the aged garlic extract protected the small intestine of rats from antitumor drugs methotrexate (MTX) and 5-Fu induced damage [44]. Chokeberry extract powder, rich in 10% anthocyanins, has strong antioxidant activity and other bioactivities. In the

last study, we demonstrated that the chokeberry ARE had the potential to increase the sensitivity of colon cancer cells to 5-Fu *in vitro*. Matsumoto *et al.* [27] demonstrated that the red pigment in black chokeberry had the potential to protect the gastric mucosa of rats from alcohol damage. Valcheva-Kuzmanova and co-workers [94] found that pretreatment the rats with the black chokeberry fruit juice decreased the gastric mucosal damage induced by indomethacin. Therefore, the aim of this study was to determine whether pretreatment with dietary chokeberry ARE could protect rats from the acute adverse side effects of 5-Fu.

3.2 Material and methods

3.2.1 Chemicals

5-Fu, powder, purchased from Sigma Chemical Company (St. Louis, MO), was diluted in 1.5% polyethylene-glycol (PEG) solution into 25mg/mL and adjusted pH to 10.0 with sodium hydroxide. Sucrose purchased from Fisher Scientific Company (Fair Lawn, NJ) was dissolved in distilled deionized water to 1.0g/mL. Food grade ARE of chokeberry (*Aronia meloncarpa E.)* was supplied by Artemis International, Inc. (Fort Wayne, IN). AIN93 diet was purchased from Dyets Inc., PA.

3.2.2 5-Fu solution preparation

The solubility of 5-Fu in water is low. It is soluble in 1 N NH4OH (50 mg/mL), which yields a clear, colorless to light yellow solution. It is also soluble in DMSO (10 - 50 mg/mL). Polyethylene-glycol (PEG) has good water solubility and can increase solubility of 5-Fu in water. In preliminary study, we found that 1.5% PEG was high enough to improve the solubility of 5-Fu to 25 mg/mL.

3.2.3 Animal treatment and housing

Twenty male Sprague Dawley (175-200 g) 6 – 8 weeks age rats were obtained from Harlan (Rockville, MD). In the first three days of acclimatization rats were slowly weaned from pellet rat chow diet to powdered AIN-93 diet. Rats were randomly allocated to four groups of 5 animals each. The groups were (1) control diet + vehicle group: AIN93 diet ip injected with vehicle solution, (2) control diet + 5-Fu group: AIN93 diet injected with 200 mg/kg body weight 5-Fu, (3) ARE diet + vehicle group: AIN93 diet containing 5 g monomeric anthocyanin/kg injected with vehicle solution, or (4) ARE diet + 5-Fu group: AIN93 diet containing 5 g monmeric anthocyanin/kg injected with 200 mg/kg 5-Fu. The 5-Fu dose was determined by preliminary study. In the preliminary study, 400 mg/kg 5-Fu was ip injected to rats. The rats were very sick and had very serious diarrhea after 5-Fu treatment. Therefore, 200 mg/kg dose was used in this study.

The animals were housed two or three in plastic cages, with wire tops. Powdered diet was provided in standard feeding cups. Diet and tap water was available *ad libtum*. Artificial light was supplied from fluorescent tubes in a 12 hour light-dark cycle. Relative humidity was maintained at 25% - 60%. Health signs were monitored daily and clinical signs for all the animals were recorded weekly. Rats were fed their assigned diets totally 14 days. On day 11, Group 2 and 4 were ip injected with 5-Fu; group 1 and 3 were ip injected with 1.5% PEG, then all the rats were gavaged with 1.0 mL of a 1.0 g/mL sucrose solution one hour after injection. Urine was collected for 6 hours in the metabolic cages right after the gavage and sucrose content in the urine was assayed as a measure of intestinal permeability. All rats were euthanized with CO_2 three days after the injection. Tissues including heart, kidney, spleen,

stomach, the entire intestine, and one femur for bone marrow extract were removed for pathological analysis. Blood was taken from the tail right before euthanizing the rats for hematocrit tests. All animal experiments were conducted in accordance with U.S. Animal Welfare Act and U.S. Public Health Service Policy. The University of Maryland Institutional Animal Care and Use Committee (IACUC) approved experimental protocols.

3.2.4 Anthocyanin containing diet preparation

The anthocyanin concentration in diet was chosen based on the previous chempreventive animal study performed in our laboratory (Lala *et. al.*, in press). Fifty g chokeberry anthocyanin extract powder (containing 10% monomeric anthocyanin; monomeric anthocoyanin content was determined by method described above) was mixed completely with 950 g of an AIN93 powdered diet, which had been prepared to be missing 50 g of cornstarch by Dyets Inc. (Bethlehem, PA). Control diet was prepared by mixing 950 g AIN93 diet with 50 g cornstarch. The diets were stored at 4 °C until use.

3.2.5 Body weight

All animals were weighed at the time point of starting the diet treatment and once a week before 5-Fu injection. The rats were also weighed on the day of 5-Fu treatment and every day after 5-Fu treatment until the last day of the experiment.

3.2.6 Urine collection

Right after sucrose gavage, samples of urine were collected for 6 hours from each animal in metabolic cages (Mini Mitter Inc., Oregon). The total volume of the urine

of each animal was measured. All samples were stored at -80°C for sucrose content test.

