INHIBITION OF INTESTINAL TUMORIGENESIS IN APC^{MIN} MICE BY TART CHERRY ANTHOCYANINS AND SULINDAC

BY

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ABSTRACT

INHIBITION OF INTESTINAL TUMORIGENESIS IN APC^{MIN} MICE BY TART CHERRY ANTHOCYANINS AND SULINDAC

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Anthocyanins, a member of the flavonoid family of phytochemicals, previously have been found to inhibit the activities of cyclooxygenase (COX) enzymes. We hypothesized that tart cherry anthocyanins suppress intestinal tumorigenesis in Apc^{Min} mice because compounds that inhibit COX potentially are protective against colon cancer development. A series of experiments were conducted to assess the influence of anthocyanins and a nonsteroidal anti-inflammatory drug, sulindac, on intestinal tumor development in Apc^{Min} mice. First, we examined time frame of intestinal adenoma development in Apc^{Min} mice. The numbers and size of adenomas in small intestine increased with age, whereas tumor number in the cecum and colon did not increase significantly with age. The volume of colonic tumors increased significantly after 75 days of age. These results characterized the early and rapid development and progression of intestinal tumors in Apc^{Min} mice. We then conducted experiments to test the potential of anthocyanins to inhibit intestinal tumor development in Apc^{Min} mice and growth of human colon cancer cell lines. Mice consuming anthocyanins and cyanidin in drinking water and a 20% tart cherry diet had fewer and smaller tumors in the cecum, whereas mice consuming sulindac in drinking water had more cecal adenomas. The mean diameter of adenomas in the small intestine was smaller in mice consuming sulindac, and

larger in mice consuming the tart cherry diet, compared to mice consuming the control diet. Anthocyanins and cyanidin also reduced the growth of human colon cancer cell lines HT 29 and HCT 116. Based on these results, a second feeding study was conducted to determine effects of dietary anthocyanins and sulindac on 1) intestinal tumorigenesis in Apc^{Min} mice, 2) expression and cellular localization of β-catenin, and 3) expression of target genes of the Wnt/β-catenin signaling pathway and global gene expression profiles. APC^{Min} mice consuming dietary anthocyanins had significantly fewer adenomas in all three intestinal sections compared to mice consuming the control diet. Small intestinal adenomas in mice consuming sulindac were fewer in number and smaller in diameter compared to mice consuming anthocyanins, but mice consuming sulindac had more cecal adenomas compared to mice consuming anthocyanins. Sulindac did not influence the expression of β-catenin protein in intestinal epithelium, whereas anthocyanins increased β-catenin expression in the small intestine and cecum. Expression of mRNA of Apc, β-catenin and most of the target genes of Wnt/β-catenin signaling was not altered by treatment. cDNA microarray analysis identified several genes that were differentially expressed in response to treatment with anthocyanins or sulindac. Based on cellular expression of β-catenin and cDNA microarray analysis, we concluded that tumor inhibition in Apc^{Min} mice by anthocyanins is unlikely to involve COX inhibition or inhibition of the Wnt/β-catenin signaling pathway. Taken together, these results suggest that tart cherry anthocyanins and their aglycone, cyanidin, may reduce the risk of colon cancer by mechanisms that are unidentified at this time.

To my family

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CHAPTER I

INTRODUCTION

Colon cancer is the second most common cause of cancer mortality and the fourth most common in incidence in the United States (American Cancer Society, 2000). It has been estimated that dietary factors may account for 29 - 41% of cancers at all sites and that 66 - 75% of colon cancer is preventable by diet (WCRF/AICR, 1998). Results from epidemiological studies have shown that consuming fruits and vegetables is associated with reduced incidences of various cancers including colon cancer. The inverse association between cancer and fruit and vegetable intake may be due to number of components, including micronutrients and phytochemicals, contained in fruits and vegetables. Phytochemicals are defined as the naturally occurring, non-nutritive substances present in plant foods (Caragay, 1992). Studies have found that approximately 14 classes of dietary phytochemicals are present in common foods. These include sulfides, phytates, flavonoids, glucarates, carotenoids, coumarins, mono-terpenes, tri-terpenes, lignans, phenolic acids, indoles, isothiocyanates, phthalides, and polyacetylenes. Food sources that are rich in flavonoids are often found to be protective against colon cancer in animal models and humans.

Anthocyanins, members of the flavonoid family of phytochemicals, are widely distributed in plants including beans, fruits, and vegetables, suggesting that plant-based diets can provide considerable amounts of anthocyanins (Harborne and Grayer, 1988). Anthocyanins primarily occur in plants as glycosides. Cyanidin is the major anthocyanin aglycone in cherries. Montmorency and Balaton™ cherries contain 0.40 and 0.80 mg/g, respectively, of anthocyanins (Wang et al., 1999a).

These anthocyanins were later found to be antioxidants and inhibit cyclooxygenase (COX) enzymes (Wang et al., 1999b). Cyanidin was intermediate in efficacy for inhibition of COX-1 and COX-2 when compared with aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) such as flurbiprofen and ibuprofen. Compounds that inhibit COX potentially are protective against colon cancer development since it has been demonstrated that NSAIDs inhibit the growth of colon tumors in animal models and reduce the risk of colon cancer in humans (Giovannucci et al., 1995; Labayle et al., 1991). Since anthocyanins have COX-2 inhibiting activity, this suggests they may have anticarcinogenic effects in the intestine.

The Apc^{Min} mouse strain is a mutant mouse lineage predisposed to multiple intestinal neoplasia (Min). This phenotype is the consequence of a mutation in the murine homolog of the adenomatous polyposis coli (APC) gene (Su et al., 1992). The APC gene is also mutated in humans who develop sporadic colon cancer as well as persons with familial adenomatous polyposis (FAP), an autosomal dominantly inherited disease that predisposes to colorectal cancer. The primary phenotype of mice carrying this mutation is the development of multiple intestinal adenomas, which progress to adenocarcinomas of the intestine in older mice. Min is transmitted by affected mice to 50% of the progeny with an unbiased sex distribution, as is characteristic of a fully penetrant autosomal dominant trait (Moser, 1990).

Research on the effects of NSAIDs sulindac and piroxicam on tumor development in Apc^{Min} mice showed that they reduced the incidence of intestinal tumors (Boobol et al., 1996; Jacoby et al., 1996). Studies have shown that NSAIDs

possessing COX-2 inhibitory activity inhibited small intestinal tumorigenesis, but not necessarily colonic tumorigenesis, in Apc^{Min} mice (McEntee et al., 1999).

In most cases, colon carcinogenesis depends on mutation of the APC gene and this gene is considered a gatekeeper in the carcinogenic process (Kinzler and Vogelstein, 1996). A major function of the APC protein is to bind cellular β-catenin and facilitate its degradation (Rubinfeld et al., 1993). Most mutations in the human APC gene have been found to occur between codons 1286 and 1513, which is within a region encoding β-catenin binding sites (Nakamura, 1993). These mutations generally result in the production of a truncated APC protein that cannot facilitate the degradation of β-catenin, suggesting that mutation of APC is essential to the process of intestinal tumor development by causing a loss of regulation of β-catenin expression. β-catenin functions as a component of the E-cadherin adhesion complex and also as a transcriptional regulator in a signaling pathway by binding T cell factor (Tcf) transcription factor (Morin et al., 1997). Aberrant accumulation of β-catenin can influence trancriptional activation and expression of target genes in this signaling pathway. A few target genes in this pathway such as peroxisome proliferator activated receptor δ (PPARδ), c-MYC, cyclin D1 (CLND1) and matrilysin (matrix metalloproteinase 7) recently have been identified (He et al., 1999; He et al, 1998; Tetsu and McCormick, 1999; Crawford et al., 1999).

Cherries, anthocyanins and sulindac may influence tumorigenesis in the intestinal tract by modulating expression of genes involved in the carcinogenesis. The purpose of this research is to determine the effects of tart cherry anthocyanins

and the NSAID sulindac on the development of intestinal adenomas and on gene expression in intestine, cecum and colon of Apc^{Min} mice.

CHAPTER II

LITERATURE REVIEW

Colon Cancer

Colon cancer is the third leading cause of cancer mortality (after lung and stomach cancers) worldwide, with mortality estimated at 492,000 in 2001 (IARC, 2001). In the United States, colon cancer is the second most common cause of cancer mortality (after lung cancer) and is the fourth most common form of cancer in terms of incidence (after breast, prostate, and lung cancers) (American Cancer Society, 2000). Five-year survival following the diagnosis of colon cancer is approximately 55% in the United States and the rest of the developed world (Potter et al., 1993).

Numerous studies have characterized the pathogenesis of colorectal cancer as a multistage process that begins with hyperproliferation of colonic mucosal epithelial cells. The intestinal epithelium has a complex architecture and undergoes continuous turnover. Maintenance of crypt structures requires control mechanisms that balance the rates of cell proliferation and cell death. Failure of the control mechanisms results in disorganization of the tissue and abnormal tissue growth. Normally, epithelial proliferation takes place at the base of mucosal glands, called the crypts of Lieberkuhn (Wright and Irvin, 1982). Quiescent (G_0) cells then migrate to the superficial or luminal zone of the crypt, where they undergo apoptosis (programmed cell death) (Potten, 1996; 1997).

Cell kinetic studies of rectal mucosal biopsies determined that patients at increased risk of developing colorectal cancer have an expansion of the proliferative compartment and distribution of S-phase cells toward the lumenal zone of the crypt

(Terpstra et al., 1987). Hyperproliferation may contribute to mutations that lead to the formation of adenomas, the precursor lesions to colonic carcinoma. To prevent development of colon cancer, it has been suggested that the multistage carcinogenesis process must be delayed or pre-empted. This could be achieved by modifying the diet to include more cancer preventive foods, since it has been estimated that 66 - 75% of colon cancer is preventable by adequate diets (WCRF/AICR, 1998). Indeed, results from epidemiological studies have shown that consuming diets rich in fruits and vegetables is associated with reduced incidences of various cancers including colon cancer (Willet, 2001).

The Multi-Step Carcinogenic Process

Carcinogenesis is a multistep process. Moolgavkar and Knudson (1981) suggested two distinct stages, preneoplastic and malignant steps. Miller and Miller (1981) summarized the carcinogenisis process as follows: "Initiation" is the cellular change forming the first preneoplastic (initiated) cell in a tissue. The chemically induced initial step from a normal cell to a premalignant cell has been shown to be irreversible in mouse skin (Berenblum and Shubik, 1947). "Promotion" can be defined as the action of any compound that increases the tumor size by expanding the pool of initiated cells. Treatment with promoting substances shortens the long latency of tumor development and the action of these substances is reversible (Miller and Miller, 1981). The irreversible processes of visible tumor formation thereafter can be termed "progression". Progression can be considered the terminal stage of neoplastic development (Pitot, 1989).

Phytochemicals as Anti-Carcinogenic Factors

It has been estimated that dietary factors may account for 29 - 41% of cancers at all sites and that 66 - 75% of colon cancer is preventable by diet (WCRF/AICR, 1998). Several human cohort and case-control studies for a number of common cancer sites have shown that increased intakes of fruits and vegetables are associated with decreased cancer incidence and mortality rates (Doll, 1990; Ocke et al, 1997). The inverse association between cancer risk and fruit and vegetable intake may be due to a number of components, including micronutrients and phytochemicals, contained in fruits and vegetables.

Phytochemicals are defined as the naturally occurring non-nutritive substances present in plant foods (Caragay, 1992). Studies have found that approximately 14 classes of dietary phytochemicals are present in common foods. These include sulfides, phytates, flavonoids, glucarates, carotenoids, coumarins, mono-terpenes, tri-terpenes, lignans, phenolic acids, indoles, isothiocyanates, phthalides, and polyacetylenes. Some of these components may potentially block metabolic pathways leading to carcinogenesis and have been found to decrease cancer risk in experimental animals. The effect of the substances may depend on animal species, structure of the carcinogen, dose regimen, timing of dosages, and many other experimental parameters. The effective doses for some anticarcinogens may exceed what humans ingest from plant sources. On the other hand, the combination of several anticarcinogens at sub-effective levels has been shown to result in antitumor effects in some cases (Renner, 1990).

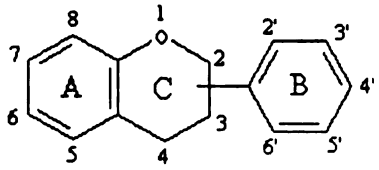
Food sources that are rich in flavonoids are often found to be protective against colon cancer. Approximately 4,000 different flavonoids have been identified in many plant families (Harborne, 1988). The flavonoid family includes a wide variety of chemical structures and are the largest group of plant phenolics (King and Young, 1999). The flavonoids (Fig. 1) are polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain. These low molecular weight compounds are usually glycosylated on one or more of their hydroxyl groups. Many flavonoids are easily recognized as flower pigments in most angiosperm families (flowering plants). However, their occurence is not restricted to flowers and in fact can be found in all parts of the plant. Major classes of flavonoids include flavanones, C-glycosylflavonoids, isoflavonoids, and anthocyanins (Fig. 1).

Anthocyanins are widely distributed in plants including beans, fruits, and vegetables (Harborne and Grayer, 1988). There are more than 240 known structures for anthocyanins based on 6 major aglycones-pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. Anthocyanins are universal plant colorants and largely responsible for colors of flower petals and fruits of higher plants (Harborne, 1988). Another function of anthocyanins includes light absorbance, thereby protecting the photosynthetic system from excess light (Timberlake, 1980). Cyanidin based anthocyanins have been found in a wide variety of berries and cherries as well as sweet potatoes, red onions and currants. Characterization of tart cherries (Wang et al., 1997) showed that the predominant anthocyanins in Balaton and Montmorency cherries were anthocyanin 1, anthocyanin 2 and anthocyanin 3 (Figure 2). Wang et al. (1999a) reported that 100 g of fresh and pitted cherries

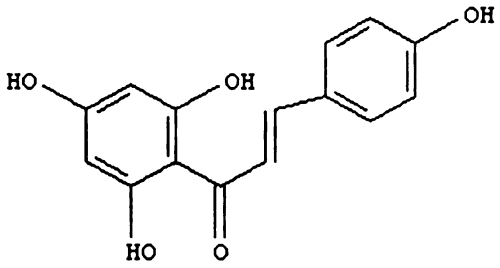
contain 12.5 - 25.0 mg of anthocyanins. Anthocyanins 1 – 3 and the parent aglycone, cyanidin, were found to possess antioxidant and anti-inflammatory activities (Wang et al., 1999b). The antioxidant activities of anthocyanins were superior to that of α-tocopherol and comparable to that of the commercial antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Cyanidin inhibited cyclooxygenase (COX) 1 and 2 enzymes with IC_{50} values of 90 and 60 μM, respectively (Wang et al., 1999b).

Cyanidin was found to be four times more powerful as an antioxidant than Trolox, a vitamin E analogue (Rice-Evans, 1995). Tsuda et al. (1996) reported inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments and cyanidin 3-O-beta-D-glucoside (C3G). Dietary cyanidin C3G also resulted in a significant decrease in generation of thiobarbituric acid reactive substances and showed a significantly lower susceptibility to lipid peroxidation in rats (Tsuda, 1988). Cyanidin has been found to function as a potent antioxidant in vivo in another recent study (Tsuda, 2000). C3G showed a potency to scavenge active oxygen species in rats fed a diet containing C3G (2 g/kg diet) for 14 days, and then subjected to hepatic ischemia-reperfusion (I/R) as an oxidative stress model (Tsuda, 2000). Ramirez-Tortosa et al. (2001) have shown that an anthocyanin-rich extract decreased lipid peroxidation and DNA damage in vitamin E-depleted rats. Seeram et al (2001) reported cyclooxygenase inhibitory and antioxidant effects of cyanidin glycosides from cherries and berries. The antioxidant activity of anthocyanins was comparable to commercial antioxidants and superior to

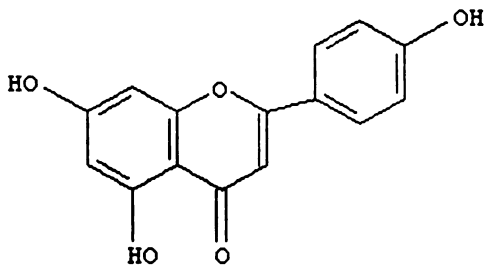
a) The skeleton structure of flavonoids



b) Chalcones



c) Flavones



d) Flavonols

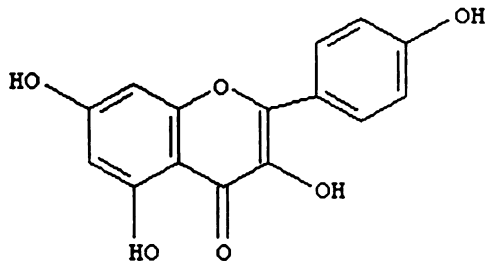
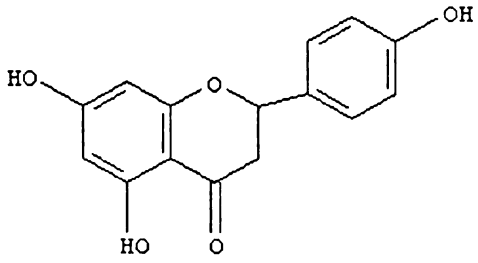
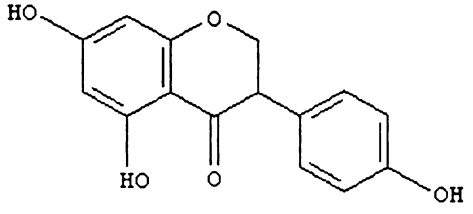


Figure 1. The chemical structures of flavonoids.

e) Flavanone



f) Isoflavones



g) Anthocyanins

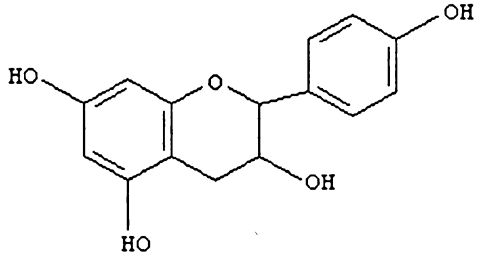


Figure 1 continued. The chemical structures of flavonoids.