3.2.7 Intestinal permeability test

Intestinal permeability was tested by urine sucrose content as described previously [8, 52]. The test was done with Sucrose Assay Kit from Sigma Chemical Company (St. Louis, MO). The urine sucrose content was expressed by the percentage of sucrose administered by mouth.

3.2.8 Histopathological analysis

Bone marrow, heart, spleen, liver, stomach and entire intestine were removed immediately and fixed in 10% neutral-buffer formaldehyde solution more than 24 hours. The entire intestine was cleaned with ice cold PBS then fixed. The fixed tissues were trimmed and imbedded in the paraffin, cut into sections and placed on microscope slides. The slides were stained with hematoxylin and eosin (H&E) (American histopathology lab) and examined under a light microscope by a pathologist (Dr. Sabine Franke-Carroll). The pathologist was blinded to the animal treatment groups until after evaluation was completed.

3.2.9 Hematological analysis

Right before the rats were euthanized, 20 μL blood per rat was collected into a capillary tube from the tails and stored at 4° C until analysis. The complete blood cells count (CBC) test was done by BioReliance Comprehensive Animal Health Services (Rockville MD) on the same day.

3.2.10 Pathological evaluation

Pathological damage was scored using descriptive scales. Zero represents within normal limits; one is minimal lesion; 2 is mild lesion, 3 is moderate lesion; 4 is severe lesion. The average score of each group was calculated.

3.2.11 Statistic analysis

For all parameters except pathological scores, the results were expressed as means±SE. Normality was tested using SAS software (Version 9.1, SAS Institute Inc, Cary, NC). Two-way ANOVA was conducted with SAS software to determine if there were significant differences between groups fed ARE diet and control diet, and groups of vehicle control and 5-Fu treated rats Pairwise comparisons adjusted with Tukeys were done once a significant difference was determined. $P<0.05$ was considered statistically significant difference.

3.3 Results

3.3.1 Clinical observation of rats with different diets

There was no difference between four groups before the 5-Fu treatment regarding body weight, activity, or food intake. All rats were very healthy and active. However, after 5-Fu treatment, the two groups receiving the 5-Fu treatment displayed diarrhea, but not the other two groups without 5-Fu treatment. Diarrhea is one of the side effects of 5- Fu as a result of chemotherapy-induced gastrointestinal toxicity and inflammation [95]. The feces of rats fed the ARE diet became purple instead of the normal brown color. There were no other clinical symptoms were observed. Therefore, 200 mg/kg 5-Fu ip injection was high enough to induce the side effects in the rats.

3.3.2 Effect of ARE and 5-Fu on the body weight of rats

Body weight is one of the indices of health status of human and experimental animals. Body weight loss is a clinical syndrome of damage induced by chemotherapy. Figure 10 shows the body weight changes of the four groups during the experiment. At the beginning of the experiment and the day of injection, there was no significant difference among the body weights of the four groups, but at the end of the experiment, there were significant body weight differences (table 2).

The average body weights of control diet + vehicle group and ARE diet + vehicle group increased an average of 91.2±3.62 and 87.0±5.15, respectively, during the whole experiment. There was no significant body weight difference between these two groups. In control diet + 5-Fu treatment group and ARE + 5-Fu group, the body weight of rats increased at the first 11 days at the same rate as the other 2 groups, but decreased dramatically after the 5-Fu treatment and this loss continued until the last day of the experiment. From the beginning to the end, the body weight changes of all groups are shown in Table 2. There was significant body weight difference between group without 5-Fu treatment and groups with 5-Fu treatment. The ARE diet + vehicle group had more weight gain than control diet + vehicle group after the injection. This suggested that the ARE diet may protect the rats from the damage of the vehicle injection, so this group gained more weight during these 3 days. There was no significant difference between ARE diet + 5-Fu group and control diet + 5-Fu group (P=0.1494). This suggests that the ARE diet could not prevent the body weight loss due to the toxicity of 5-Fu.
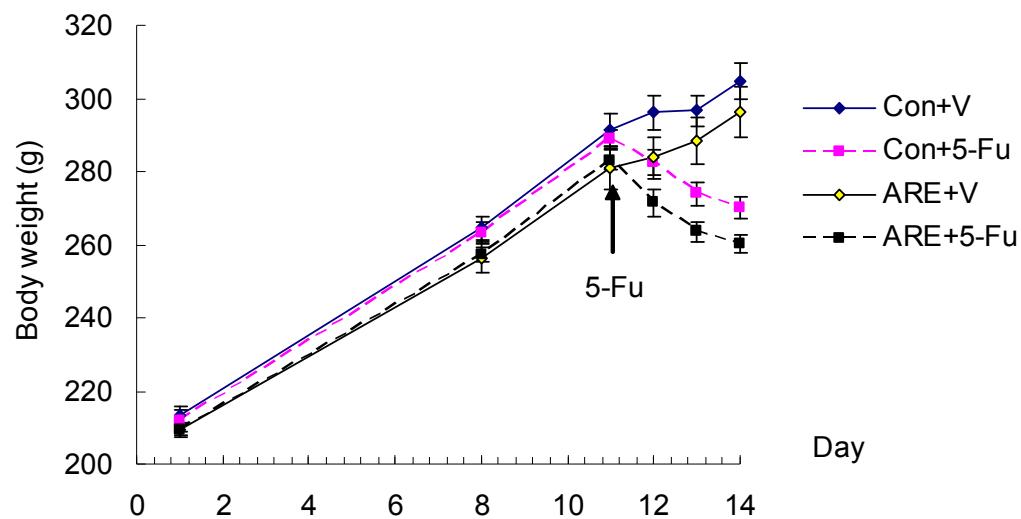
Figure 11 Effects of 5-Fu and ARE on the body weight of rats.

The body weight of rat was measured on day 1, day 8 and day 11 to day 14. On day

11, rats were ip injected with either 5-Fu or vehicle. Values are means ±SE.

Table 2 Weight change of rats during the experiment

Difference of body weight (g)	Control + Vehicle	Control + 5-Fu	ARE + Vehicle	ARE + 5-Fu
Day 14-Day 11	8.7±1.3a	-19.0±1.2b	15.4±1.6c	-23.0±3.1b
Day 14-Day 1	91.2±3.6a	58.0±1.5b	87.0±5.2a	50.6±3.2b
Day 11-Day 1	77.7 ±2.6a	77.1±1.5a	71.2±4.4a	73.5±2.8a

The data sharing the same letter do not have significant difference

3.3.3 Effect of ARE and 5-Fu on the organs

Single dose 200 mg/kg 5-Fu significantly reduced the weight of spleen and the ratio of spleen weight to body weight compared with control normal rats (Table 3). ARE diet had no effect on spleen weight. Pretreatment with ARE did not block the spleen weight reduction due to the toxicity of 5-Fu in the experiment period. Both ARE diet and 5-Fu had no damaging effect on liver and heart weights. There was also no visible effect on their morphological appearance (data not shown).

In order to test the degree of adverse side effects of 5-Fu, a pathological analysis of the bone marrow, kidney, stomach, duodenum, jejunum and ileum, was conducted. The cellularity of bone marrow of all rats without 5-Fu treatment was within normal limits. All rats treated with 5-Fu displayed severe to moderate damage to their bone marrow (Table 4). 5-Fu treatment also resulted in the dilation, necrosis of the GI tract and villi denudation of the small intestine (Table 4 and Figure 11). 5-Fu treatment was more damaging to the intestine than the stomach. However, ARE itself seemed to have resulted in some minor to moderate lesions in the stomach and small intestine (Table 4), but no severe damage. The small intestine (duodenum, jejunum and ileum) was more sensitive to the 5-Fu damage than large intestine (cecum and colon). The average lesions of 5-Fu to duodenum, jejunum and ileum regarding to crypt necrosis were 3.5, 3.7, and 3.5. The average lesions to cecum and colon were 2.3, and 1.7. 5-Fu also caused damage on kidney. The average lesion of acute toxic nephropathy was 3.8. ARE diet itself also had some damage on kidney. The average lesion of acute toxic nephropathy was 2.8. The damage was less severe than the 5-Fu.

Table 3 The ratios of organ weights to body weight

	Control + Vehicle	Control + 5-Fu	ARE + Vehicle	ARE + 5-Fu
Spleen	0.29±0.01	0.16±0.01**	0.27±0.01	0.16±0.01**
Liver	4.10±0.09	4.00±0.01	4.30±0.11	4.30±0.11
Heart	0.42±0.01	0.37±0.01	0.38±0.01	0.39±0.02

**P<0.0001 compared with control group

Table 4 Organ lesion pathology scores

Group	Organ	Normal (0)	Minimal (1)	Mild (2)	Moderate (3)	Severe (4)
Control+Vehicle	Bone marrow	7/7	0/7	0/7	0/7	0/7
	Kidney	7/7	0/6	0/6	0/6	0/6
	Stomach	6/6	0/6	0/6	0/6	0/6
	Duodenum	7/7	0/7	0/7	0/7	0/7
	Jejunum	7/7	0/7	0/7	0/7	0/7
	Ileum	7/7	0/7	0/7	0/7	0/7
	Cecum	7/7	0/7	0/7	0/7	0/7
	Colon	7/7	0/7	0/7	0/7	0/7
Control + 5-Fu	Bone marrow	0/6	0/6	0/6	1/6	5/6
	Kidney (Acute toxic nephropathy)	0/6	0/6	2/6	4/6	0/6
	Stomach	3/6	1/6	2/6	0/6	0/6
	Duodenum (Denuded villi)	0/6	0/6	0/6	1/6	5/6
	Duodenum (Crypt necrosis)	0/6	0/6	0/6	3/6	3/6
	Jejunum (Denuded villi)	0/6	0/6	2/6	0/6	4/6
	Jejunum (Crypt necrosis)	0/6	0/6	0/6	2/6	4/6
	Ileum (Denuded villi)	0/6	0/6	2/6	3/6	1/6
	Ileum (Crypt necrosis)	0/6	0/6	0/6	3/6	3/6
	Cecum (Crypt necrosis)	0/5	0/5	2/5	2/5	1/5
	Colon (Crypt necrosis)	0/6	3/6	2/6	1/6	0/6
ARE+Vehicle	Bone marrow	5/5	0/5	0/5	0/5	0/5
	Kidney (Acute toxic nephropathy)	0/5	0/5	1/5	4/5	0/5
	Stomach (Glandular dilation)	1/4	0/4	3/4	0/4	0/4
	Duodenum (Vacuolation crypt)	0/5	0/5	5/5	0/5	0/5
	Jejunum (Vacuolation crypt)	3/5	0/5	2/5	0/5	0/5
	Ileum (Vacillation villi)	3/5	0/5	0/5	2/5	0/5
	Cecum (Crypt necrosis)	5/5	0/5	0/5	0/5	0/5
	Colon (Crypt necrosis)	4/4	0/4	0/4	0/4	0/4
ARE +5-Fu	Bone marrow	0/5	0/5	0/5	0/5	5/5
	Kidney (Acute toxic nephropathy)	0/5	0/5	2/5	3/5	0/5
	Stomach (Glandular dilation)	2/5	1/5	1/5	1/5	0/5
	Duodenum (Denuded villi)	0/5	0/5	2/5	3/5	0/5
	Duodenum (Crypt necrosis)	0/5	0/5	4/5	1/5	0/5
	Jejunum (denuded villi)	0/5	0/5	2/5	2/5	0/5
	Jejunum (Crypt necrosis)	0/5	0/5	1/5	4/5	0/5
	Ileum (Denuded villi)	0/5	1/5	3/5	1/5	0/5
	Ileum (Crypt necrosis)	0/5	0/5	1/5	3/5	1/5
	Cecum (Crypt drop out)	0/5	1/5	1/5	3/5	0/5