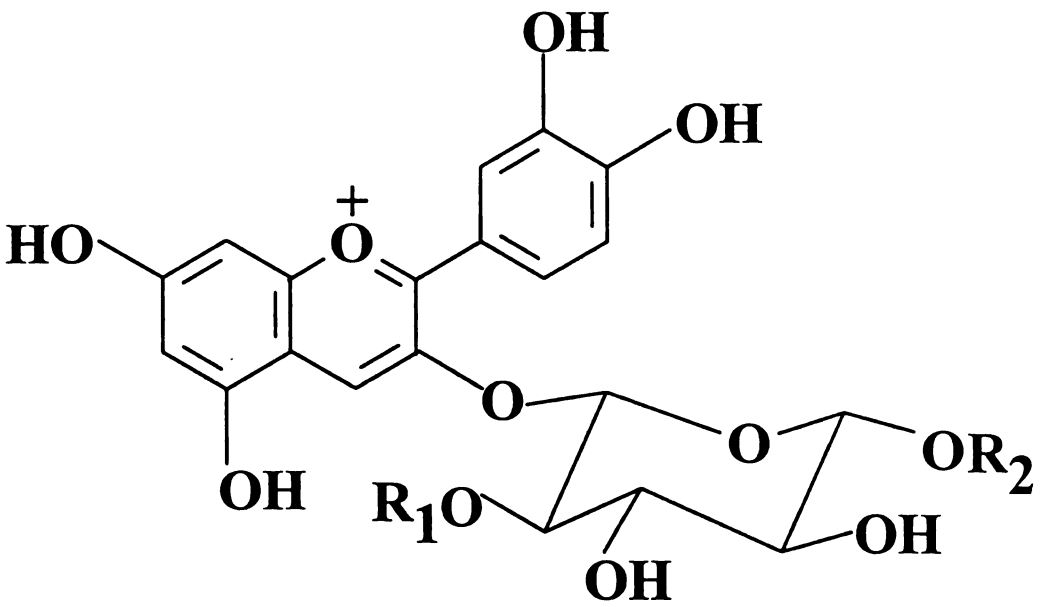


Figure 2. Anthocyanins from tart cherries

Anthocyanin 1. R_1=glucose, R_2= rhamnose,

[3-cyanin 2''-O-β-D-glucopyranosyl-6''-O-α-L-rhamnopyranosyl-β-D-glucopyranoside]

Anthocyanin 2. R_1=H, R_2= rhamnose,

[3-cyanin 6''-O-α-L-rhamnopyranosyl-β-D-glucopyranoside]

Anthocyanin 3. R_1=R_2=H,

[3-cyanin O-β-D-glucopyranoside]

vitamin E whereas the COX inhibitory activities were comparable to those of ibuprofen and naproxen at 10 μM concentrations (Seeram et al, 2001). In general, anthocyanins are stable in acidic conditions (below pH 7) and unstable in alkaline conditions. Anthocyanins can be hydrated at the C-2 position and degraded to chalcones in alkaline conditions of pH 7 or higher. A mechanistic study done by Tsuda et al. (1996) showed that C3G had strong antioxidative activity even at neutral pH. These results indicate that the flavylium cation form, which predominates at acidic pH, may not be essential for the antioxidative activity of anthocyanins. The study also showed that C3G reacted with azo-compounds to generate alkylperoxyl radicals. The reaction products were identified as 4,6-dihydroxy-2-*O*-*b*-*D*-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid (Tsuda et al., 1996). Thus, degradation of anthocyanin pigments could produce additional radical scavengers. A subsequent study on absorption and metabolism of C3G (Tsuda et al., 1999) showed that C3G was rapidly found in the plasma in rats ingesting 0.9 mmol/kg body weight of C3G by direct stomach intubation, indicating that the glycoside can be absorbed intact. Protocatechuic acid was found in the plasma at a concentration 8-fold higher than that of C3G, whereas cyanidin was not detected in plasma (Tsuda et al., 1999). Miyazawa et al. (1999) also found that oral supplementation of C3G and cyanine-3, 5-diglucode incorporated into plasma and liver of rats and human plasma by UV-HPLC.

Little research has been conducted for the effect of anthocyanins and cyanidin on colon carcinogenesis. Koide et al. (1996) first reported that feeding red rice prolonged survival of Balb/C mice inoculated i.p. with Meth/A lymphoma cells

and that hydrolyzed anthocyanins decreased the growth of HCT-15 cancer cells. Later, they found that sugar-bonded bioflavonoids extracted from red soybeans and red beans prolonged survival of Balb/C mice bearing tumor Meth/A cells and that anthocyanin fractions extracted from red soybeans inhibited the growth of HCT-15 cells (Koide et al., 1997). Pool-Zobol et al. (1999) have shown that anthocyanins reduced H_2O_2-induced DNA strand breaks but did not prevent endogenous generation of oxidized DNA bases in HT 29 clone 19A human colon cancer cells.

Recently, Meiers et al. (2001) investigated the influence of anthocyanins on the growth of human tumor cells. They reported that the aglycones of anthocyanins, cyanidin and delphinidin inhibited the growth of human vulva carcinoma cell line A431 and their IC_{50} values were 42 and 18 μM, respectively. Cyanidin (0.8 ± 0.2 μM) and delphinidin (1.3 ± 0.2 μM) were potent inhibitors of the tyrosine kinase activity of the epithermal growth-factor receptor (EGFR). Treatment with an irreversible inhibitor of the EGFR-kinase, EKI-569, in combination with sulindac showed remarkable protection from tumor development (95% reduction in polyp number) in Apc^{Min} mice (Torrance et al., 2000). Recently, it has been reported that purple corn anthocyanins suppressed the promotion of colon tumors caused by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats initiated with dimethyl hydrazine. However, these researchers did not test the potential of purple corn color anthocyanins to directly suppress tumor development (Hagiwara et al., 2001).

Apc^{Min} Mouse Model

The Apc^{Min} (mutiple intestinal neoplasia) mouse has been proposed to be a useful model for the study of human colorectal cancer. Moser et al. (1990) reported a mutation in the mouse genome that resulted in the development of multiple intestinal tumors in all carriers of the mutation. The mutation was identified in a pedigree established during a mutagenesis project in which C57BL/6J (B6) males were treated with ethylnitrosourea and then mated to AKR/J (AKR) females. The primary phenotype of Apc^{Min} mice is the development of multiple adenomas that progress to adenocarcinomas of the intestine in older mice. Min is transmitted by affected mice to 50% of progeny with an unbiased sex distribution, as is characteristic of a fully penetrant autosomal dominant trait (Moser, 1990). The mutation is currently maintained by crossing Min/+ males with B6 females. Tumors usually are present throughout both the small and large intestine and the cecum, but are not present in the stomach.

Apc^{Min} mice are heterozygous for a nonsense mutation (Apc^{Min}) at the Apc locus, the murine homologue of the adenomatous polyposis coli (APC) gene (Su et al., 1992). Two candidate tumor suppressor genes, APC and MCC (mutated colon cancer) on human chromosome 5q21 frequently are mutated in sporadic colorectal cancers (Kinzler et al., 1991). The APC gene is also mutated in humans with familial adenomaous polyposis (FAP), an autosomal dominantly inherited disease that predisposes to colorectal cancer. The frequency of somatic APC mutations is the same in adenomas and carcinomas, suggesting that mutation of APC is an early

event in sporadic colorectal cancer (Levy et al., 1994). Using a site-specific quantitative polymerase chain reaction assay, Luongo et al. (1994) found extensive loss of the remaining wild-type Apc (Apc^{+}) allele at the Apc locus in spontaneously-occurring intestinal adenomas from mice heterozygous for the Apc^{Min} nonsense mutation.

Examination of human FAP kindreds demonstrates that family members who inherit the same APC mutation may differ dramatically in tumor burden (Leppert et al., 1990). Although environmental factors may be partially responsible, modifier genes have been proposed to account for some of this variability (Paul et al., 1993; Giardiello et al., 1994). Further study of Min-induced neoplasia by manipulation of the mouse germline identified genes modifying the Min phenotype: Mom1(modifier of Min 1), genes for the 5-cytosine DNA methyltransferase, and DNA mismatch repair factor Msh 2 (Bilger et al., 1996). Quantitative trait loci mapping identified the Mom 1 locus in the distal region of mouse chromosome 4 (McPhee et al., 1995). Mom 1 also resides in a region of synteny with human chromosome 1p35, a region frequently associated with loss of heterozygosity in neuroblastoma and colon cancer (Martinsson et al., 1989; Leister et al., 1990). The gene for secretory type II phospholipase A2 (sPLA2) maps to the same region that contains Mom 1 (McPhee et al., 1995). The sPLA2 are extracellular enzymes that, upon activation, cleave acyl ester bonds in the sn-2 position in glycerophospholipid. These enzymes play a crucial role in the generation of arachidonates and eicosanoids, proinflammatory mediators.

Therefore, the Min mutation exerts a decisive effect in a pathway leading to intestinal tumor formation. However, the fact that all cells of a Apc^{Min} mouse carry this predisposing germline alteration, but only a limited number of tumors develop, indicates that somatic events are also necessary for tumor formation. Min-dependent tumorigenesis also occurs with less frequency in mammary glands, the pancreas, and the body wall (Bilger et al., 1996). Mouse models such as the Min mutant should provide a major resource for studying the anticarcinogenic potential of dietary factors, pathways of tumorigenesis, and the molecular identification of genes that can control particular neoplastic processes.

There has been great interest in studying the effects of dietary or other interventions on colon carcinogenesis. However, most research has been conducted using animal models of carcinogenesis with tumors initiated by injecting large doses of carcinogens and using different experimental conditions (i.e. different carcinogens, injection doses, etc). This makes it difficult to compare results from studies conducted in different laboratories. The Apc^{Min} mouse has been used as a model to study effects of dietary factors on human colorectal cancer.

Several studies have been conducted to study the potential of diet to inhibit the formation of intestinal tumors in Apc^{Min} mice. Bowman-Birk protease inhibitor concentrate (0.5%) from soybeans reduced colonic tumors and small intestinal tumors in Min mice by approximately 40% (Kennedy et al., 1996). Paulsen et al (1997) showed that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-enriched fish oil suppressed tumor number and burdon in Apc^{Min} mice. Rye-bran diet also has been shown to prevent the formation of intestinal polyps in Apc^{Min} mice

(Mutanen et al., 2000). Mahmoud et al. (2000) reported that plant phenolics, including caffeic acid phenethyl ester and curcumin, decreased intestinal tumor formation by 63% in Apc^{Min} mice. Green tea extract and sulindac acted synergistically to inhibit development of intestinal tumors in Apc^{Min} mice (Suganuma et al., 2001). Treatment with both green tea extract and sulindac significantly reduced the number of tumors from 72 to 32 tumors whereas green tea extract or sulindac alone reduced tumor number to 57 or 49, respectively (Suganuma et al, 2001).

Results from studies using Apc^{Min} mice have not always supported the findings of epidemiological studies and studies with carcinogen-injected animal models. Some dietary factors do not influence tumor development when administered to Apc^{Min} mice. Pierre et al. (1997) reported that addition of short chain fructooligosaccharides to the diet reduced colonic tumor incidence but had no effect on small intestinal tumors in Apc^{Min} mice. Van Kranen et al. (1998) found no effect of dietary fat or a fruit and vegetable mixture on tumor formation in Apc^{Min} mice. Sorenson et al. (1998) reported that soy isoflavones had no effect on tumor formation in Apc^{Min} mice.

Nonsteroidal Anti-Infammatory Drugs (NSAIDs) and Colorectal Cancer

Several lines of investigation have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the growth of colon tumors in animal models and reduce the risk of colon cancer in humans. Giovannucci et al. (1995) found that four to six tablets of aspirin per week sustained over years substantially reduced the

risk of colorectal cancer. Other NSAIDs such as sulindac and ibuprofen have been shown to reduce the relative risk of colorectal cancer after 2 or more years of continuous use (Muscat et al., 1994).

The mechanism of the anticarcinogenic effect of these drugs has not been fully established. One potential explanation for this protective effect is inhibition of intestinal epithelial growth. It has been demonstrated that NSAIDs, including indomethacin and sulindac, inhibit the rate of growth of carcinogen-induced colon tumors in animal models (Olsson et al., 1984; Skinner et al., 1991). Levi et al. (1990) showed that indomethacin inhibits the gastric epithelial cell hyperproliferation that normally restores mucosal integrity after injury in rodent models. Studies of sulindac in FAP patients provide further evidence that NSAIDs inhibit epithelial cell proliferation in the gastrointestinal tract. Regression of colorectal polyps was observed after 6 months of treatment with oral sulindac (Labayle et al., 1991; Rigau et al.; 1991). Polyps returned after treatment was discontinued. Reductions of mucosal prostaglandin E_2 levels paralleled polyp regression (Labayle et al., 1991).

Most hypotheses for explaining the anticarcinogenic effects of NSAIDs have focused on the common property of NSAIDs to inhibit COX enzymes and thereby cause a subsequent reduction in levels of prostaglandins in tissue. Inducible COX-2 is overexpressed in human colon cancers. Sulindac inhibited tumor formation and reduced overexpression of COX-2 level in Min mice (Boolbol et al., 1996). Jacoby et al. (1996) demonstrated that dietary piroxicam significantly reduced intestinal tumor multiplicity in Min mice to 12% of that observed in control animals. Plasma

thromboxane B_2 levels were reduced in parallel with tumor inhibition (Jacoby et al., 1996). Ritland and Gendler (1999) confirmed chemopreventive effects of proxicam on intestinal adenomas in Apc^{Min} mice. Short-term administration of proxicam (200 ppm) reduced tumor multiplicity by 90%. Treatment of Apc^{Min} mice with the NSAID nabumetone resulted in a dose dependent suppression of intestinal tumorigenesis (Roy et al., 2001). Nabumetone induced apoptosis by downregulating the anti-apoptotic protein, Bcl-2, in the intestine of Apc^{Min} mice.

McCracken et al. (1996) investigated the effect of COX-inhibiting (S-form) and non-COX-inhibiting (R-form) enantiomers of flurbiprofen on rat colonocyte proliferation. Sprague Dawley rats were treated orally with R- or S-flurbiprofen or vehicle. Both R and S forms significantly reduced colonocyte labeling index, by 34% and 23%, respectively, compared with vehicle. R-flurbiprofen caused minimal ulcer formation (4.48 mm^2) compared with S-flurbiprofen (94.4 mm^2). These findings suggest that R-flurbiprofen-mediated control of colonocyte proliferation is independent of prostaglandin biosynthesis. Shiff et al. (1995) showed a possible alternate mechanism by which NSAIDs inhibit gastrointestinal cell proliferation. They demonstrated that both sulindac and sulindac sulfide caused cell cycle quiescence and induced apoptosis by inhibition of the p33 cdk protein, a key regulator of the cell cycle. Sulindac was effective despite its relative weak COX-inhibitory activity compared to sulindac sulfide. Notably, disruption of the mouse sPLA2 locus has been shown to increase tumor number and restoring sPLA2 expression through transgenic constructs decreases tumor number in Apc^{Min} mice,

despite the prediction that sPLA2 should decrease prostaglandin production (Kennedy et al., 1995; Cormier et al., 1997).

The clinical and experimental evidence that NSAIDs are anticarcinogenic is strong, and several studies have suggested COX-independent mechanisms for this action. The exact relationship of prostaglandin synthase inhibition by NSAIDs to tumor prevention remains uncertain.

Genetics of Intestinal Neoplasia: APC Mutation and its consequences

Different forms of human colon cancer share mutations in APC and may also share other genetic features (Fearon and Vogelstein, 1990). Certain mutations are common in colon cancer, but the sequence of events may vary. Temporal series of somatic changes including mutation of APC, K-RAS, DCC and P53 and hypomethylation of DNA have been demonstrated by molecular analysis of human intestinal cancer (Fig. 3). This observation shows that a particular molecular change is correlated with a particular stage in the carcinogenesis. It generates the hypothesis that certain gene mutations are necessary for particular carcinogenic steps in colon carcinogenesis.

In most cases, colon carcinogenesis depends on mutation of the APC gene and it is considered a gatekeeper in the carcinogenic process (Kinzler and Vogelstein, 1996). APC encodes a cytoplasmic polypeptide of 2843 residues (Fig. 4). The N-terminus contains oligomerization sites indicating that the molecule acts as a polymer (Su et al., 1993a). The central part of the molecule contains sites for 20 amino acid β-catenin binding repeats and also contains heptad repeats and armadillo (Arm) repeats

(Su et al., 1993b). The heptad repeats lead to supercoiling of helix and arm repeats composed of three alpha helices mediate protein-protein interactions. The truncations in the midpoint cluster region, which eliminate all the axin-binding and most of the β-catenin-binding repeats, account for more than 60% of oncogenic mutations in APC (Miyoshi et al., 1992). The C terminal region contains tubulin binding sites (Munemitsu et al., 1994; Smith et al., 1994). Tubulin is the protein that polymerizes into long chains or filaments that form microtubules, hollow fibers which serve as a skeletal system for processes such as cell division and the movement of materials within cells. The region also contains binding sites for the human homologue of the Dlg (Drosophila large discs) tumor supressor protein.

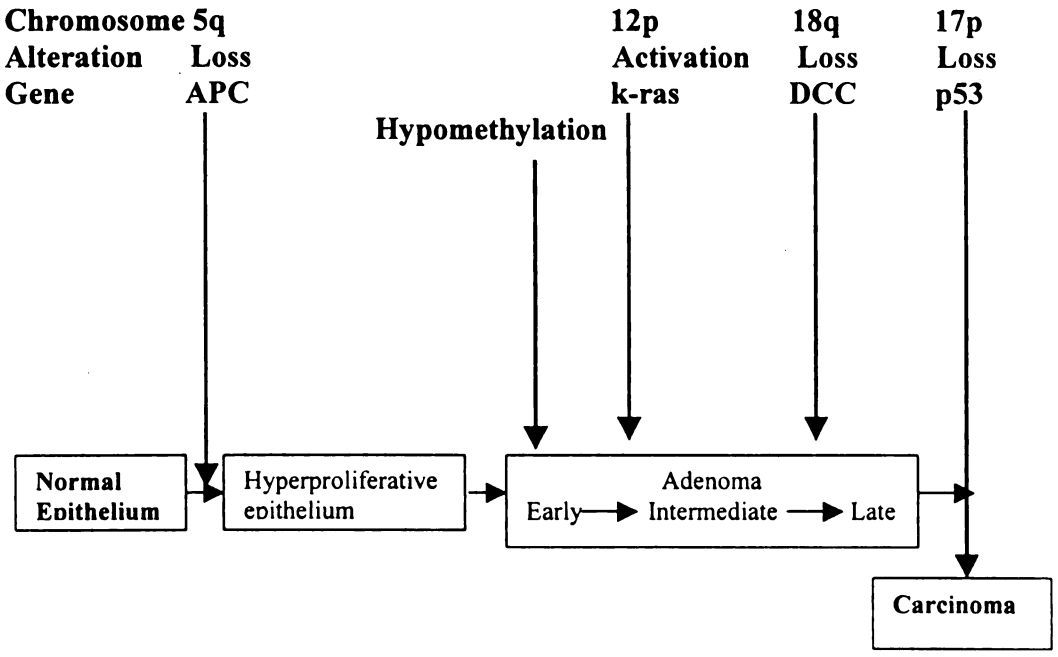


Figure 3. A genetic model for colorectal tumorigenesis (modified from Vogelstein and Kinzler, 1993)

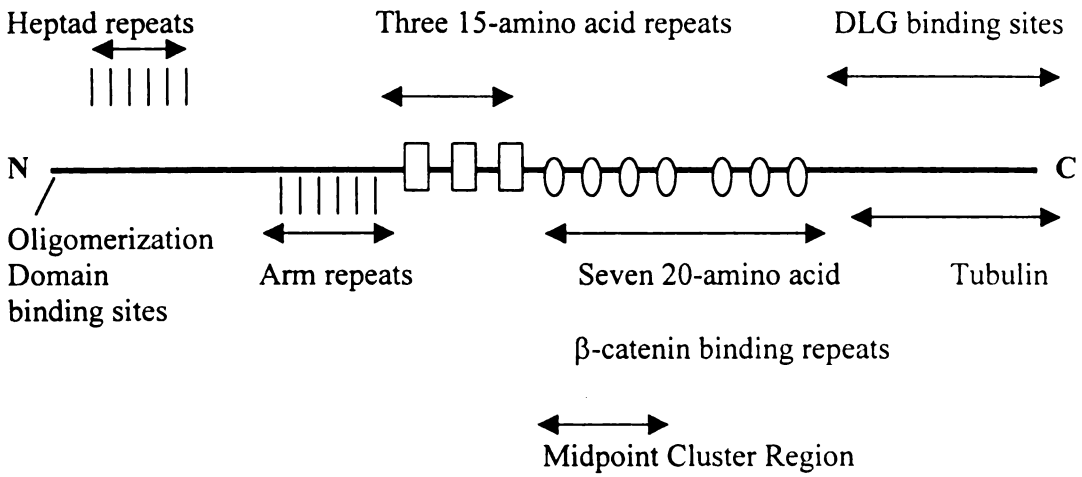


Figure 4. Schematic of the APC protein primary structure (modified from Spink et al., 2001)

It has been shown that intestinal adenomas in Apc^{Min} mice and human colon tumor cell lines have lost the wild type allele or express only short N-terminal fragments of the Apc polypeptide (Luongo et al., 1994). The Min allele contains a nonsense codon at position 850 (Moser et al., 1990) and other targeted mutant Apc alleles have been developed that result in Apc polypeptide truncation of codons 716, 1638 and 474 by Oshima et al. (1995), Fodde et al. (1994), and Sasai et al. (2000), respectively. Apc^{716} mice are predisposed to develop 200 - 500 adenomas mainly in the small intestine, whereas Apc^{1638} are predisposed to develop 5 - 6 adenomas and adenocarcinomas along the intestinal tract and a number of extra-intestinal lesions including mammary tumors (Oshima et al., 1995; Fodde et al., 1994). Apc^{474} mice develop around 100 intestinal polyps and 18% of them manifested mammary tumors (Sasai et al., 2000).

The APC cytoplasmic polypeptide plays a regulatory part in the cell and participates in several protein assemblies. Most human APC mutations are found between codons 1286 and 1513 and this midpoint mutation cluster region occurs near the β-catenin binding sites (Nakamura, 1993). Mutations result in the production of a truncated protein which cannot facilitate the degradation of β-catenin, suggesting that mutated APC is essential in the process of tumor development by causing a loss of regulation of β-catenin.

β-catenin performs different functions within the cell. Cytoplasmic and nuclear β-catenin accumulate only in response to activation of the Wnt/Wingless growth factor signaling pathway whereas a membrane associated pool is bound to E-

cadherins and α-catenin in the adherins junction (Yap et al., 1997). E-cadherin is one of a large family of genes which code for calcium-dependent cell adhesion molecules (Takeichi et al., 1988). Any free β-catenin not bound to E-cadherens is targeted for degradation in the absence of a Wnt signaling. The APC gene product is required in the β-catenin degradation process as an essential part of a mutiprotein complex. A complex of glycogen synthase kinase (GSK)-3β and conductin/axin phosphorylates the APC/β-catenin complex, which induces ubiquitination and degradation of β-catenin by proteosomes (Easwaran et al., 1999). The ubiquitination and degradation of β-catenin is a highly regulated process and requires a multi-molecular complex with APC, conductin/axin, and GSK-3β.

APC mutation allows increased levels of cytoplasmic β-catenin, which may then trigger a cascade of events resulting in the initiation of adenomas. The cytoplasmic β-catenin is translocated to the nucleus and functions as a transcription factor in intracellular signaling. β-catenin binds to T cell factor(Tcf)/lymphoid enhancer factor (Lef) transcription factors in the nucleus (Ilyas and Tomlinson, 1997). Thus, APC mutation causes aberrant accumulation of β-catenin, which then translocates to the nucleus and binds Tcf/Lef , causing increased transcriptional activation of some genes. These events ultimately result in abnormal cell growth since normal APC function is essential for maintaining normal cell architecture and growth.

Genes that might be targets for transcriptional activation by β-catenin/Tcf transactivation have been identified. Crawford et al. (1999) suggested that matrilysin

is a target gene for β-catenin/Tcf transactivation. Matrilysin is a matrix metalloproteinase 7 (MMP 7) expressed in approximately 90% of human colonic adenocarcinomas and in many human colonic tumor cell lines (Newell et al., 1994). Cyclin D1 (CLND1) has also been identified as a target gene of β-catenin transactivation (Tetsu and McCormick, 1999). CLND1 is a regulator of the cell cycle and is highly expressed in adenomatous polyps and colorectal adenocarcinomas (Zhang et al., 1997). Overexpression of CLND 1 may derange the cell cycle machinery and contribute inappropriate neoplastic cell growth (Hartwell and Kastan, 1994). He et al. (1998) identified c-MYC as a target gene in the signaling pathway of APC mutation. C-MYC expression mediated through Tcf-4 binding sites in the promoter region is repressed by APC and activated by β-catenin. He et al. (1999) also reported that peroxisome proliferator activated receptor δ (PPARδ) is a target of the APC tumor suppressor pathway and is an APC-regulated target of NSAID drugs. They found that APC repressed PPARδ expression and that PPARδ activity was regulated by the APC/β-catenin/Tcf-4 pathway when APC was introduced in HT-29 cell lines. Interestingly, it has been suggested that PPARs may be a target of NSAIDs in suppressing tumorigenesis since NSAIDs can perturb eicosanoid metabolism and mediators involved in eicosanoid metabolism are known ligands for PPARs (Prescott and White, 1996). Sulindac sulfide resulted in a dose-dependent repression of PPARδ activity in HCT116 and SW480 cell lines by disrupting the DNA binding ability of PPARδ/RXRα (retinoid X receptor α) heterodimers (He et al., 1999). Recently, the role of PPARδ in colorectal

tumorigenesis was evaluated by Park et al. (2001). They created PPARδ null cell lines (PPARδ -/-) by deleting the PPARδ gene in a human colon cancer cell line using targeted homologous recombination. When inoculated as xenografts in nude mice, PPARδ -/- exhibited a decreased ability to form tumors compared with PPARδ +/- and wild-type controls, suggesting that suppression of PPARδ expression contributes to the growth-inhibitory effects of the APC tumor suppressor (Park et al., 2001).

Recent immunohistochemical analysis of β-catenin showed that β-catenin staining was mainly localized to the cell membrane in normal epithelial cells of stomach, duodenum, appendix, and rectum, whereas β-catenin accumulated in the cytoplasm and/or nucleus in 57 cases (79.2 %) out of 72 cases of human gastrointestinal carcinoid tumors (Fujimori et al., 2001). McEntee et al. (1999) reported that sulindac regressed small intestinal adenomas and reduced β-catenin expression in adenomas of Apc^{Min} mice. Mutanen et al. (2000) also reported that rye bran diet prevented tumor formation in Apc^{Min} by decreasing the cytosolic β-catenin level. Sheng et al. (1998) found nuclear localization of β-catenin in hereditary and carcinogen-induced intestinal adenomas. β-catenin was expressed in the cell membrane and cytoplasm of morphologically normal intestinal cells whereas it was found in the nucleus of adenomas from Apc^{Min} mice and Apc^{716} mice. Cellular distribution of β-catenin was also modified by sphingolipid consumption (Schmelz et al., 2001). Sphingolipid feeding reduced adenoma numbers in all regions of intestine in Apc^{Min} mice and sphingosine reduced cytosolic and nuclear β-catenin and

inhibited growth of human colon cancer cell lines SW 480 and T84 (Schmelz et al, 2001). He et al (1999) showed that sulindac sulfide repressed PPARδ activity in HCT116 and SW480 cell lines in a dose-dependent manner.

A proposed model for APC- and NSAID-mediated suppression of intestinal tumorigenesis (Fig. 5) shows several features of NSAID-mediated chemopreventive action. First, numerous studies have shown that NSAIDs inhibit COX activity and subsequently decrease levels of prostaglandins. However, apoptosis-inducing activities of NSAIDs are not entirely related to the inhibition of COX, suggesting additional cellular targets. Second, NSAIDs inhibit PPARδ activity (He et al., 1999). The pro-apoptotic effect of NSAIDs may be explained by direct inhibition of PPARδ. Third, NSAID-induced regression of intestinal adenomas is associated with reduced levels of β-catenin (McEntee et al., 1999). McEntee et al. (1999) observed a 50% decrease in β-catenin as well as Bcl-2 in small intestinal tumors from Apc^{Min} mice treated with sulindac. Rosin-Arbesfeld et al. (2000) identified a nuclear export function of the APC gene. They showed that APC contains highly conserved nuclear export signals 3' and adjacent to the mutation cluster region that enable it to export β-catenin from the nucleus. When this ability is lost in APC mutant cancer, the result is accumulation of β-catenin in the nucleus.

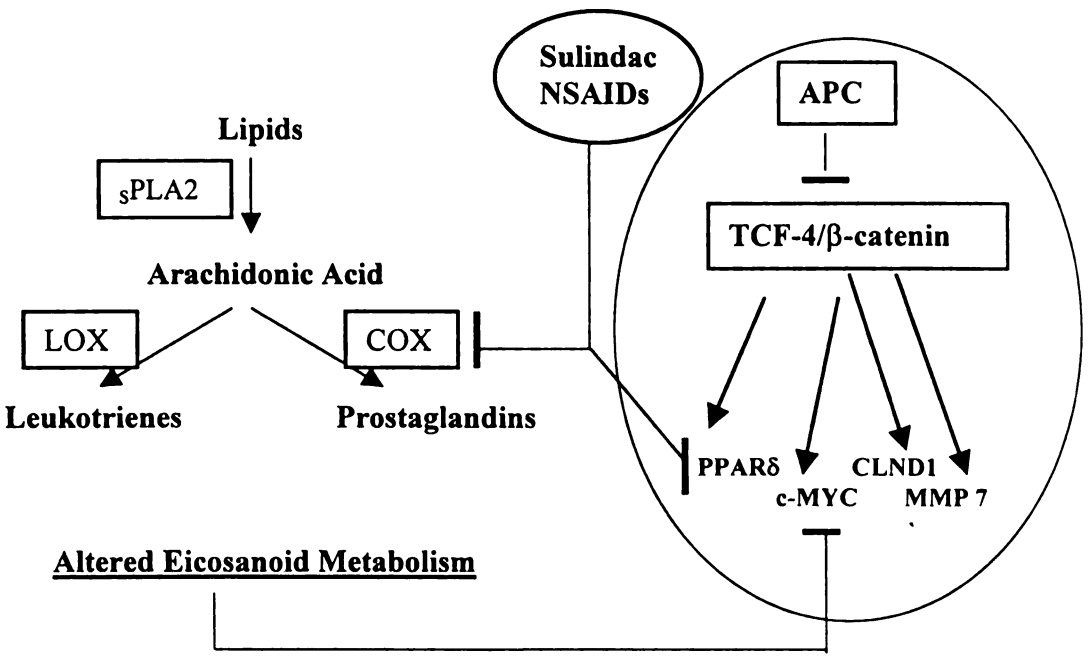


Figure 5. APC-and NSAID-mediated suppression of intestinal tumorigenesis

(Modified from He et al., 1999)

Hypotheses and Research Objectives

Tart cherry anthocyanins may suppress intestinal tumorigenesis because they inhibit the activities of COX enzymes. However, the potential of the anthocyanins and cyanidin to inhibit colon cancer development has not been fully established. Few studies have examined the chemopreventive potential of anthocyanins and cyanidin. Cyanidin inhibited epidermal growth factor receptor (EGFR) kinase activity in cancer cells (Meiers, 2001). Treatment with an irreversible inhibitor of the EGFR-kinase reduced tumor development in Apc^{Min} mice (Torrance et al., 2000). Hagiwara et al. (2001) demonstrated that anthocyanins in purple corn color reduced the promotion of colon tumors caused by PhIP in rats initiated with dimethylhydrazine. However, they did not test the potential of purple corn color anthocyanins to directly suppress tumor development (Hagiwara et al, 2001).

The overall objective of the study is to determine the effects of tart cherry anthocyanins on the development of intestinal adenomas and on global gene expression in intestine, cecum and colon of Apc^{Min} mice.

The hypotheses to be tested and specific aims are:

1. Tart cherry anthocyanins inhibit intestinal tumorigenesis in Apc^{Min} mice.

Specific aims:

a. Examine ontogeny of intestinal adenoma development in mouse model (Apc^{Min} mouse) to obtain baseline data on tumor development.
b. Examine effects of tart cherry anthocyanins, their aglycone, cyanidin, and their degradation products on growth of human colon cancer cell lines HT 29 and HCT 116.
c. Examine effects of tart cherry diets, anthocyanins and their aglycone, cyanidin on multiplicity and size of intestinal adenomas in Apc^{Min} mice.

2. The inhibiton of intestinal tumorigenesis caused by tart cherry anthocyanins is related to changes in gene and protein expression in Apc^{Min} mice.

Specific aims:

a. Examine the expression and cellular distribution of β-catenin in intestinal sections of Apc^{Min} mice fed tart cherry anthocyanins in comparison to Apc^{Min} mice fed control or sulindac-supplemented diets.
b. Examine the expression of cancer-related genes in intestinal epithelial cells of Apc^{Min} mice fed tart cherry anthocyanins in comparison to Apc^{Min} mice fed control or sulindac-supplemented diets. I will specifically determine the expression of the predicted target genes of the Wnt/β-catenin signaling pathway such as PPARδ, C-Myc, CLND1 and MMP7.

The Apc^{Min} mouse strain is an excellent model for studying colon cancer development and the anticarcinogenic potential of dietary factors. The time frame of tumor development in Apc^{Min} mice will influence experimental protocols. First, we will examine the timing of small intestinal and colonic tumor development in Apc^{Min} mice and determine the influence of sex on small intestinal and colonic adenoma multiplicity and size in Apc^{Min} mice. We will then determine the effects of tart cherry anthocyanins, cyanidin and sulindac on intestinal adenoma multiplicity and size in the small intestine, cecum and colon in Apc^{Min} mice. The ability of tart cherry anthocyanins to directly inhibit the growth of human colon cancer cell lines HT 29 and HCT 116 also will be determined.

Expression of thousands of genes can be examined simultaneously using a novel technique called cDNA microarray analysis. cDNA microarrays are capable of profiling gene expression patterns of tens of thousands of genes in a single experiment (Duggan et al., 1999). Microarrays consist of a collection of immobilized genes which can be simultaneously analyzed to ascertain their expression in tissue.

DNA targets, in the form of 3' expressed sequence tags (ESTs), are arrayed onto glass slides and probed with fluorescent-labeled cDNAs. Premade microarrays of gene-specific cDNA fragments immobilized on glass slides for the mouse will be used to analyze 15,000 genes that survey major biological pathways (Tanaka et al., 2000).

Screening changes in expression of such target genes as well as oncogenes and tumor suppressor genes in the different sections of intestine may yield insights about the mechanisms responsible for tumor modulation by anthocyanins and sulindac. We speculate anthocyanins may inhibit expression of PPARδ, c-MYC, CLND1 or matrilysin and influence β-catenin expression and distribution in the cell membrane, cytoplasm and nucleus in the intestinal cells.

CHAPTER III

ONTOGENY OF INTESTINAL ADENOMA DEVELOPMENT IN APC^{MIN} MICE.

ABSTRACT

Apc^{Min} mice (C57BL/6J $Apc^{min/+}$) develop small intestinal and colonic adenomas spontaneously. This experiment was conducted to determine the influence of age on the numbers and sizes of small intestinal, cecal and colonic tumors in Apc^{Min} mice. Apc^{Min} mice were weaned at 28 days of age and fed Teklad 8640 rodent diet throughout the experiment. Mice were sacrificed at 30, 45, 60, 75, 90 and 120 days of age. The entire small intestine, cecum, and colon were removed and the small intestine was separated into proximal, middle and distal segments. Intestinal sections were fixed in 10% neutral buffered formalin and stained with 0.2% methylene blue. Intestinal tumors were counted and measured. Body weights and the lengths of the small intestine and the colon increased with age. The numbers and sizes of adenomas in the small intestine also increased with age ($p<0.05$). The numbers of adenomas in the cecum and colon did not increase significantly with age, but the volume of cecal tumors was not influenced by age. Colonic tumors increased significantly after 75 days of age. Sex did not influence the numbers of small intestinal or colonic tumors. Mouse body weights, colon length, and colon tumor volume were influenced by sex. Male Apc^{Min} mice had greater body weights, colon lengths and colon tumor volumes than female Apc^{Min} mice. Tumors were already present in 30-day-old mice. These results indicate that intestinal tumors develop early in life and progress in size with increasing age in Apc^{Min} mice. Experiments examining the effects of dietary or other interventions in Apc^{Min} mice should take into account their early and rapid tumor development.

INTRODUCTION

Colon cancer is the second most common cause of cancer mortality and the fourth most common in incidence in the United States (American Cancer Society, 2000). It has been estimated that 66-75% of colon cancer could be preventable by proper diets (WICR/AICR, 1999). Hence, there has been great interest in studying the effects of dietary or other interventions on colon carcinogenesis. Most research on colon cancer prevention by diet has been conducted using animal models of carcinogenesis. Most of these studies rely on tumors initiated by injecting large doses of carcinogens and often have used different experimental conditions (i.e. different carcinogens, injection doses, etc). This makes it difficult to compare results from studies conducted in different laboratories.

The Apc^{Min} mouse has been proposed to be a model for the study of human colorectal cancer (Moser et al., 1990). Apc^{Min} mice are a mutant mouse lineage predisposed to multiple intestinal neoplasia (Min) resulting from a mutation in the murine homolog of the adenomatous polyposis coli (APC) gene (Su et al., 1992). The APC gene also is frequently mutated in humans who develop sporadic colon cancer and is the mutation responsible for causing familial adenomatous polyposis (FAP), an autosomal dominantly inherited disease that predisposes persons to colorectal cancer. The primary phenotype of Apc^{Min} mice is the development of multiple adenomas which progress to adenocarcinomas of the intestine in older mice. Min is transmitted by affected mice to 50% of progeny with an unbiased sex distribution, as is characteristic of a fully penetrant autosomal dominant trait (Moser et al., 1990).