The table header "Number of rats /total number evaluated" spans the five score columns.

Colon (Crypt drop out)	0/5	0/5	4/5	1/5	0/5

3.3.4 Myelotoxicity of 5-Fu

In order to test the effect of ARE on bone marrow suppression of 5-Fu, hematological analysis was done. As shown in Table 4, rats receiving an ip injection of 200mg/kg body weight 5-Fu had blood parameters that indicated that 5-Fu was high enough to inhibit the bone marrow function of rats. Rats treated with 5-Fu had significantly lower peripheral blood total leukocyte[2] counts including neutrophilia, lymphocyte, and monocyte counts. The number of platelets, or thrombocytes (functioning to stop the loss of blood from wounds) also was decreased compared with rats without 5-Fu treatment. Rats fed the ARE diet had no significant effect on the blood cell counts during the experiment comparing with control diet.

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[2] **Leukocytes, or white cells, are responsible for the defense of the organism. In the blood, they are much less numerous than red cells. The density of the leukocytes in the blood is 5000-7000 /mm^3. Leukocytes divide in two categories: granulocytes and lymphoid cells or agranulocytes. Granulocytes distinguish themselves in neutrophil, eosinophil and basophil. The main function of neutrophilia cells is phagcytosis of bacteria. The lymphoid cells, distinguish themselves in lymphocytes and monocytes. Lymphocytes defend against the attack of pathogenic microorganisms. Monocytes are (precursors of macrophage cells and are active in phagocytosis.**

Table 5 Myelotoxicity of 5-Fu in rats

Treatment	WBC (K/µL)	Neutrophils (K/µL)	Lymphocytes (K/µL)	Monocytes (K/µL)	Platelets (K/µL)
Control + Vehicle	20.1±1.2	9.2±0.7	9.3±0.6	1.1±0.1	743.1±39.2
Control + 5-Fu	9.1±0.3 *	4.0±0.8*	4.2±0.4*	0.6±0.1*	512.2±81.4*
ARE + Vehicle	20.4±2.0	8.6±0.9	10.2±0.7	0.9±0.1	700.6±46.4
ARE + 5-Fu	9.2±1.4 *	4.2±0.9*	4.0±0.4*	0.6±0.1*	523.6 ±89.2*

WBC, white blood count. *$P < 0.05$ compared with control group. K/µL =1000/µL

3.3.5 Intestinal permeability

By assaying the ratio (excretion amount/administration amount) of the sucrose marker in the urinary excretion, the damage of the intestinal epithelia was tested. The sucrose percentages in the urine of rats were 0.09%, 0.82%, 0.21% and 1.03% respectively in the control diet + vehicle, control diet + 5-Fu, ARE diet + vehicle, and ARE diet + 5-Fu groups. The sucrose percentage in the urine of rats treated with 5-Fu was significant higher than that of the rats without 5-Fu treatment, either control diet with 5-Fu or ARE diet with 5-Fu. Sucrose excretion in rats treated with control diet + 5-Fu was 9 times of the control + vehicle group.
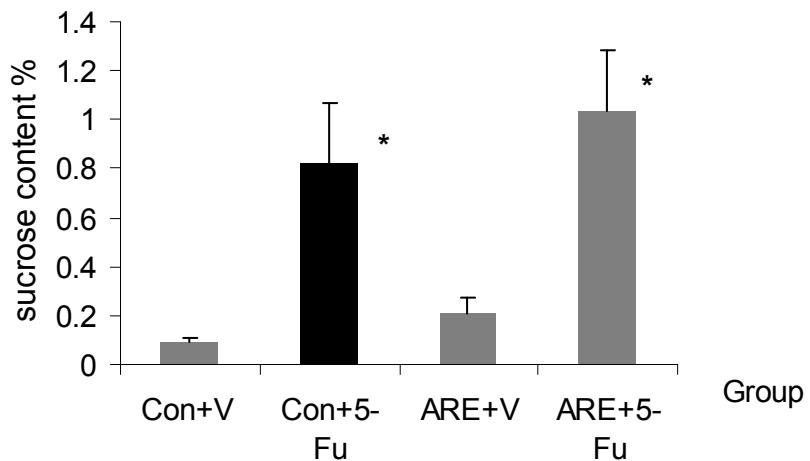
Figure 12 Sucrose content of the urine expressed as a percent of gavaged dose

One milliliter 1.0 mg/mL sucrose solution was gavaged to rats one hour after 5-Fu or vehicle treatment. Urine was collected for 6 hours right after the gavage in metabolic cages. Urine volume was determined and sucrose content was measured by sucrose testing kit according to the manufacture's instruction. The data represent the mean±SE. * significantly different from control+Vehicle group, P<0.001.