The Min mouse strain may be an excellent model for studying colon cancer development and the anticarcinogenic potential of dietary factors. However, the time frame of tumor development in Apc^{Min} needs to be more thoroughly investigated to allow optimization of experimental protocols. Therefore, the purpose of this study was to examine the timing of small intestinal and colonic tumor development in Apc^{Min} mice and to determine the influence of sex on small intestinal and colonic adenoma multiplicity and size in Apc^{Min} mice.

MATERIALS AND METHODS

Animals and Diets. All research was conducted with approval of the MSU All University Committee on Animal Use and Care. Mice were housed in MSU University Laboratory Animal Resources maintained facilities. A colony of Apc^{Min} mice was maintained by crossing carrier (C57BL/6J $Apc^{Min/+}$) male mice with normal adult female C57BL/6J mice. Mice were housed in a temperature and humidity-controlled room (20-22 °C, 50%) with a 12-h light/dark cycle. Mice were weaned at 28 days of age and fed Teklad 8640 rodent diet throughout the experiment. Mice were not genotyped before being randomly assigned to study groups (ages). We anticipated that 50% of the mice allotted to each group would carry the Apc^{Min} mutation and 50% would be normal with respect to Apc gene status. Body weights were measured when mice were sacrificed at 30, 45, 60, 75, 90 and 120 days of age.

Tumor Number and Size. Upon sacrifice, a section of liver was removed and frozen immediately for Apc genotyping using a PCR-based procedure (Su et al., 1992) followed by gel electrophoresis. The intestine was removed and tissues were separated into the following sections: proximal one-third of small intestine, middle one-third of small intestine, distal one-third of small intestine, cecum, and colon. All intestinal sections were opened longitudinally, rinsed thoroughly with water, fixed in neutral-buffered formalin, and then stained with 0.2% methylene blue. Tumor numbers and sizes were determined in each intestinal segment by direct counting with the aid of a dissecting microscope. Because small intestinal tumors are typically sessile and cecal and colonic tumors are typically polypoid, the tumor sizes were determined by measuring the mean diameter of flat tumors in the small

intestine and by measuring volume of three-dimensional tumors in the cecum and colon. Spheric volumes were calculated for cecal and colonic tumors using the formula V = 0.523(width x length x height). Tumor numbers in each small intestinal segment were summed to obtain a total small intestinal tumor burden for each mouse.

Body weights, tissue lengths, tumor numbers and tumor diameters in the small intestine were analyzed by two-way analysis of variance to detect the effects and potential interactions of age and sex. For tumor numbers and volumes in the cecum and colon, data were transformed to ranks and then analyzed by two-way analysis of variance (ANOVA). When significant treatment effects were detected ($P<0.05$), means were compared using the Least Significant Difference (LSD) method.

RESULTS

Body weights of mice were influenced by age, sex, and Apc status (Min vs. normal). Body weights of all mice increased with increasing age ($p<0.05$) (Fig. 6a). Male mice weighed approximately 3 grams heavier than female mice for ages 45 through 120 days. Body weights of Apc^{Min} mice (Fig. 6b) averaged about 1 gram less than those of normal $Apc^{+/+}$ mice at all ages. Small intestine and colon length increased with age ($p<0.05$) (Table 1). Apc^{Min} mice had small intestine lengths about 9 mm less than normal $Apc^{+/+}$ mice. Small intestine length was not influenced by sex. However, colons were approximately 4 mm longer in males than females. Carrier status did not influence colon length.

Intestinal tumors were only detected in Apc^{Min} mice. Tumor numbers (Fig. 7) in the small intestine of Apc^{Min} mice also increased with age ($p<0.05$). Mice averaged 13.6 small intestinal tumors at 30 days of age. Small intestinal tumor numbers increased steadily to a maximum of 70-80 tumors per mouse by 90 days of age. As mice aged, the numbers of adenomas in the distal small intestine increased to a greater magnitude compared to those in the proximal and middle small intestine. The proportion of small intestinal adenomas in proximal, middle, and distal segments was 28, 34, and 38%, respectively, in 30-d old mice and 10, 34, and 56%, respectively, in 120-d old mice. The average diameter of the small intestinal adenomas (Fig. 8) was relatively constant from 30-75 days of age, averaging 0.94 mm per tumor. This diameter increased significantly in 90-d old mice (1.2 mm) and increased dramatically in 120-d old mice to 1.8 mm per tumor. There was no sex effect on small intestinal tumor numbers or diameters.

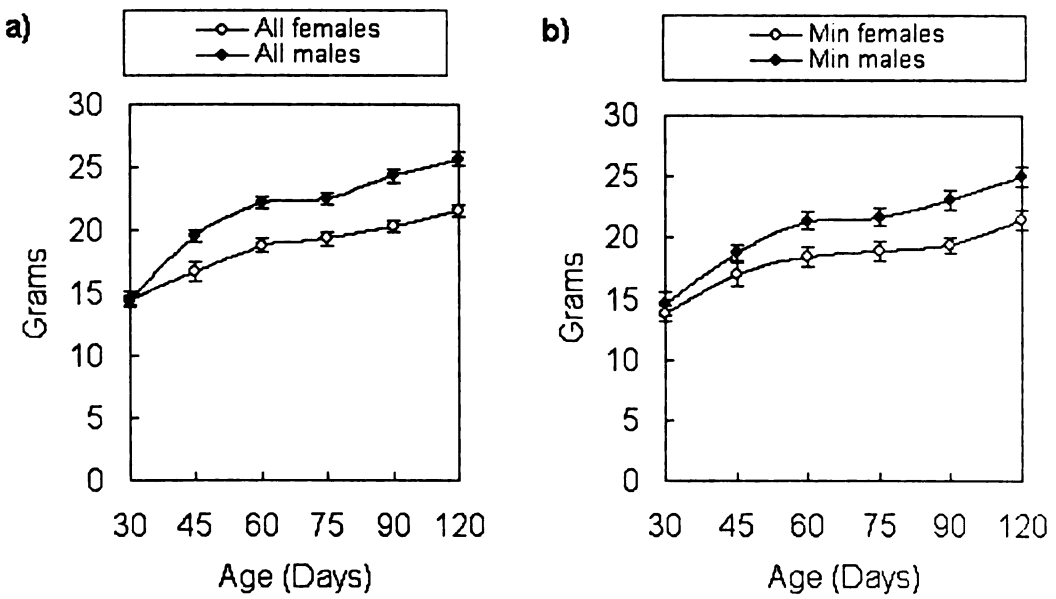


Figure 6. Influence of age on body weights **a)** Influence of age on body weights of all mice. Each point represents the mean of the age groups (30 days; n=22, 45 days; n=17; 60 days; n=17; 75 days; n=22; 90 days, n=23,120 days; n=23). Body weight increased with age at all time points except between 60 and 75 days. Error bars indicate SEM. Males were significantly larger ($p<0.05$) than females after 30 days of age. **b)** Influence of age on body weights of Apc^{Min} mice. Each point represents the mean of the age groups (30 days; n=8, 45 days; n=10; 60 days; n=10; 75 days; n=9; 90 days, n=9,120 days; n=8). Error bars indicate SEM. Body weights increased with age at all time points except between 60 and 75 days. Body weights were significantly different between males and females after 30 days of age.

Table 1. Lengths of small intestine and colon of all mice (30 days; n=22, 45 days; n=17, 60 days; n=17, 75 days; n=22, 90 days; n=23,120 days; n=23) (Normal; n=54, Apc^{Min}; n=70) (Female; n=56, male; n=68). Each value represents mean ± SEM. Means in the same column within a category not sharing a common superscript are significantly different ($p<0.05$).

Age (days)	Small intestine (mm)	Colon (mm)
30	308 ± 4^{a}	68 ± 2^{a}
45	319 ± 5^{a}	76 ± 2^{b}
60	339 ± 4^{b}	79 ± 2^{bc}
75	349 ± 4^{b}	80 ± 2^{bc}
90	349 ± 4^{b}	78 ± 2^{b}
120	378 ± 4^{c}	84 ± 2^{c}

Apc Status	Small intestine (mm)	Colon (mm)
Normal	345 ± 2^{b}	78 ± 1
Min	336 ± 2^{a}	78 ± 1

Sex	Small intestine (mm)	Colon (mm)
Female	338 ± 2	75 ± 1^{a}
Male	343 ± 2	80 ± 1^{b}

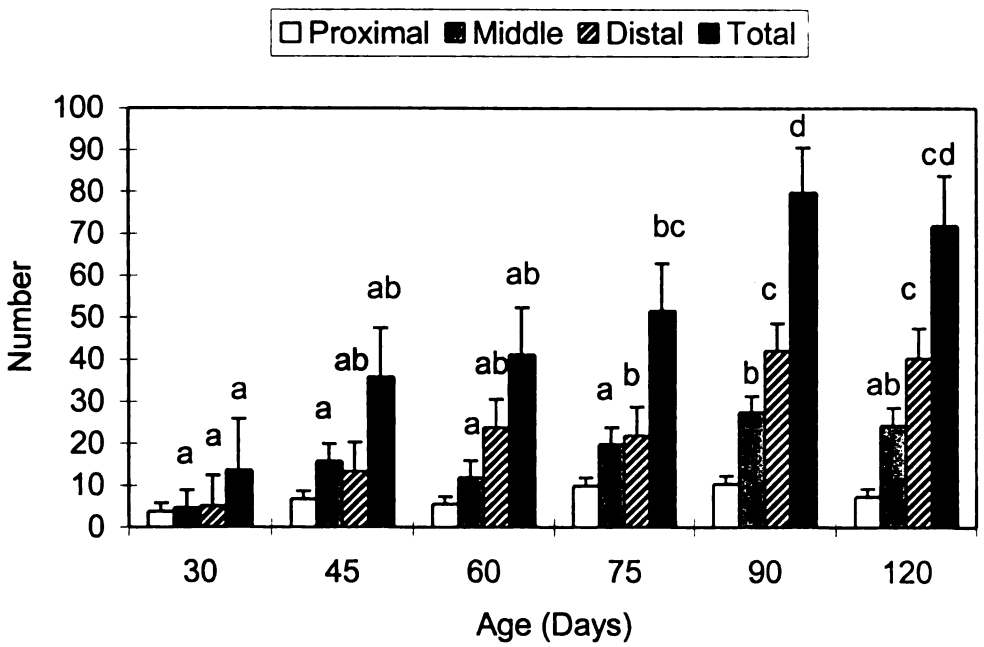


Figure 7. Influence of age on adenoma numbers in proximal (white), middle (gray) and distal (hatched) segments of the small intestine and total (black) numbers in the small intestine (30 days; n=8, 45 days; n=10; 60 days; n=10; 75 days; n=9; 90 days, n=9,120 days; n=8). Error bars indicate SEM. Columns of the same shade not sharing a common superscript are significantly different ($p<0.05$).

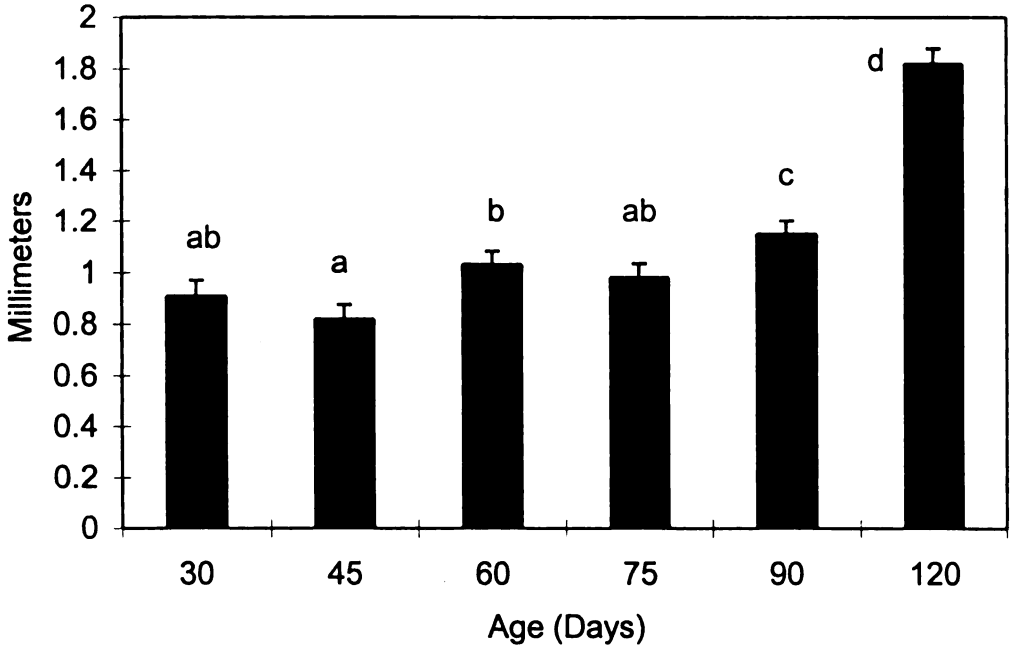


Figure 8. Influence of age on the average diameter of small intestinal adenomas (30 days; n=8, 45 days; n=10; 60 days; n=10; 75 days; n=9; 90 days, n=9, 120 days; n=8). Error bars indicate SEM. Columns not sharing a common superscript are significantly different ($p<0.05$).

The numbers (Fig. 9) of adenomas in the cecum were not significantly influenced by age. The mean volume of cecal adenomas was larger in older mice (Fig. 10), but this effect did not reach statistical significance. There was no sex effect on cecal tumor number or volume. The number of adenomas in the colon (Fig. 9) was influenced by age, but not in a consistent manner. Mice of 90-d of age had significantly more colonic tumors than mice at 45 or 60 days. The total volume of colonic tumors was significantly influenced by age (Fig. 11). Colonic tumors were small in size through 75 days of age, and then increased in volume dramatically after this time. There was also a significant sex effect on colon tumor volume. Colon adenoma volume averaged 28.9 mm^3 larger in males (43.8 mm^3) than in females (14.9 mm^3) at 120 days of age. This sex effect was not detected in the Apc^{Min} mice from the subsequent studies.

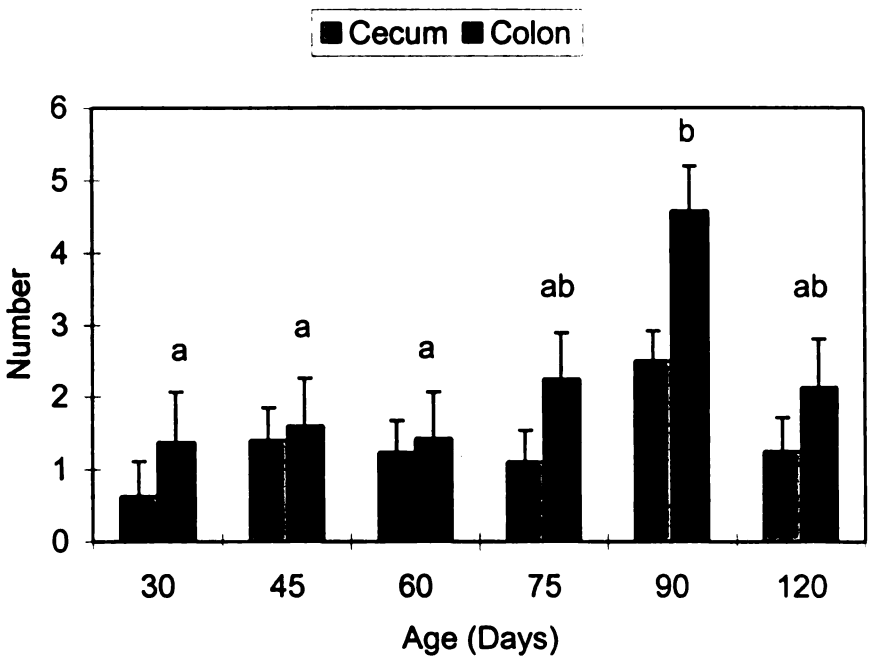


Figure 9. Influence of age on the average numbers in the cecum (gray) and colon (black) (30 days; n=8, 45 days; n=10; 60 days; n=10; 75 days; n=9; 90 days, n=9, 120 days; n=8). Error bars indicate SEM. Columns of the same shade not sharing a common superscript are significantly different ($p<0.05$).

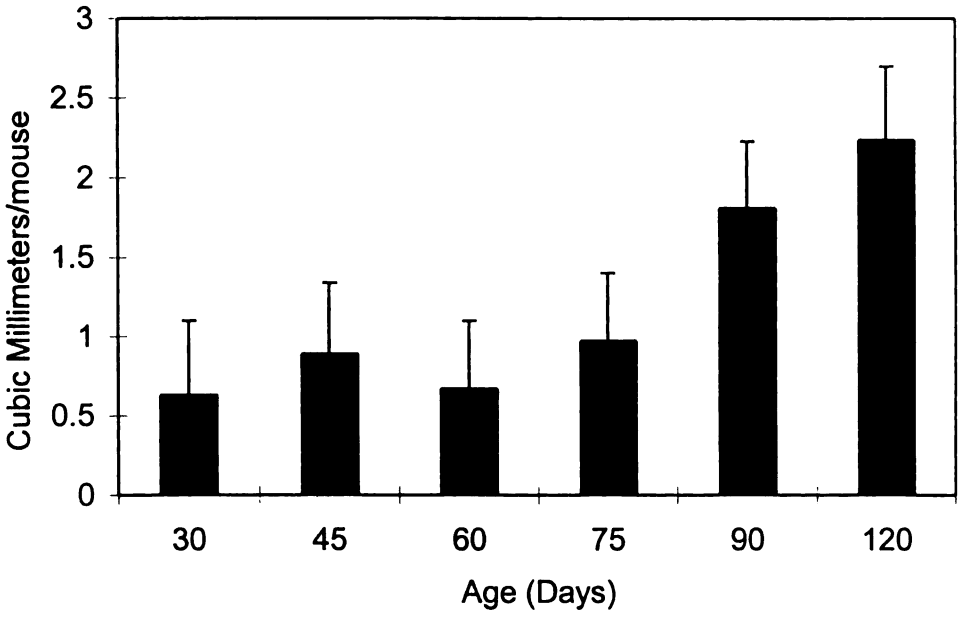


Figure 10. Influence of age on adenoma volume (mm^3) in the cecum (30 days; n=8, 45 days; n=10; 60 days; n=10; 75 days; n=9; 90 days, n=9, 120 days; n=8). Error bars indicate SEM.