3.3.6 Reduction of gastrointestinal damage from 5-fu by ARE

The small intestine is one of targets damaged by 5-Fu. Rats treated with 5-Fu had evidence of damage in small intestine based on the histopathological analysis. The damages consisted of villi length reduction due to degeneration and necrosis of villi epithelium. Although the character of this change was similar in the control diet + 5-Fu group and the ARE diet + 5-Fu group, the severity of the lesions in the ileum appeared to be overall less in rats of the ARE diet + 5-Fu group (Figure 11). The averaged score of denuded villi lesion damage of ileum was 2.0 for ARE diet + 5-Fu group, and 2.8 for control diet + 5-Fu group according to the lesion evaluation of hispathological analysis (Table 5). Ileal tissues from the ARE diet + vehicle group had normal morphology, with average score of 0.0.

Duodenum was very sensitive to 5-Fu toxicity. The average duodenum score was 3.8 of villi denusion, and 3.5 of crypt necrosis (Table 5) in control diet +5-Fu group. The score of normal rats was 0.0. Rats fed ARE diet and treated with 5-Fu had reduced duodenal crypt epithelium damage as compared to rats fed the control diet and treated with 5-Fu (Figure 12). The average score was 2.6 of villi denusion, and 2.2 of crypt necrosis in ARE diet + 5-Fu group (Table 5). Duodenal tissues from ARE diet + vehicle only group had normal morphology with the average score of 0.0. The average jejunum crypt necrosis lesion score was 3.7 of control diet + 5-Fu group, and 2.8 of ARE diet + 5-Fu group.

a   b   c
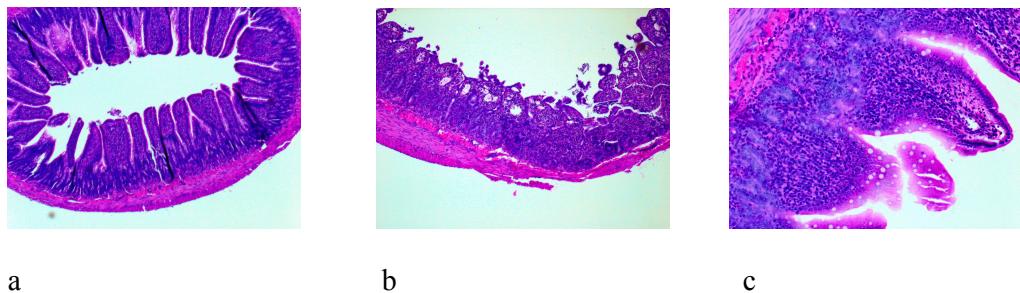
Figure 13 Photomicrographs of ileal sections of demonstrating differences in intestinal damage. Representative crosssections of ileum from various treatment groups (a) Control diet group with vehicle, 4 x magnification. (b) Control diet with 5-Fu treatment group, 4 x magnification. (c) ARE diet + 5-Fu treated group, 10 x magnification. Sections stained with H&E. According to the lesion evaluation, the score of picture a is 0, b 4, c 1-2.
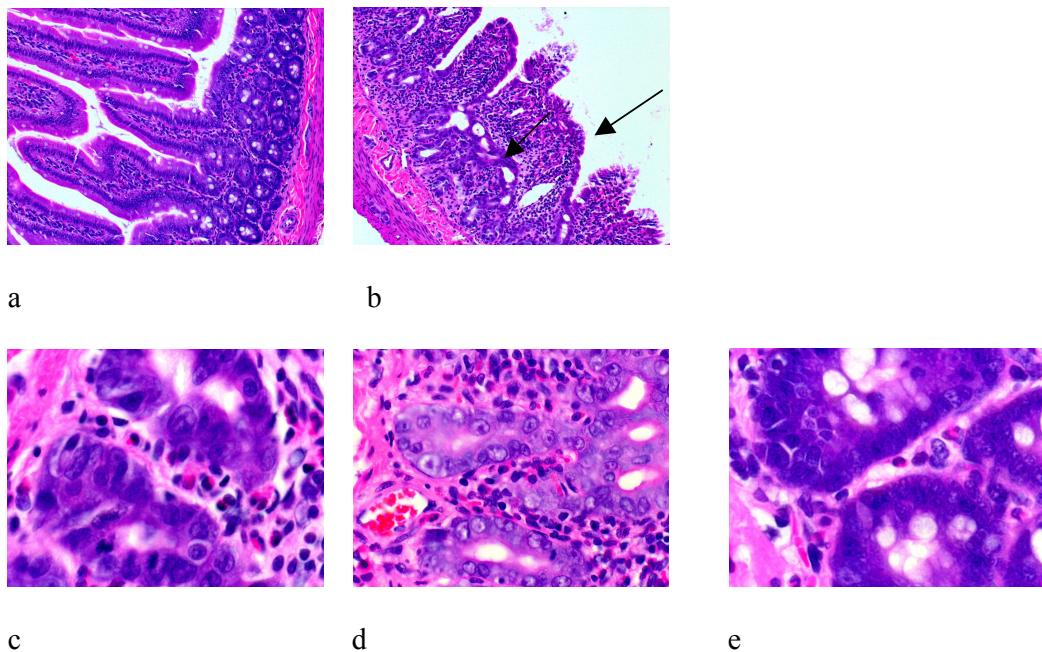
Figure 14 Photomicrographs of duodenum sections of demonstrating differences in intestinal damage. Representative crosssections of duodenum from various treatment groups (a) Villi of control diet group without 5-Fu, 10 x magnification. (b) Villi of control diet with 5-Fu treatment group, 10 x magnification. (c) Control diet with 5-Fu treated crypt epithelium, 60 x magnification. (d) ARE diet + 5-Fu treated crypt epithelium, 60 x magnification. (e) Normal control diet group without 5-Fu treatment crypt epithelium, 60 x magnification.

3.4 Discussion

Dietary modulation is one of the promised approaches to modulate the toxicity of cancer chemotherapy. Dietary folic acid, fiber, lipid, vitamins, fruit juice and some other extracts from edible plants such as soybean and garlic have been reported to influence the efficacy and toxicity of cancer chemotherapy [27, 44, 61, 62, 89, 90, 94, 96, 97]. Red pigments in black chokeberry mainly consisting of anthocyanins had a significant protective effect on gastric damage induced by ethanol [27]. ARE from chokeberry with 10% anthocyanins, may also have the similar bioactivity.

3.4.1 Effects of ARE on the normal rats

In this study, 5 g/kg ARE added to the diet had no visible effect on the clinical signs of rats during the experimental period. Two groups without 5-Fu treatment were very healthy and active. The only visible difference was that the color of the feces of ARE diet group was dyed to dark purple by anthocyanins. The tissues of ARE diet + vehicle group were overall similar to those of control diet + vehicle group. However, some changes were observed more frequently in the ARE diet + vehicle group than the control diet + vehicle group. These included dilation of gastric gland, renal interstitial edema, bone marrow vacuolation and vacuolation of the small intestinal crypt and /or villi epithelium. These changes may be some type of adaptation to the high levels of ARE. The significance of these morphological changes is unknown and need to be determined.

3.4.2 The myelotoxicity of 5-fu on rats

The dose of 200mg/kg body weight 5-Fu was high enough to induce the effects of myelosuppression, gastrointestinal toxicity and immunotoxicity in rats. Rats treated

with 5-Fu had minor to severe damage to kidney, spleen, bone marrow, and GI tract. Bone marrow is the most important organ for production of blood cells. 5-Fu inhibits the proliferation of rapid growing cells, including the normal bone marrow cells. As a result, it induces serious myelotoxicity, such as leucopenia (white blood reduction), peripheral thrombocytopenia (platelet reduction), and medullary erythrocytopenia (red blood cell reduction) [62]. In order to solve this problem, a variety of myelopoietic growth factors were used with 5-Fu clinically to recover or ameliorate myelopeiteic function. These factors include granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL) -3 and IL-6, IL-11 and stem cell factor [62, 70]. One study reported that the polysaccharide of black soybean promoted (PSBS) myelopoiesis and reconstituted bone marrow after 5-Fu and irradiation-induced myelosuppression [62]. Single dose 5-Fu 150 mg/kg was ip injected into ICR mice. Six hours later, 400 mg/kg body weight/day of PSBS was orally administrated for five days. The results showed that PSBS treatment could restore the leukocyte counts reduced by 5-Fu treatment and enhance CFU-GM colony formation of bone marrow cells. All these indicated that PSBS had myelopreventive activity and may be used to reconstruct the damaged bone marrow system by 5-Fu or other chemotherapeutic agents. In present study, the rats were ip injected a single dose 200 mg/kg 5-Fu. The total white blood cell count, lymphocyte, neutrophile, and monocyte counts were reduced by 5-Fu treatment. The total blood cell count was reduced significantly from 20.1 ± 1.20 to 9.1 ± 0.26. The platelet count was significantly decreased from 743.1 ± 39.23 to 512.2 ± 81.42. It also severely reduced the precursor and mature cell number in bone marrow showed in histopathological study. 5-Fu treatment also reduced the weight of spleen and the ratio of spleen weight to body

weight. However, pretreatment the rats with ARE diet did not prevent the inhibition of the bone marrow function induced by 5-Fu toxicity. One reason may be that the dose of 5-Fu was too high, inducing a high level of toxicity that cannot be prevented. In the current study, the Sprague Dawley rats were used and treated with 200 mg/kg body weight 5-Fu. In some other studies, different strains of rats (Fischer 344) and different doses of 5-Fu were used, with doses as high as 546 mg/kg body weight [97]. There may be a tolerance difference between different strains of rats, or there may be a difference in formulations as some studies used the injection liquid formulation for humans. In this study, 5-Fu powder was dissolved with 1.5 PEG. The PEG vehicle may also had some effect on 5-Fu. All these uncertain factors are needed to be further determined.