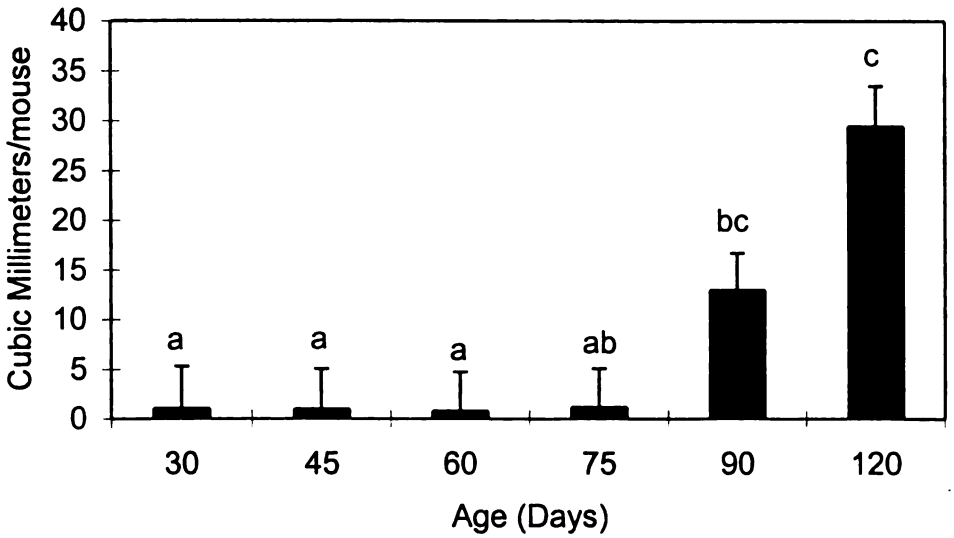


Figure 11. Influence of age on adenoma volume (mm^3) in the colon (30 days; n=8, 45 days; n=10; 60 days; n=10; 75 days; n=9; 90 days, n=9, 120 days; n=8). Error bars indicate SEM. Columns not sharing a common superscript are significantly different ($p<0.05$).

DISCUSSION

Mouse models such as the Min mutant should provide a major resource for studying the anticarcinogenic potential of dietary factors, pathways of tumorigenesis, and the molecular identification of genes that can control particular neoplastic processes. The Min mouse has been and will continue to be used as a model to study effects of dietary factors on human colorectal cancer. Effective use of Apc^{Min} mice in such studies requires a thorough assessment of their normal process of tumor development.

A few concerns have been identified concerning the use of Apc^{Min} mice as a model for research of colorectal cancer. Unlike human intestinal cancers, which are predominantly colorectal, Apc^{Min} mice develop the majority of their tumors in the small intestine. The utility of Apc^{Min} mice may also be limited by their lack of large numbers of colonic adenomas and aberrant crypt foci, the putative precursors of large-bowel polyps and cancers. These potential limitations also would pertain to various other Apc knockout mouse strains such as Apc^{1638}, $Apc^{\Delta 716}$ and Apc^{474} (Fodde et al., 1994; Oshima et al., 1995; Sasai et al., 2000). Accelerated polyposis models have been developed to increase the observed numbers of tumors and aberrant crypts. For example, additional carcinogen injections (Shoemaker et al., 1995; Steffensen et al., 1997) or genetic mutation in mismatch repair genes have been added to Apc^{Min} (Baker et al., 1998; Lal et al., 2001). However, the majority still uses the standard Apc^{Min} mouse model for the study of colorectal cancer development.

Realtively little research has been conducted to determine the tumor development characteristics of Apc^{Min} mice. Shoemaker et al. (1995) have shown age-related effects on the formation of adenomas in Apc^{Min} mice after additional treatment with ethylnitrosourea (ENU). Their results demonstrate that ENU-induced intestinal tumors in Apc^{Min} mice are most likely to be initiated during the first 2 weeks of life.

In the present study, the time frame of tumor development in Min mice was determined. Our results provide baseline data for the influence of age and sex on body weight and intestinal tissue length of Apc^{Min} mice in comparison with those of normal $Apc^{+/+}$ mice. These results also describe the influence of age and sex on adenoma development of Apc^{Min} mice. Small intestinal, cecal and colonic adenomas were already present in 30 day-old mice. Tumor volumes remained relatively small throughout the intestine for the first 75 days of age. After this time, tumor volumes increased rapidly up to 120 days of age.

We chose 120 days as the final time point in this study because it has been observed previously that Apc^{Min} mice suffer considerable morbidity and mortality beyond this age (Moser, 1990). Morbidity and mortality in Apc^{Min} mice is typically due to anemia caused by hemorrhraging associated with the small intestinal tumors after they progress beyond a certain size. We have observed that there is little morbidity in Apc^{Min} mice that have small intestinal tumors less than 1.5 mm in diameter. However, small intestinal tumors greater than 1.5-2.0 mm in diameter tend to perfolate and hemorrhrage. Hence, the choice of an end point (age) for feeding studies conducted using Apc^{Min} mice must be fully considered. Tumor progression

occurs predominantly in the age range of 75-120 days, with significant morbidity observed thereafter. These results suggest that experiments examining the effects of dietary or other interventions in Apc^{Min} mice should take into account their early and rapid tumor development.

CHAPTER IV

TART CHERRY ANTHOCYANINS INHIBIT CECAL TUMOR DEVELOPMENT IN APC^{MIN} MICE AND REDUCE GROWTH OF HUMAN COLON CANCER CELLS

ABSTRACT

Anthocyanins, which are bioactive phytochemicals, are widely distributed in plants and especially enriched in tart cherries. Based on previous observations that tart cherry anthocyanins and their respective aglycone, cyanidin, can inhibit cyclooxygenase enzymes, we conducted experiments to test the potential of anthocyanins to inhibit intestinal tumor development in Apc^{Min} mice and growth of human colon cancer cell lines. Mice consuming the cherry diet, anthocyanins, or cyanidin had significantly fewer and smaller cecal adenomas than mice consuming the control diet or sulindac. Colonic tumor numbers and volume were not significantly influenced by treatment. Anthocyanins and cyanidin also reduced cell growth of human colon cancer cell lines HT 29 and HCT 116. The IC_{50} of anthocyanins and cyanidin was 780 μM and 63 μM for HT 29 cells, respectively and 285 μM and 85 μM for HCT 116 cells, respectively. These results suggest that tart cherry anthocyanins and cyanidin may reduce the risk of colon cancer.

INTRODUCTION

Tart cherries contain substantial quantities of anthocyanins in addition to other bioflavonoids (Wang et al., 1997). Anthocyanins, a member of the bioactive phytochemicals, are widely distributed in fruits, vegetables and beans, suggesting that plant-based diets can provide considerable amounts of anthocyanins (Wang et al., 1999; Seeram et al., 2001). Like the vast majority of flavonoids, anthocyanins primarily occur in plants as glycosides. Cyanidin is the major anthocyanin aglycone in tart cherries. Montmorency and Balaton™ tart cherries contain 0.40 to 0.80 mg/g, respectively, of anthocyanins (Wang et al., 1997). These anthocyanins were found to function as antioxidants and cyanidin was shown to inhibit the activities of COX enzymes *in vitro* (Wang et al., 1999a; Wang et al., 1999b). Several studies have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the growth of colon tumors in animal models and reduce the risk of colon cancer in humans (Giovannucci et al., 1995; Labayle et al., 1991). In most cases, colon carcinogenesis depends on mutation of the APC gene, which is considered a gatekeeper in the carcinogenic process (Kinzler and Vogelstein, 1996). Human APC gene germline mutations cause familial adenomatous polyposis, an autosomal dominantly inherited disease that predisposes affected individuals to develop numerous adenomatous polyps and, ultimately, colorectal cancer (Su et al., 1992). APC gene mutations also are a frequent and early event in sporadic colon cancer. 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 (Moser et al., 1990). The primary phenotype of Apc^{Min} mice is the

development of multiple intestinal adenomas that progress to adenocarcinomas of the intestine in older mice.

The objectives of this research were to determine the potential of tart cherry anthocyanins and cyanidin to inhibit intestinal tumor development in Apc^{Min} mice and to determine the potential of anthocyanins and cyanidin to directly inhibit the growth of human colon cancer cells.

MATERIALS AND METHODS

Animals and Diets. This research was conducted with approval of the Michigan State University All-University Committee on Animal Use and Care. Mice were housed in a temperature and humidity-controlled room (20-22 °C, 50%) with a 12-h light/dark cycle. Apc^{Min} progeny were identified by a PCR-based assay (Su et al, 1992) and were randomly assigned to five treatment groups (n = 10 per group; equal numbers of males and females) at 4 - 5 weeks of age and fed treatment diets for 10 weeks. Deionized water and diets was provided ad libitum. The treatments (Table 2) were; 1) control diet (modified American Institute of Nutrition 93G diet containing 220 g/kg protein, 150 g/kg soybean oil and 50 g/kg cellulose), 2) control diet + 800 mg/L anthocyanins in the drinking water, 3) control diet + 200 mg/L cyanidin in the drinking water, 4) control diet + 200 mg/L sulindac (an NSAID) in the drinking water, and 5) modified control diet containing 200 g/kg freeze-dried pitted tart cherries. Cherries were frozen, freeze-dried and ground before they were incorporated into the diet. Anthocyanins were isolated from tart cherries and were a mixture of 3-cyanidin 2''-*O*-β-D-glucopyranosyl-6''-*O*-α-L-rhamnopyransyl-β-D-glucopyranoside and 3-cyanidin 6''-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (Wang et al, 1999) at 65 and 35%, respectively. The aglycone, cyanidin, was prepared from the anthocyanins (Wang et al, 1997). Ascorbic acid (50 mg/L) was added to the drinking water of all mice to enhance the stability of anthocyanins and cyanidin in solution by lowering the pH.

Table 2. Composition of the experimental diets (Percentage of diet)

Ingredient	Modified AIN-93G diet	20% Cherries diet
Casein	22.12	22.12
Soybean Oil	15.00	15.00
Corn Starch	31.72	24.22
Dyetrose	10.57	8.07
Sucrose	10.00	0.00
AIN-93G-MX	3.87	3.87
AIN-93G-VX	1.11	1.11
L-Cystine	0.33	0.33
Choline Bitartrate	0.28	0.28
Tert-Butylhydroquinone	0.003	0.003
Cellulose	5.00	5.00
Dried Cherries	0.00	20.00

Tumor Number and Size. The mice were sacrificed after 10 weeks of treatment and the numbers and sizes of adenomas in the intestinal sections were measured. The entire small intestine, cecum, and colon were removed from each mouse to determine the number and size of adenomas. Intestinal sections were opened longitudinally, rinsed thoroughly with water, fixed overnight in 10% neutral-buffered formalin, and then stained with 0.2% methylene blue. Tumor numbers and dimensions for each intestinal segment were determined by direct counting with the aid of a dissecting microscope and measuring grid. The tumor sizes were determined by measuring the spherical (three dimensional) volume of adenomas in the cecum and colon and the average diameter of tumors in the small intestine. Tumors in the cecum and colon of Apc^{Min} mice typically are polypoid in appearance, whereas the small intestinal tumors are sessile. Spherical volumes of cecal and colonic tumors were calculated by the formula: Volume = 0.532 x (width x length x height of tumor).

Tumor numbers and diameters in the small intestine were analyzed by two-way analysis of variance (treatment, sex). For tumor numbers and volumes in cecum and colon, data were transformed to ranks and then ranks were analyzed by two-way analysis of variance. When significant treatment effects were detected ($P<0.05$), treatment means were compared using the Least Significant Difference method. Six mice (one from the control group, two from the anthocyanin group, and three from the cyanidin group) were excluded from the final statistical analysis because it was determined at the end of the experiment that these animals did not carry the APC^{Min} gene mutation, and therefore did not develop intestinal tumors.

Cell Culture and Growth Assays. The human colorectal cancer cell lines HCT 116 and HT 29 (American Type Culture Collection) were cultivated in McCoy's 5A media supplemented with 10% fetal bovine serum. Cells were harvested for growth assays when they had reached 50-80% confluence by trypsin:EDTA treatment and counted using a hemacytometer. Cells were then seeded at 15,000 cells/well in 24-well tissue culture plates. Plates were incubated overnight at 37°C and 5% CO_2 to allow cells to attach and begin proliferating.

At the beginning of treatments, the media was gently aspirated from each of the wells, which were then rinsed with PBS. One ml of treatment media was added to each well (n = 8-12 wells per treatment level per cell line) and plates were incubated for 72 h. Treatment media was McCoy's 5A media supplemented with 10% fetal bovine serum and containing the respective concentrations of anthocyanins (0-1000 μM) or cyanidin (0-250 μM). Anthocyanins were dissolved in distilled water before addition to the treatment media, whereas cyanidin was solubilized in DMSO before addition. When used, the DMSO concentration was equalized for all treatment media and never exceeded 0.1% (v/v) of the final treatment media.

Total cell numbers in each well were quantified after 72 h of incubation in treatment media. Cell numbers were calculated based on total DNA content in each well using a procedure that quantifies DNA based on fluorescence of bound Hoescht 33258 (Labarca et al, 1980). Fluorescence was measured by a Cytofluor II fluorimeter (Applied Biosystems; Foster City, CA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Fluorescence readings were converted to DNA by comparison to standard solutions of Salmon testis DNA

(Sigma Chemical Company; St. Louis, MO). The cell numbers in each well were calculated by converting the quantity of DNA in each well by the amount of DNA present in each cell (determined experimentally) for HCT 116 and HT 29 cells.

Cell numbers observed in each well after 72 h of growth were corrected for initial cell number (determined at the time treatment media was added). These data were then subjected to multiple regression analysis to develop least-squares polynomial equations describing the influence of anthocyanins or cyanidin concentration on cell number. These equations were then used to iteratively calculate the concentration of anthocyanins or cyanidin required to cause a 50% reduction (IC_{50}) in growth (cell number) for each cell line.

RESULTS

Anthocyanins, Cyanidin and Tart Cherries Influence Intestinal Tumor Development. Final body weights of mice were significantly influenced by treatment and averaged 22.8, 24.1, 21.3, 19.7, and 25.5 grams for mice consuming control diet, anthocyanins, cyanidin, tart cherries, and sulindac, respectively (Fig. 12). Final body weights for mice consuming anthocyanins and sulindac were greater ($P<0.05$) than for mice consuming tart cherries. Averaged across all treatments, male mice weighed significantly greater ($P<0.05$) than female mice both at the beginning (18.3 versus 15.6 grams) and the end (24.6 versus 20.8 grams) of the experiment. Male mice weighed approximately 4 grams heavier than female mice.

Treatments had differential effects on tumor incidence and burden in the various sections of the intestinal tract. Mice consuming anthocyanins, cyanidin, or tart cherries had fewer ($P<0.05$) adenomas in the cecum than mice consuming the control diet or sulindac (Fig. 13). The average number of adenomas in the cecum of mice that consumed anthocyanins, cyanidin and tart cherries was 0.6, 0.6, and 0.6, respectively, whereas mice that consumed the control diet or sulindac had an average of 2.4 and 3.8 cecal tumors, respectively. Colonic adenoma number was not influenced by treatment (Fig. 13). Average adenoma numbers in the colon were 3.6, 3.1, 3.6, 3.3, and 5.2 for mice that consumed the control diet, anthocyanins, cyanidin, tart cherries, and sulindac, respectively. The total burden (volume) of cecal adenomas was less ($P<0.05$) in mice consuming anthocyanins, cyanidin or tart cherries when compared to mice consuming the control diet or sulindac (Fig. 14). Adenoma volumes in the cecum of mice that consumed anthocyanins, cyanidin, or

tart cherries were 0.7, 0.6, and 1.8 mm^3 respectively, whereas those in mice consumed the control diet and sulindac were 3.0 and 4.0 mm^3, respectively (Fig. 13). Although mice that consumed tart cherries had the greatest adenoma burden in the colon (8.4 mm^3), this was not statistically greater than that observed in mice consuming the other treatments (Fig. 14).

Tumor multiplicity in the small intestine was not significantly influenced by treatment and averaged 48 tumors per mouse (Fig. 15). Mice that consumed sulindac had the smallest number of small intestinal adenomas (28 per mouse), but this was not statistically different than small intestinal tumor numbers observed for the other treatments. The average size of small intestinal adenomas (Fig. 16) was increased ($P<0.05$) by feeding tart cherries (1.7 mm) and reduced ($P<0.05$) by sulindac (0.9 mm) relative to that observed in mice consuming the control diet (1.4 mm), anthocyanins (1.3 mm), or cyanidin (1.3 mm).

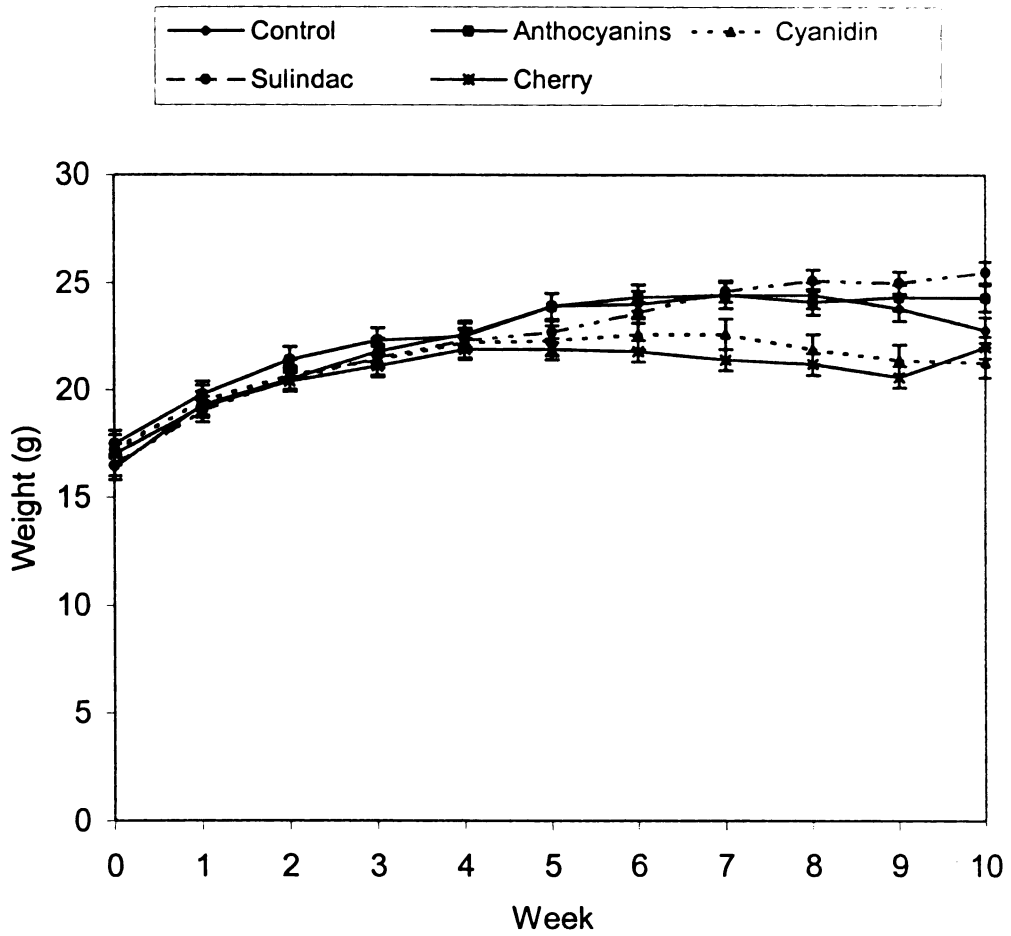


Figure 12. Influence of anthocyanins, cyanidin, tart cherries and sulindac on body weights (g) of mice throughout the experimental period. Error bars indicate SEM.