In this study period, ARE diet pretreatment did not prevent some damages of 5-Fu on rats. There are several possibilities. One may be that the dose of 5-Fu was too high and the toxicity was too strong and can not be recovered in such short time. Another possibility may be that the ARE diet percentage was not high or treatment time was not long enough to exert its function. The last one may be that some unknown factors. So, in future studies, lower 5-Fu doses should be investigated. Also, we killed the animals three days after the rats were exposed to 5 Fu, when the peak damage would be present. It may be possible that animals on the ARE diet treatment would recover faster, and/or show differences in myelotoxicity if the treatment time of anthocyanins was extended.

3.4.3 Intestine permeability increased by 5-Fu treatment

Intestinal permeability increase is a common clinical sign of side effects of chemotherapy because chemotherapy agents destroy the structure and integrity of the intestine. In the normal condition, sucrose is rapidly degraded in small intestine by sucrase and is therefore not excreted into urine. Healthy gastrointestinal mucosa is relatively impermeable to sucrose. The presence of orally administrated sucrose in the urine indicates injury of the small intestine and increased permeability of the small intestine [23]. We found that 5-Fu significantly increased the excretion of sucrose in urine more than 9 times compared with control diet group. However, pretreatment with ARE diets for 11 days did not prevent the sucrose permeability in rats treated with 5-Fu. This could be for the same reasons as for myelotoxicity.

3.4.4 Intestine damage from 5-Fu protected by ARE

The colonic mucosa is a highly organized dynamic structure with rapid cell turnover. Proliferation stem cells at the base of the crypt produce precursor cells that migrate up to the crypt and differentiate into functional cells [89]. Chemotherapy-induced intestinal injury is a major cause of morbidity and mortality during therapy for cancer. As a result, the oncologists have intensified researches into combinational chemotherapeutic regimens to improve long term survival and decrease the side effects [56]. 5-Fu is well documented to seriously damage the gastrointestinal tract, by inhibiting the proliferation of crypt cells and inducing apoptosis of the crypt cells. 5-Fu also results in the decreased intake of food and decreased intestinal absorptive capacity [61]. Furthermore, the patients develop anorexia and malnutrition. Recently, interest in diets moderating the toxicity of chemotherapy has increased greatly. Kimura *et al.* [90] reported that chitosan could prevent the myelotoxicity,

gastrointestinal toxicity and immunocompetent organic toxicity induced by 5-Fu without loss of antitumor activity in mice. The balance between intestinal epithelial cell proliferation and cell death maintains intestinal mucosal integrity. The ratio of villi height to crypt depth is one of parameter that can be use as a measure of the integrity of mucosa [96]. This ratio is altered only when the spontaneous or induced apoptosis occurs. When the tissues are under abnormal or damaged due to chemotherapy, the ratio will change and the shape of villi also will be affected [96]. In this study, the 5-Fu toxic change in the small intestine consisted of reduction of the apparent villi length due to degeneration and necrosis of the villi epithelium. The denuded duodenum villi and duodenum crypt necrosis scores of organ lesion by histopathological analysis of 5-Fu treatment increased to 3.8 and 3.5 from 0.0. By comparison, the average score was 2.6 of villi denusion, and 2.2 of crypt necrosis in the duodenum of ARE diet + 5-Fu group (Table 5). The average jejunum Crypt necrosis lesion score was 3.7 of control + 5-Fu only group, and 2.8 of ARE diet + 5-Fu group. The organ lesion evaluation (Table 4) and histological analysis (Figure 11 and Figure 12) results both showed that the ARE diet could prevent the damage of intestine by 5-Fu at some extent. It may be either because the damage of the 5-Fu was too serious to be totally recovered in such a short time, or because the dose of ARE and feeding time were not high and long enough. This result suggested that ARE can be a potential intestine protective reagent for chemotherapy drugs, but regarding to the mechanism and the appropriate dose of anthocyanins still need to be determined.

One of possible intestinal protection mechanisms of ARE could be prevention of apoptosis of intestinal epithelial cells by increasing antioxidant status [96]. Cellular lipid peroxidation and production of free radicals are the primary mediators of

apoptosis [96]. Anthocyanins are strong antioxidants. They have the potential to increase the antioxidative level of the body, scavenge reactive oxygen radicals, prevent the formation of lipid peroxides, and suppress membrane peroxidation, so they may have the potential to prevent the apoptosis of the intestinal epithelial cells.

Kimura *et al.* [90] suggested that the co-administration of 5-Fu plus orally administrated dietary chitosan 750 mg/kg twice a day to sarcoma 180 transplanting ICR mice lowered the 5-Fu level in blood in the first 30 min and also prolonged the transiting time of 5-Fu in the intestine. This could be one of reasons that chitoson prevented the side effects of 5-Fu such as myelotoxicity and gastrointestinal toxicity. Chitosan reduced the 5-Fu incorporation into RNA of small intestine and spleen but not into the tumor in the tumor bearing mice. Anthocyanins are charged molecules and have the capacity to form complexes with other compounds. Therefore anthocyanins may form a complex with 5-Fu, decrease the blood concentration of 5-Fu. This also could be one possible mechanism to prevent the absorption of 5-Fu in the small intestine and lower the toxicity. In this case, we are not sure if they will lower the effectiveness of 5-Fu because of the low concentration in blood. This should be further determined.