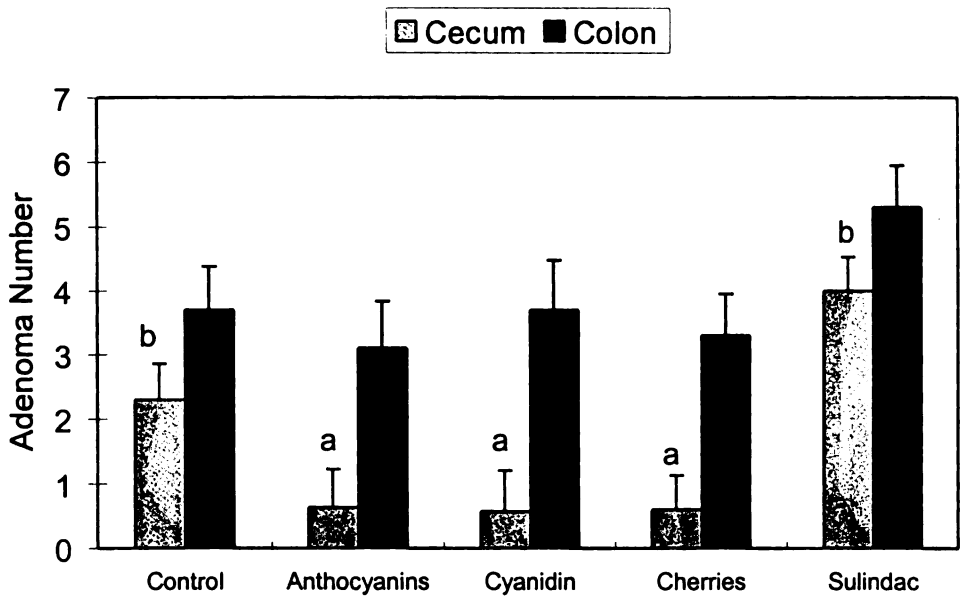


Figure 13. Influence of anthocyanins, cyanidin, tart cherries and sulindac on adenoma numbers in cecum (gray) and colon (black). Error bars indicate SEM. Columns of the same shade not sharing a common superscript are significantly different ($P<0.05$).

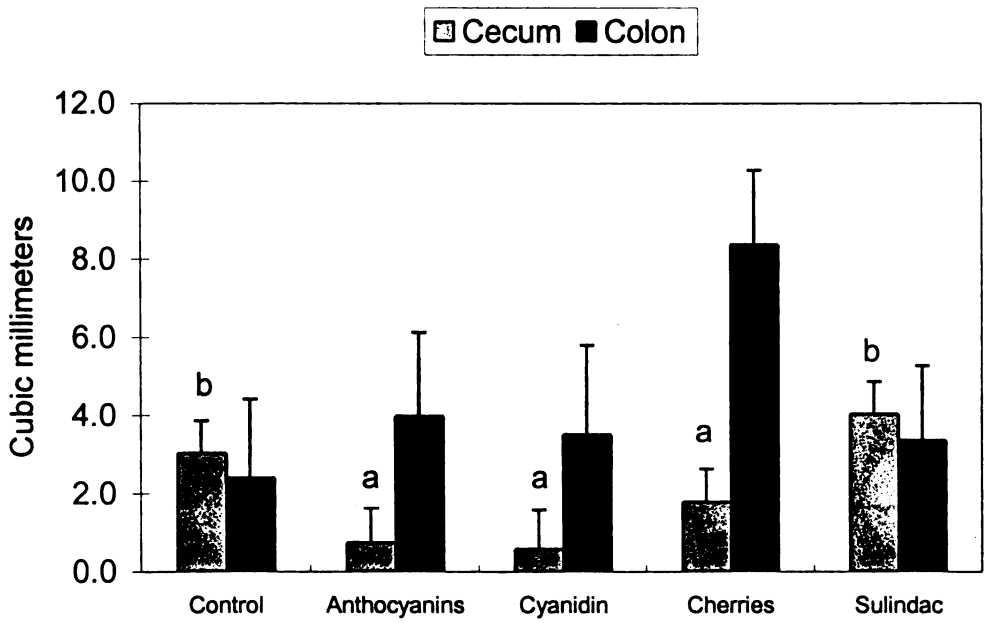


Figure 14. Influence of anthocyanins, cyanidin, tart cherries and sulindac on adenoma volumes (mm^3) in cecum (gray) and colon (black). Error bars indicate SEM. Columns of the same shade not sharing a common superscript are significantly different ($P<0.05$).

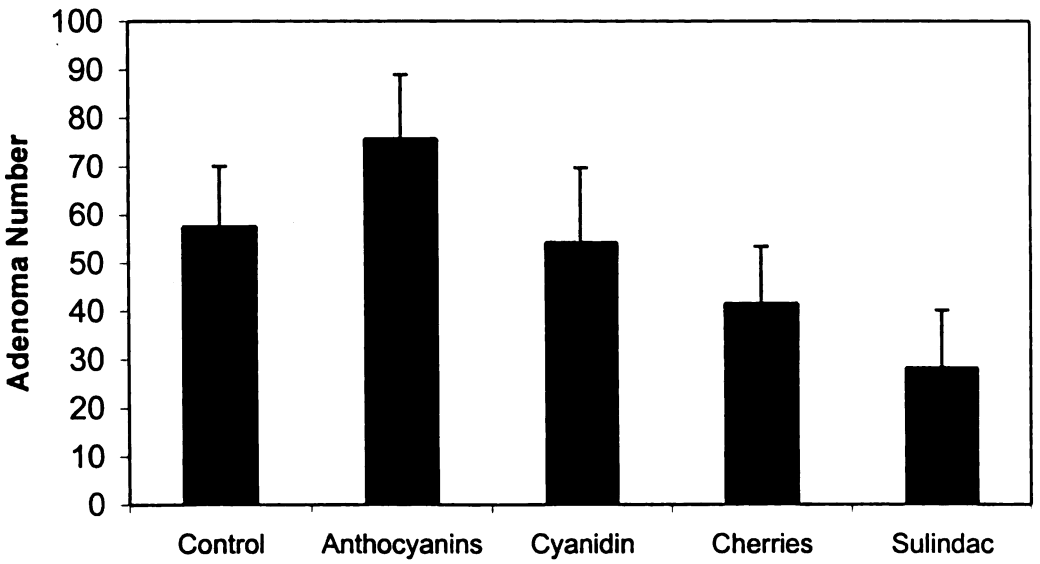


Figure 15. Influence of anthocyanins, cyanidin, tart cherries and sulindac on adenoma numbers in small intestine. Error bars indicate SEM.

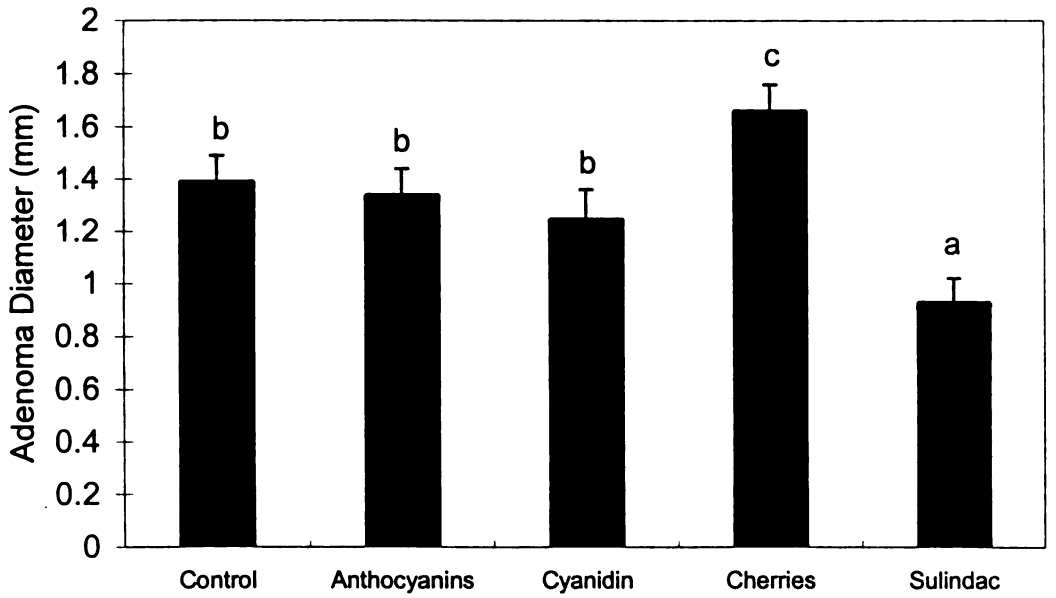


Figure 16. Influence of anthocyanins, cyanidin, tart cherries and sulindac on average diameters (mm) of adenomas in small intestine. Error bars indicate SEM. Columns not sharing a common superscript are significantly different ($P<0.05$).

Anthocyanins and Cyanidin Inhibit Human Colon Cancer Cell Growth.

Treatment with anthocyanins (Fig. 17) or cyanidin (Fig. 18) caused a dose-dependent reduction in cell numbers for both HCT 116 and HT 29 cells. Neither anthocyanins nor cyanidin caused cytotoxicity even at the highest concentrations tested, as indicated by little or no dead cells. Cyanidin was far more effective in inhibiting the growth of these cancer cell lines than anthocyanins. The observed IC_{50} values for cyanidin were 85 and 63 μM for HCT 116 and HT 29 cells, respectively, whereas those for anthocyanins were 260 and 585 μM for HCT 116 and HT 29 cells, respectively.

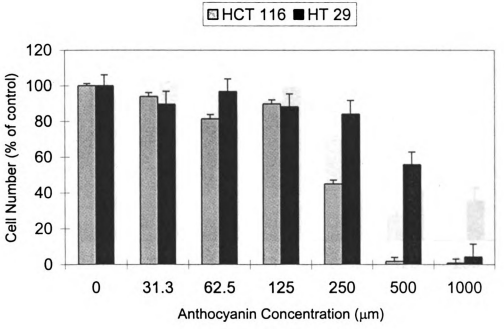


Figure 17. Influence of anthocyanins on growth of human cancer cells, HCT 116 (gray) and HT 29 (black). Error bars indicate SEM.

Cell Number (% of control)

Figure

and H

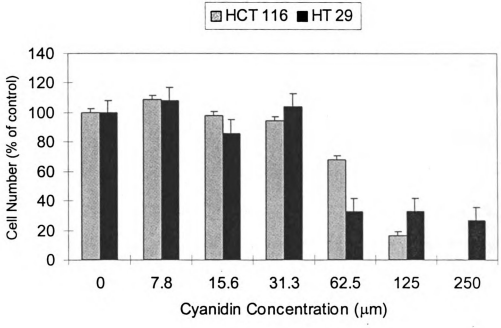


Figure 18. Influence of cyanidin on growth of human cancer cells, HCT 116 (gray) and HT 29 (black). Error bars indicate SEM.

DISCUSSION

Our interest in testing the potential of tart cherry anthocyanins and cyanidin to inhibit tumor development in Apc^{Min} mice stemmed from the observation that these compounds inhibit the activities of COX enzymes (Wang et al., 1999). Other studies have demonstrated that sulindac (and other NSAIDs) reduce small intestinal tumor multiplicity and size in Apc^{Min} mice (Boolbol et al., 1996; Chiu et al., 1997; Mahmoud et al., 2000). In this study, we found that anthocyanins, cyanidin, and tart cherries (presumably as a source of anthocyanins) all significantly reduced the number and burden of tumors in the cecum of Apc^{Min} mice. Conversely, sulindac did not influence tumor development in the cecum. None of the treatments tested influenced the numbers of tumors in the small intestine or the numbers or burden of tumors in the colon. Our inability to detect a significant reduction in small intestinal adenoma number by sulindac was likely due to a number of factors, including the relatively small numbers of mice per treatment group and large variations among individual mice in adenoma development. We also have observed that the ability of sulindac to inhibit small intestinal tumor development decreases as Apc^{Min} mice age (Bourquin, L. D., unpublished research). The lack of effect of anthocyanins or cyanidin on colonic tumor development may be a consequence of their metabolism by intestinal bacteria.

The average size of small intestinal tumors was smaller in mice consuming sulindac and larger in mice consuming tart cherries when compared to that in mice consuming the control diet, anthocyanins or cyanidin. Progression in the size of small intestinal tumors is highly correlated with morbidity in Apc^{Min} mice. We have

observed that significant morbidity and weight loss occur when small intestinal tumors reach an average size of 1.5 mm diameter. At this stage, the tumors tend to hemorrhage and may perforate the small intestine. The observed differences in final body weight in this experiment likely are a consequence of these differences in small intestinal tumor promotion. It is well documented that sulindac and other NSAIDs consistently reduce the size of small intestinal tumors in Apc^{Min} mice (Boolbol et al., 1996; Chiu et al., 1997; Mahmoud et al., 2000). The mechanism for the larger small intestinal tumor size in mice consuming rat cherries is not known.

The effects of the treatments on tumor development were not consistent throughout the intestinal tract. Sulindac and other NSAIDs typically reduce small intestinal tumor development in Apc^{Min} mice, but often have little effect on tumor development in the cecum and colon (Barnes et al., 1998; also Bourquin, L. D., unpublished research). Mice consuming anthocyanins, cyanidin and tart cherries had fewer and smaller tumors in the cecum, but these compounds did not afford any protection to the small intestine. These results indicate that although anthocyanins and cyanidin inhibit the activities of COX enzymes *in vitro* (Wang et al., 1999; Seeram et al., 2001), they likely do not influence intestinal tumor development in Apc^{Min} mice through a pathway involving COX inhibition.

Both anthocyanins and cyanidin inhibited the growth of the colon cancer cell lines HT 29 and HCT 116, although cyanidin was much more effective. We have identified three degradation products from anthocyanins and cyanidin in cell culture medium. These were protocatechuic acid, 2,4-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acids (Seeram et al., 2001). In addition, we have detected trace

quantities of cyanidin-3-glucoside and cyanidin in culture medium after 72 h of cell growth in anthocyanin treatments. We have evaluated the potential of these degradation products to inhibit the growth of HCT 116 and HT 29 cells. None of the degradation compounds assayed demonstrated any inhibition of cell growth at concentrations ranging up to 250 μM (Seeram et al., 2001).

Anthocyanins and cyanidin are unstable at pH 7.0 and spontaneously degrade to chalcone and benzoic acid derivatives. The red cyanidin cation is stable at pH < 3, but deprotonates and produces ketoquinonoidal bases and finally an ionized quinonoid base at pH > 7 (Seeram et al., 2001). At pH 3-6, the cyanidin cation forms a carbinol pseudobase or chalcone pseudobase.

Anthocyanins are highly water-soluble and considered to be structurally similar to a number of strong DNA intercalators (Mas et al., 2000). Both DNA and RNA act as strong copigments for anthocyanins (Mistry et al., 1997). Also, anthocyanins protect DNA against oxidative damage (Sarma et al., 1999). Under *in vivo* and cell culture conditions, both anthocyanins and cyanidin potentially form corresponding pseudobases due to pH variations. These pseudobases are transition compounds and may be stable *in vivo* as protein bound complexes. Given these results, we predict that the anthocyanins, the aglycone cyanidin, or its varying pseudobases directly suppress cell growth and subsequent tumor development.

We believe that our results are the first to demonstrate that anthocyanins and cyanidin have the potential to directly interfere with intestinal tumor development. Hagiwara et al. (Hagiwara et al., 2001) demonstrated that anthocyanins in purple corn color reduced the promotion of colon tumors caused by 2-amino-1-methyl-6-

phenylimidazo[4,5-*b*]pyridine (PhIP) in rats initiated with dimethyl hydrazine. However, they did not test the potential of purple corn color anthocyanins to directly suppress tumor development (Hagiwara et al., 2001).

In summary, we have demonstrated that tart cherry anthocyanins and their aglycone cyanidin significantly reduced tumor development in the cecum of Apc^{Min} mice. These compounds also directly inhibited the growth of human colon cancer cells *in vitro*, with the aglycone cyanidin being far more effective than the anthocyanin glycosides. Benzoic acid derivatives yielded from the degradation of anthocyanins and cyanidin had no influence on colon cancer cell growth. Taken together, these results suggest that cyanidin or its corresponding pseudobase is directly inhibiting tumor development in the cecum of Apc^{Min} mice. Anthocyanins also are effective presumably due to their deglycosylation to cyanidin by cecal bacteria. The lack of a clear suppression of tumor development in the colon probably is due to further degradation of the cyanidin molecule by elevated pH in the intestinal lumen and bacterial metabolism.

CHAPTER V

TART CHERRY ANTHOCYANINS REDUCE INTESTINAL TUMORIGENESIS AND ALTER INTESTINAL EPITHELIAL CELL GENE EXPRESSION, BUT DO NOT INFLUENCE INTRACELLULAR DISTRIBUTION OF β-CATENIN IN INTESTINE OF APC^{MIN} MICE.