3.4.5 Antitumor activity of 5-Fu may be affected by ARE

There were studies that the chitosan could selectively inhibit the uptake of 5-Fu into small intestine and spleen tissue by adsorption to the cationic polymer chitosan, but without affecting the 5-Fu incorporation into tumor tissue [90]. In the current study, the absorption of 5-Fu and the blood levels of 5-Fu were not determined. It is not possible to know if the ARE would affect the absorption and metabolism of 5-Fu in

the body. It is possible that the ARE had the similar effect forming the complex with anthocyanins polymers, reducing the blood level of 5-Fu and releasing the 5-Fu later without affecting the antitumor effect of 5-Fu. It also was possible that ARE would change the metabolism pathways of 5-Fu, lower the blood level of 5-Fu and lower the antitumor effect of 5-Fu. In the present study, tumor model was not used. The physiological status of rats may change after the rats bearing tumor and it also would influence the uptake, metabolism of 5-Fu and the interaction of 5-Fu and ARE, so tumor model should be selected in future study and conduct the same experiment to confirm the interaction between 5-Fu and ARE.

3.4.6 Bioavailability of anthocyanins

McDougall *et al.* [85] used *in vitro* digestion system to investigate the digestion of the raspberry anthocyanins. They found that anthocyanins can survive gastric digestion, but only 5% can go into serum, and 70% of total anthocyanins can be recovered. Some of them are metabolized. The absorption of anthocyanins was influenced by other foodstuffs, but the effect was very little. We did not measure the diet intake of rats during the experiment. However, we monitored the body weight change and the condition of the feces of the animals. Feces of rats with ARE diets were purple colored after consuming ARE diets. Feces of rats with AIN93 diets were normal brown color. It showed at least some of anthocyanins were not metabolized inside the body and excreted by the rats. We know the absorption of the anthocyanins by humans and animals are very low. Some reports showed the bacteria in small intestine and pH increase lead to the degradation of anthocyanins. Therefore, there still is plenty of work to be done for the bioavailability of anthocyanins.

3.5 Summary

During the whole experiment period, rats were pretreated with 5% ARE diet for 11 days, and then ip injected 200 mg/kg 5-Fu and vehicle. ARE diet was continued to the last day of experiment. Three days after injection, rats were killed. Rats with AIN93 diet and ARE diet without 5-Fu treatment were active and had no visible damage. The rats treated with 200 mg/kg 5-Fu with AIN93 diet and ARE diet had some significant difference with rats without 5-Fu treatment. The body weight of rats decreased dramatically after 5-Fu treatment. Rats treated with 5-Fu also had lower white blood cell counts comparing with the rats without 5-Fu treatment. The intestine permeability of rats with 5-Fu treatment increased significantly induced by 5-Fu injection. The small intestine was damaged by 5-Fu expressed as villi denusion, crypt necrosis. Even though the rats treated with 5-Fu+ARE had similar intestinal damage, the overall damage was less severe showed from the histopathological analysis. One of most serious adverse effects of 5-Fu chemotherapy is intestinal toxicity. This study showed that the ARE diet could partially prevent the small intestine from the 5-Fu damage. It could be a potential agent to reduce the side effect of 5-Fu treatment. However, further tumor studies and longer treatment period are still needed.

Chapter 4: Conclusions

4.1 The sensitivity of colon cancer cell lines, HT29 and SW620, to 5-Fu was significantly increased in the presence of chokeberry ARE, when the monomeric anthocyanin concentration was 50 μg/mL. The addition of ARE simultaneously with 5-Fu increased the growth inhibition of IC50 5-Fu more than 20% for both cell lines when the concentration of ARE was 50 μg/mL. However, the same dose ARE had little or no effect on the growth inhibition induced by 5-Fu in normal colon cell line NCM460. This indicated that ARE may modulate the positive effect of 5-Fu on colon tumor cells but not increase the side effect on normal tissues. So ARE could be a potential sensitizer of 5-Fu chemotherapy with increasing therapeutic index, but with same level side effect.

4.2 Rats treated with 200 mg/kg 5-Fu lost body weight dramatically. The white blood cell counts and platelet counts were significantly reduced by 5-Fu myelotoxicity. The small intestine permeability was increased significantly by 5-Fu treatment compared to rats without 5-Fu treatment. The morphology of small intestine also was changed by 5-Fu treatment. The villi height and integrity of mucosa was damaged by 5-Fu treatment. Pretreatment of rats with ARE diet for 11 days did not prevent the reduction of white blood cell count and platelet counts or body weight lost. So further study with longer treatment is needed to fully determine the potential the function of ARE.

4.3 Even though the rats treated with ARE diet + 5-Fu had similar small intestinal damage as the rats with control diet + 5-Fu, 5 g/kg ARE diet partially protected the

small intestine from the damage induced by 5-Fu treatment, including protection of the villi denudation and crypt necrosis based on the histopathological analysis. Therefore, ARE diet could be a modulator to reduce the intestinal toxicity during the 5-Fu chemotherapy.

4.4 A tumor-bearing animal model should be used in future studies because of the potential differences in the physiological responses during normal and diseased conditions. Maybe the response of rats to 5-Fu will change in rats bearing tumors. The optimal ARE diet concentration and feeding time also need to be further determined.

In conclusion, this represents the first report of evidence of an anthocyanin-rich extract enhancing the cytotoxicity of 5-Fu to colon cancer cells *in vitro*, and reduction of 5-Fu-induced intestinal damage by in rats *in vivo*.

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