ABSTRACT

Anthocyanins are flavonoid compounds that impart color to fruits and vegetables including cherries. Previous research in our laboratory has demonstrated the potential of tart cherry anthocyanins to inhibit tumor development in Apc^{Min} mice and reduce proliferation of human colon cancer cell lines. The objective of this research was to determine the extent to which dietary anthocyanins inhibit intestinal tumorigenesis, alter β-catenin expression and distribution, and influence global gene expression profiles in intestinal epithelial cells of Apc^{Min} mice. Mice were randomly assigned to one of three treatment groups at 4 weeks of age (n = 25 normal + 25 Apc^{Min} mice per treatment). The treatments were 1) AIN 93G control diet, 2) the control diet + 200 mg/kg sulindac, and 3) the control diet + 750 mg/kg anthocyanins. The number of adenomas in the small intestine was reduced ($P<0.05$) by diets containing either sulindac ($\overline{X} = 9$) or anthocyanins ($\overline{X} = 17$) relative to the controls ($\overline{X} = 27$). The number and volume of cecal adenomas were reduced ($P<0.05$) in mice that consumed anthocyanins (0.6, 0.7 mm^3) compared to mice consuming sulindac (2.1, 4.7 mm^3) or the control diet (1.0, 1.5 mm^3). Mice consuming anthocyanins had significantly fewer colonic adenomas (0.8) than those consuming sulindac (1.6) or the control diet (2.2). Colonic adenoma burden was not influnced by treatment. Dietary anthocyanins reduced tumor development throughout the intestinal tract of Apc^{Min} mice, whereas tumor inhibition by sulindac was specific to the small intestine. Anthocyanins increased ($P<0.05$) β-catenin mRNA expression in middle small intestine and cecum. Intracellular distribution of β-catenin mRNA was primarily in the epithelial cell membrane for mice on all dietary treatments. cDNA

microarray analysis showed that expression of mRNA of β-catenin was not altered by anthocyanins or sulindac. Anthocyanins reduced the expression of cyclin D1 mRNA compared to sulindac in cecum, but expression of mRNA for other predicted target genes of the Wnt/β-catenin signaling pathway – proxisome proliferator activated receptor δ, C-Myc and matrix metalloproteinase 7 – were not altered by anthocyanins or sulindac. These observations suggest that the tumor inhibition caused by feeding anthocyanins or sulindac was not a consequence of any reduction in the cytoplasmic level and translocation of β-catenin to the nucleus and subsequent influences on target gene expression. cDNA microarray analysis identified a total of 1,492 genes that were influenced by any treatment in the small intestine, colon or ceum. Genes influenced by anthocyanins and sulindac are involved in a wide variety of cellular and molecular processes including DNA synthesis and repair, transcription, mRNA processing, translation, protein interactions and turnover, cell proliferation and differentiation, cell cycle control, and programmed cell death. The patterns of gene expression changes suggest that alterations of cellular and molecular processes by anthocyanins and sulindac likely are different in Apc^{Min} mice. Additional confirmatory analysis of these gene expression results will be necessary to further establish potential mechanisms whereby anthocyanins and sulindac influence tumor development.

INTRODUCTION

Tart cherries contain substantial quantities of anthocyanins in addition to other bioflavonoids (Wang et al., 1997). Anthocyanins, a member of the bioactive phytochemicals, are widely distributed in fruits, vegetables and beans, suggesting that plant-based diets can provide considerable amounts of anthocyanins (Wang et al., 1999; Seeram et al., 2001). Like the vast majority of flavonoids, anthocyanins primarily occur in plants as glycosides. Cyanidin is the major anthocyanin aglycone in tart cherries. Montmorency and Balaton™ tart cherries contain 0.40 to 0.80 mg/g, respectively, of anthocyanins (Wang et al., 1997). These anthocyanins were found to function as antioxidants and cyanidin was shown to inhibit the activities of COX enzymes in vitro (Wang et al., 1999a; Wang et al., 1999b). Several studies have demonstrated that NSAIDs inhibit the growth of colon tumors in animal models and reduce the risk of colon cancer in humans (Giovannucci et al., 1995; Labayle et al., 1991). In previous research, we demonstrated that anthocyanins and cyanidin, when administered in drinking water, reduced the numbers and total burden of tumors in the cecum of Apc^{Min} mice (Chapter IV). Cyanidin and anthocyanins also inhibited the growth of human colon cancer cell lines HT 29 and HCT 116 in vitro (Chapter IV).

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 (Su et al., 1992). The primary phenotype of Apc^{Min} mice is the development of multiple intestinal adenomas that progress to adenocarcinomas of the intestine in older mice. In most cases, colon carcinogenesis depends on

mutation of APC gene and this gene is considered a gatekeeper in the carcinogenic process (Kinzler and Vogelstein, 1996). Most mutations in the human APC gene are found to occur between codons 1286 and 1513, which is within a region encoding β-catenin binding sites (Nakamura, 1993). These mutations result in the production of a truncated APC protein product which cannot facilitate the degradation of β-catenin. Recently, a nuclear export function of the APC gene has been identified (Rosin-Arbesfeld et al., 2000). APC contains highly conserved nuclear export signals 3' adjacent to the mutation cluster region that enable it to bind and export β-catenin from the nucleus. When this ability is lost due to APC mutation, β-catenin accumulates in the nucleus.

β-catenin functions as a component of the E-cadherin adhesion complex and also as a transcriptional regulator in a signaling pathway by binding to T cell factor (Tcf) transcription factor in the nucleus. Aberrant accumulation and nuclear localization of β-catenin can enhance trancriptional activation and expression of target genes in this signaling pathway. A few target genes in this pathway – peroxisome proliferator activated receptor δ (PPARδ), c-MYC, cyclin D 1 (CLND1) and matrilysin (matrix metalloproteinase 7) – have been recently identified (He et al., 1999; He at al., 1998; Tetsu and McCormick, 1999; Crawford et al., 1999). Screening changes in expression of these target genes as well as full genome in the different sections of intestine should yield insights into the potential mechanisms responsible for tumor modulation by anthocyanins and sulindac.

Expression of thousands of genes can be examined simultaneously using a novel technique called cDNA microarray. Microarrays consist of a collection of gene-specific cDNA fragments immobilized on glass slides which can be simultaneously analyzed to ascertain their expression in tissue. Anthocyanins and sulindac likely influence tumorigenesis in the intestinal tract by modulating the expression of genes involved in carcinogenesis. The objectives of this study were to determine the extent to which dietary anthocyanins and sulindac 1) inhibit intestinal tumorigenesis, 2) influence the expression and celluar localization of β-catenin, and 3) influence global gene expression profiles in intestine of Apc^{Min} mice.

MATERIALS AND METHODS

Animals and Diets. This research was conducted with approval of the Michigan State University All-University Committee on Animal Use and Care. Apc^{Min} mice were produced by mating normal C57BL/6J ($Apc^{+/+}$) female mice with Min C57BL/6J ($Apc^{Min/+}$) male mice. One-hundred-fifty mice were grouped by sex, weight and litter and then randomly assigned to three treatment groups (n= 50 per group) at 4 weeks of age. Mice were not genotyped to determine Apc status before the experiment, but it was expected that approximately 25 Min mice and 25 normal mice would be randomly allotted to each treatment. Deionized water and diets were provided ad libitum. Body weights were measured weekly. The dietary treatments were; 1) control diet (modified American Institute of Nutrition 93G diet containing 220 g/kg protein, 150 g/kg soybean oil and 50 g/kg cellulose), 2) control diet + 200 ppm sulindac, and 3) control diet + 750 ppm anthocyanins. Sulindac and anthocyanins were incorporated into the appropriate diets upon formulation. The concentrations of sulindac (200 ppm) and anthocyanins (750 ppm) were based on efficacious concentrations used in a previous experiment conducted in our laboratory (Chapter IV). Anthocyanins were isolated from tart cherries and were a mixture of 3-cyanidin 2″-*O*-*β*-D-glucopyranosyl-6″-*O*-*α*-L-rhamnopyransyl-*β*-D-glucopyranoside and 3-cyanidin 6″-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside (Wang et al., 1997) at 65 and 35%, respectively.

Tumor Number and Size. The mice were sacrificed after 8 weeks of dietary treatment. A sample of liver tissue was obtained from each mouse for confirmation of Apc gene status by a PCR-based procedure (Su et al., 1992). The entire small

intestine, cecum, and colon were removed from each mouse. Intestinal sections were opened longitudinally and rinsed thoroughly with water. Sixteen Apc^{Min} mice from each treatment were used for cDNA microarray analysis (8 males and 8 females). The middle and distal two-thirds of the small intestine were fixed overnight in 10% neutral buffered formalin (NBF) and then stained with 0.2% methylene blue. Tumor numbers and dimensions for the cecum and colon were determined immediately on unfixed tissue sections – these sections and proximal small intestine were then used for microarray analysis. For the remaining mice, all tissues were fixed in 10% NBF prior to tumor measurement and embedded for immunohistochemistry. Tumor numbers and dimensions for each intestinal segment were determined by direct counting with the aid of a dissecting microscope and measuring grid. The tumor sizes were determined by measuring the spherical (three dimensional) volume of adenomas in the cecum and colon and the average diameter of tumors in the small intestine. Tumors in the cecum and colon of Apc^{Min} mice typically are polypoid in appearance, whereas the small intestinal tumors are sessile. Spherical volumes of cecal and colonic tumors were calculated by the formula: Volume = 0.523 x (width x length x height of tumor).

Weekly body weights were statistically analyzed by repeated-measures analysis of variance as a split-plot design. Main plot terms included treatment, sex, Apc status and their interactions. Sub-plot terms included time and its interactions with main plot effects. Tissue lengths were analyzed by two-way analysis of variance (ANOVA) to detect the effects and potential interactions of treatment and sex. Tumor numbers and diameters in the small intestine were analyzed by two-way

ANOVA (treatment, sex). For tumor numbers and volumes in cecum and colon, data were transformed to ranks and then ranks were analyzed by two-way ANOVA. When significant effects were detected ($P<0.05$), appropriate means were compared using the Least Significant Difference method. Mice which did not carry the APC^{Min} gene mutation, and therefore did not develop intestinal tumors, were excluded from the final statistical analyses of tumor number and size.

Microarray Analysis. RNA Isolation and Extraction Sixteen (8 males and 8 females) Apc^{Min} mice from each treatment were used for microarray analysis. Total RNA was extracted from pooled samples (4 mice with same sex for each pooled sample) of the proximal small intestine, cecum and colon of these 16 Apc^{Min} mice for each treatment. Tumors in the cecum and colon were quantified under a dissecting microscope and excised prior to epithelial cell isolation. Colon and cecum were scraped with a glass slide to obtain epithelial cells. RNAlater™ (200 to 1000 µl, Ambion, Austin, TX) was added and cells were stored at –80 °C. Approximately 800 mg of cells were obtained from the proximal small intestine section of each mouse, and approximately 200 mg and 250 mg of cells were obtained from the cecum and colon of each mouse, respectively. Equal amounts of cells from each mouse were pooled within same treatment, sex and tissue and used for RNA extraction and microarray analysis.

The improved single step RNA isolation method (Chomczynski and Sacchi, 1987) was used to isolate total RNA from epithelial cells. RNA was prepared with Trizol LS reagent (Gibco BRL, Grand Island, NY) via manufacturer's instructions. Briefly, cell pellets were resuspended in Trizol and incubated 5 minutes at room

temperature. Chloroform was added to the suspension to a final concentration of 16 % (V/V) and phases were separated by centrifugation for 5 minutes (10,000 x g). The aqueous phase was removed and RNA precipitated with isopropyl alcohol. The total RNA pellet was washed with 75% ethanol and concentration determined by spectrophotometry. RNA integrity was monitored by agarose gel electrophoresis.

Fluorescence Labeling of Microarray Probes. Four pooled RNA samples for each tissue site (2 males and 2 females) were prepared from 8 male and 8 female Apc^{Min} mice for each treatment. Methods for fluorescence labeling followed the protocol developed by Hegde et al. (2000). cDNA was prepared by reverse transcription of total RNA samples (15 to 40 μg) in the presence of aminoallyl modified dUTP (aa-dUTP). Reverse transcription was performed with Superscript II (Gibco) via manufacturer's instructions. Briefly, the secondary structure of RNA was relaxed in the presence of anchored oligo dT ($T_{21}VN$). cDNA was produced upon addition of dNTPs (aa-dUTP: dTTP 2:1) and Superscript II (20 units) and incubation at 42 °C for 2 hours. Unincorporated dNTPs were removed using commercially available kit (Qiagen PCR, Qiagen Inc., Valencia, CA) and probes were evaporated in a speed vaccum for 1 hour.

Dye coupling was done by incubation of cDNA with the monoreactive dyes cyanine 3 (Cy3) or cyanin 5 (Cy5) (Amersham, Buckinghamshire, UK) for 1 hour at room temperature in the dark. Cy3 and Cy5 labeled samples for a given slide were mixed following addition of hydroxylamine at a final concentration of 2M. Unincorporated dyes were removed using the Qiagen cleanup kit (Quiagen Inc.). Each probe (Cy3 and Cy5 labeled pair) was dried in a speed vacuum and

resuspended in buffer (50% formamide, 5X SSC, 0.1% SDS + 20 μg COT-1 DNA and 20 μg polyA DNA). Each probe mixture then was applied to microarray slides in a loop design (Fig. 19). After adding the probes, the slides were coverslipped and placed in a hybridization oven at 42 °C for 20 hr. The slides then were removed and washed with 1X SSC /0.2 % SDS until the cover slips were released. The slides were again washed two times with 0.1X SSC/0.2% SDS for 5 min and 0.1XSSC for 1 min to remove residual SDS.

Preparation of the microarray slides. Microarray slides were printed with 15,360 genes received from the National Institute on Aging's (NIA) mouse 15,264-gene (15K) set. The 15K cDNA clones were rearrayed from 52,374 ESTs (expressed sequence tags) derived from various embryonic and neonatal tissues. Full details on development on the 15K gene set are described by Tanaka et al. (2000). The 15K gene set was supplemented with an additional 96 known genes to allow symmetrical printing of genes in the slides. The cDNA library contains sequences coding for genes with known function, no hit genes (genes with no sequence information), unknown genes (genes with sequence information only), and genes coding for hypothetical protein (genes encoding a hypothetical protein based on high BLAST [basic local alignment search tool] score).

Printing of this array was carried out using a superaldehyde substrate according to the manufacturer's protocol (Telechem). Briefly, PCR amplification of inserts from each plasmid was performed using primers that contain a C6 amine group coupled to the 5' end. The amplicons were precipitated and resuspended in spotting solution (3XSSC). The solutions were robotically spotted (Omnigrid robot,

GeneMachines, CA) onto superaldehyde coated glass slides at the Plant Research Laboratory at Michigan State University. The unbound DNA was removed and the bound probes denatured at 100°C. Free aldehydes were reduced with sodium borohydride and the slides washed extensively with 0.1X SSC/0.2% SDS and 0.1XSSC.

Scanning, Immage Quantitation, Normalization and Statistical Analysis. The slides were scanned in an array scanner (Affymetrix 428™ tray, Santa Clara, CA) and array image files were generated using the Jaguar 2.0 program ™ (Affymetrix). Both Cy3 and Cy5 channels for each of the 15,360 genes (spots) on each slide were recognized, quantitated, and normalized against overall and peripheral background intensity level using the GenImage program developed by Dr. Sorin Draghici's Bioinformatics lab at Wayne State University. These data were again normalized for dye variation using the GP3 R Script developed by Dr. Timothy Zachrawski's lab at Michigan State University.

A total of 36 pooled RNA samples were used in this analysis (2 male and 2 female samples for Apc^{Min} mice for each of the three treatments for each tissue [small intestine, cecum, colon]). The full dataset, based on Cy3 and Cy5 channels in 72 slides, includes four independent estimates of expression for each of the 15,360 genes for each pooled RNA sample. Outlier observations were excluded by deleting any of the four independent observations of expression for each gene (for each RNA sample) that differed from the mean by more than one standard deviation. The remaining observations were averaged to obtain a single expression value for each gene for each RNA sample. These data were then statistically analyzed using SAS

(version 8.1) by tissue site and by gene using a 2-way analysis of variance (treatment, sex). When significant treatment effects were detected ($P<0.05$), mean values were compared using the Least Significant Difference method.

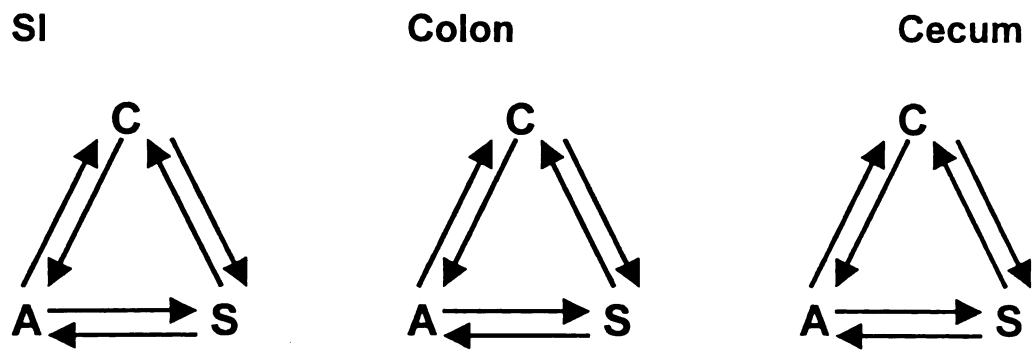


Figure 19. Aminoallyl labeling loop of RNA for microarray analysis

C, A and S represent three different dietary treatments, control, anthocyanins and sulindac, respectively. Each arrow represents a single microarray slide. In each slide the sample was labeled with Cy3 (tail of arrow) and also with Cy5 (head of arrow). The entire scheme was repeated four times, corresponding to the four pooled RNA samples (2 male, 2 female) obtained from animals on each treatment at each tissue site. (SI; small intestine)

β-catenin Immunohistochemistry Analysis. Expression of β-catenin in intestinal sections of normal and Apc^{Min} mice exposed to the various dietary treatments was conducted. Four micrometer paraffin-embedded sections of small intestine, colon and cecum were deparaffinized and stained for β-catenin by indirect peroxidase biotin-streptavidin immunohistochemistry. Sections were pretreated by an antigen retrieval procedure using 10 mM citrate buffer (pH 6) for 20 minutes at 95 °C and preblocked with 3% H_2O_2 in PBS followed by normal goat serum. After incubation of sections for 14 hr at 4 °C with anti-β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY) at a dilution of 1:250, biotinylated anti-mouse immunoglobulin (DAKO Laboratories, Carpinteria, CA) was applied to the sections at room temperature for 40 minutes. Sections then were treated with streptavidin-peroxidase (DAKO) at room temperature for 40 minutes. Linked peroxidase was visualized by staining with aminoethylcarbazole (DAKO) for 30 minutes. The β-catenin immunostaining results for different locations within the tissue (top and bottom for small intestine and top, middle, and bottom for colon and cecum) and distribution within the cells (membrane and cytoplasm) were evaluated by visually comparing the staining intensities. The intensity of β-catenin immunostaining was scored using a scale ranging from absent (0) to very high (4).

The scores for intensity of β-catenin immunostaining were transformed to ranks and then ranks were analyzed by four-way analysis of variance (treatment, Apc status, region within the tissue, and distribution within the cells). When significant

effects were detected ($P<0.05$), means were compared using the Least Significant Difference method. Images in this dissertation are presented in color.

RESULTS

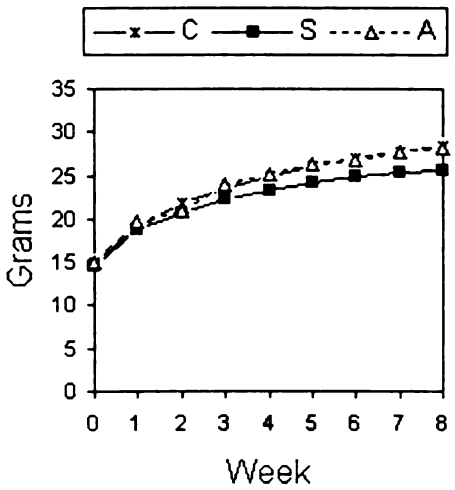
Anthocyanins Inhibit Intestinal Tumor Development. Averaged across all treatments, sexes and time points, mice consuming control (21.8 g) and anthocyanins (21.3 g) weighed more than mice consuming sulindac (20.3 g) ($P<0.05$). Male mice weighed significantly more ($P<0.01$) than female mice both at the beginning (14.0 versus 13.0 grams) and the end (26.4 versus 22.3 grams) of the experiment (Fig. 20). Final body weights of Apc^{Min} mice consuming control diet, anthocyanins, and sulindac averaged 24.8, 23.5, and 24.9 grams , respectively. The lengths of the small intestine, cecum and colon were not influenced by dietary treatment (data not shown).

Mice consuming sulindac had more ($P<0.05$) adenomas in the cecum than mice consuming control diet or anthocyanins (Fig. 21). Cecal adenoma number for mice consuming control diet also was greater ($P<0.05$) than in mice consuming anthocyanins. The average number of adenomas in the cecum of mice that consumed anthocyanins, sulindac and control diet was 0.6, 2.1, and 1.0, respectively. Colonic adenoma number also was influenced by treatment (Fig. 21), with mice consuming control or sulindac having more tumors ($P<0.05$) than mice consuming anthocyanins. Average adenoma numbers in the colon were 0.8, 1.6, and 2.2 for mice that consumed anthocyanins, sulindac and control diet, respectively. The total burden (volume) of cecal adenomas was smallest ($P<0.01$) in mice consuming anthocyanins when compared to mice consuming control diet or sulindac (Fig. 22). Adenoma volume in the cecum of mice that consumed anthocyanins was 0.7 mm^3, whereas adenoma volumes in mice consuming control diet and sulindac were 1.5 and 4.7 mm^3, respectively (Fig. 22). The total burden of colonic adenomas was not

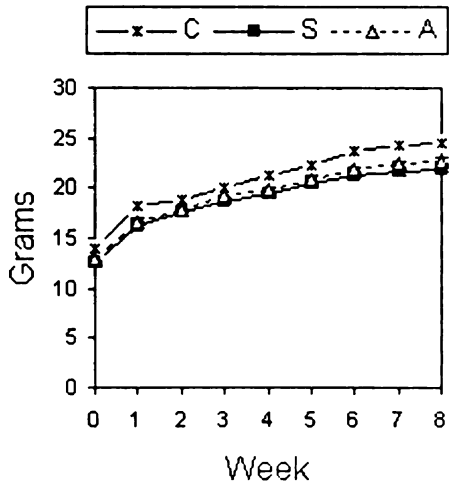
statistically significant at the $P<0.05$ level, but there was a trend that anthocyanins and sulindac reduced the total burden of colonic adenomas relative to mice consuming the control diet ($P=0.08$). Adenoma volumes in the colon of mice that consumed anthocyanins, control, and sulindac were 2.6, 5.6, and 3.0 mm^3, respectively (Fig. 22).

Tumor multiplicity in the small intestine was significantly influenced by treatment ($P<0.05$). Adenoma number of the small intestine was obtained by combing adenoma numbers of the middle and distal small intestine segments. Mice that consumed sulindac had the smallest number of small intestinal adenomas (9 per mouse) whereas mice that consumed the control diet had the greatest number of small intestinal adenomas (27 per mouse) (Fig. 23). Adenoma number of small intestine was intermediate in mice consuming anthocyanins (17 per mouse) (Fig. 23). Mice consuming sulindac had significantly fewer adenomas in both middle and distal small intestine segments than mice consuming control diet, and significantly fewer adenomas in the distal small intestine than mice consuming anthocyanins ($P<0.05$) (Fig. 23). The average size of small intestinal adenomas (Fig. 24) was reduced ($P<0.05$) by feeding sulindac (1.1 mm) relative to that observed in mice consuming the control diet (1.3 mm). The average size of small intestinal adenomas was intermediate in mice consuming anthocyanins (1.2 mm) (Fig. 24).

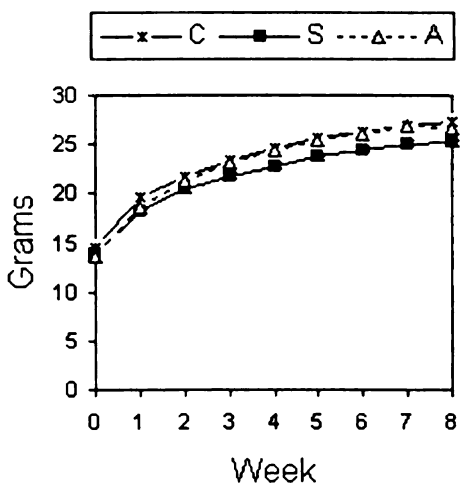
a) Male normal mice



b) Female normal mice



c) Male Apc^{Min} mice



d) Female Apc^{Min} mice

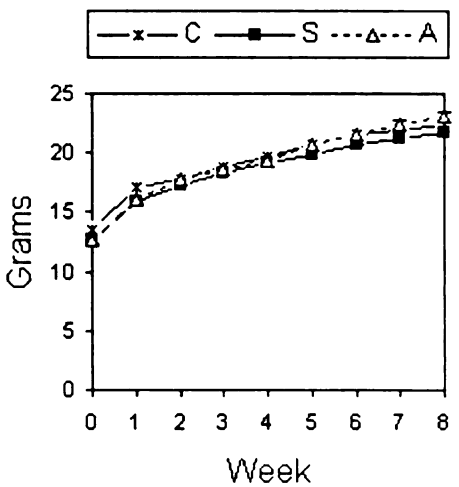


Figure 20. Influence of treatment on body weights (g) of mice throughout the experimental period. C; control, S; sulindac, A; anthocyanins. Error bars indicate SEM. **a)** Body weights of male normal mice **b)** Body weights of female normal mice **c)** Body weights of male Apc^{Min} mice **d)** Body weights of female Apc^{Min} mice

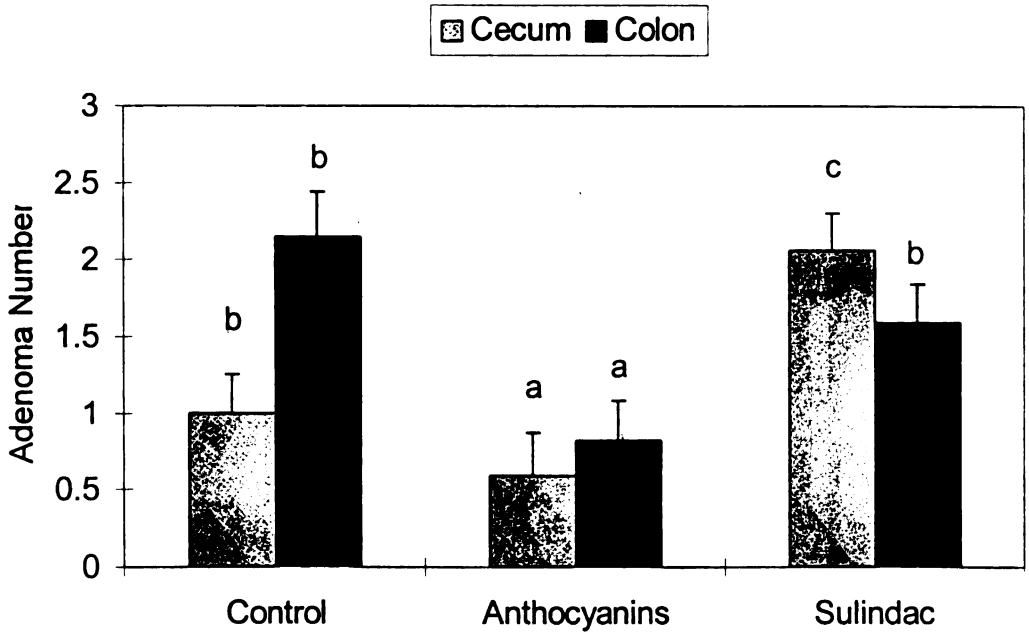


Figure 21. Influence of anthocyanins and sulindac on adenoma number in cecum (gray) and colon (black). Error bars indicate SEM. Columns of the same shade not sharing a common superscript are significantly different ($P<0.05$).

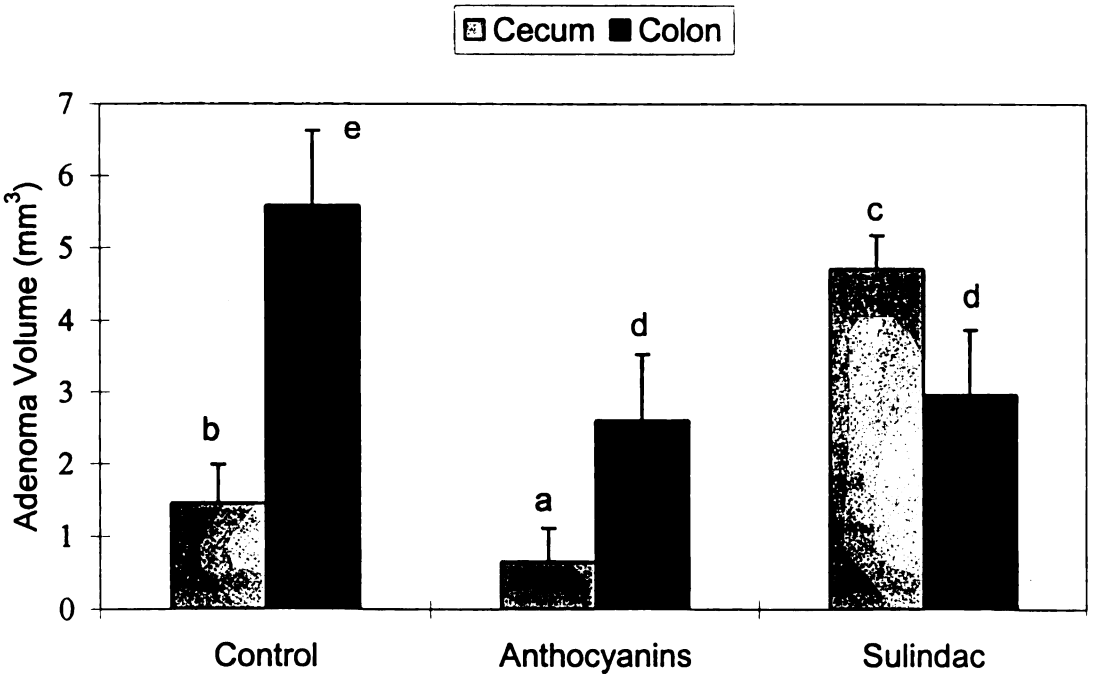


Figure 22. Influence of anthocyanins and sulindac on total adenoma volumes (mm^3) in cecum (gray) and colon (black). Error bars indicate SEM.

[a, b, c]Significant treatment effect ($P<0.05$). Columns not sharing a common superscript are significantly different.

[d, e]Treatment effect trend ($P=0.08$).

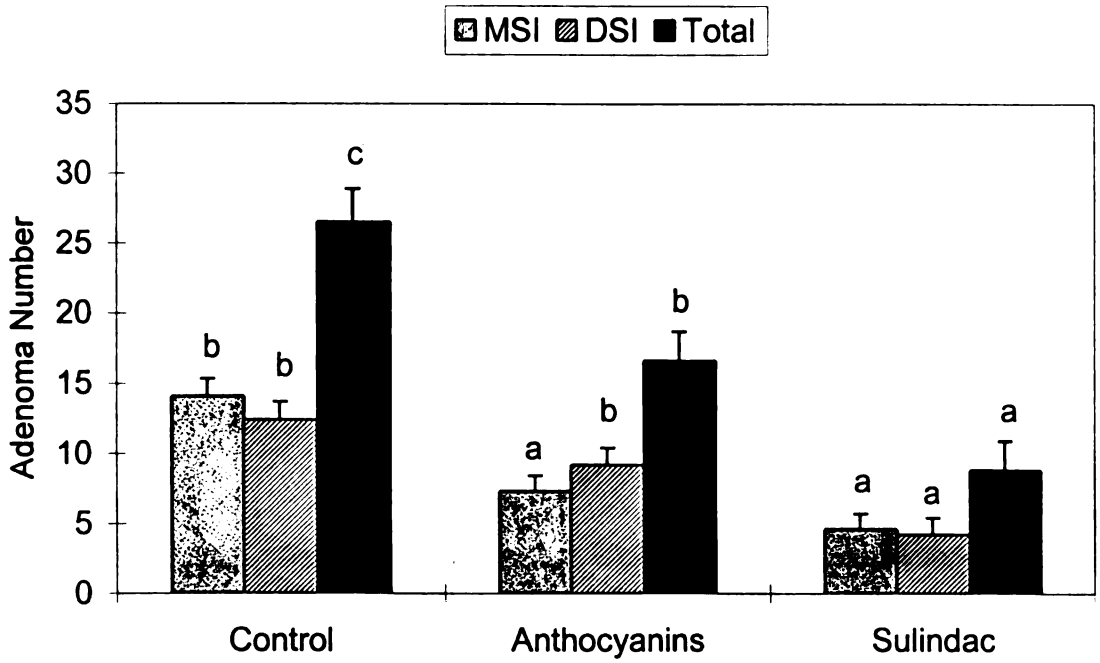


Figure 23. Influence of anthocyanins and sulindac on adenoma number in small intestine. MSI; middle small intestine, DSI: distal small intestine. Error bars indicate SEM. Columns of the same shade not sharing a common superscript are significantly different ($P<0.05$).

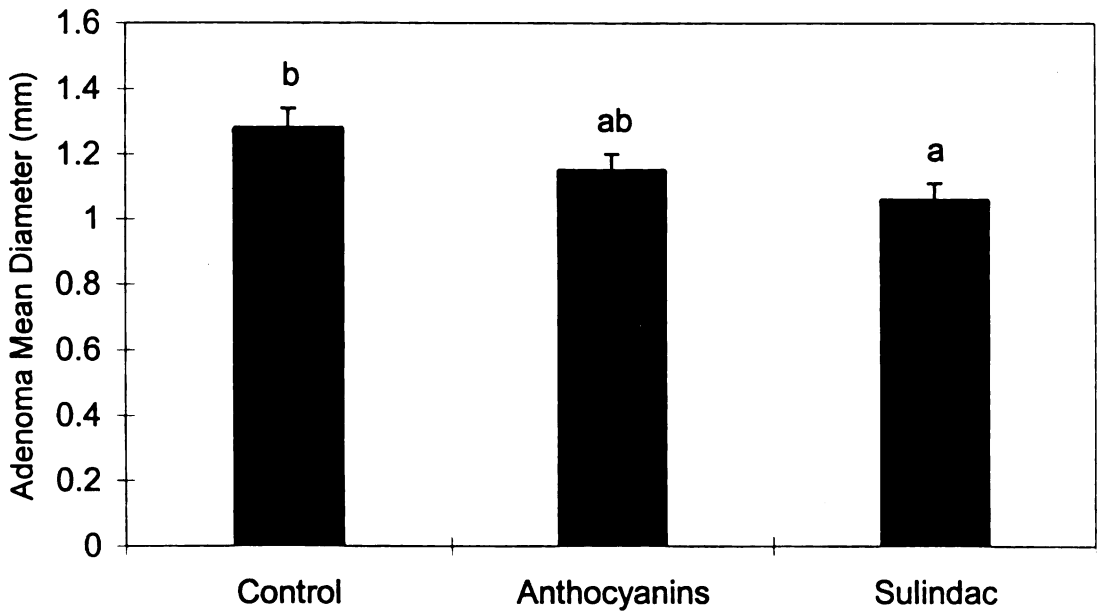


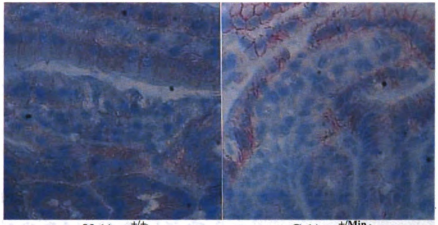
Figure 24. Influence of anthocyanins and sulindac on mean diameters (mm) of adenomas in small intestine. Error bars indicate SEM. Columns not sharing a common superscript are significantly different ($P<0.05$).

Anthocyanins Do Not influence Intracellular Distribution of β-catenin.

When the score of intensity of β-catenin immunostaining was compared, the expression of β-catenin protein was consistently higher at the top of villi in the small intestine and top one third of crypts in cecum and colon of all carrier (Apc^{Min}) and normal ($Apc^{+/+}$) mice ($P<0.05$) (Fig. 25, 26 and 27). Within epithelial cells, expression of β-catenin protein was higher in cell membrane than in cytoplasm in all tissue sections ($P<0.05$) (Fig. 25, 26 and 27). Nuclear expression of β-catenin protein was negligible in all tissue sections. The expression of β-catenin protein was greater ($P<0.05$) in intestinal tissues of Apc^{Min} mice than those of normal mice in all sites (Fig. 25, 26 and 27).

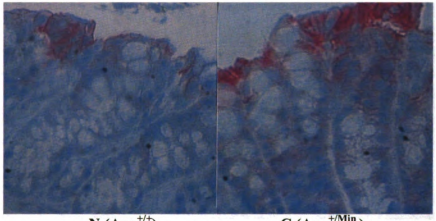
Mice consuming anthocyanins had increased expression of β-catenin protein in the cecum relative to mice consuming control diet or sulindac ($P<0.05$) (Table 4). Significant interactions between treatment and Apc status were found in the proximal and middle small intestinal sections. The expression of β-catenin protein was increased in the proximal small intestine as well as in the middle small intestine of Apc^{Min} mice consuming anthocyanins compared with those consuming control diet ($P<0.05$) (Table 4).

(a) Small Intestine



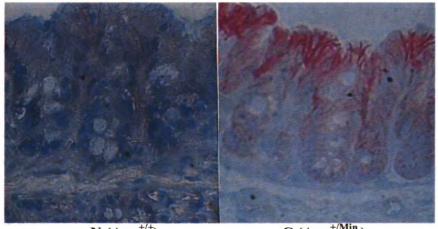
N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

(b) Colon



N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

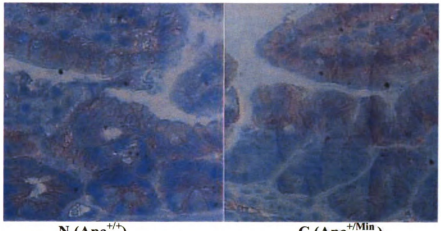
(c) Cecum



N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

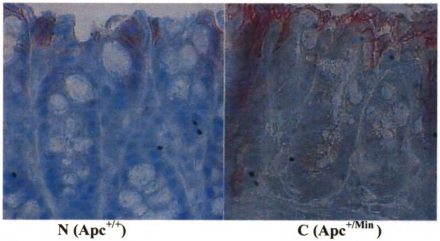
Figure 25. Expression of β-catenin in mice consuming the control diet.

(a) Small Intestine



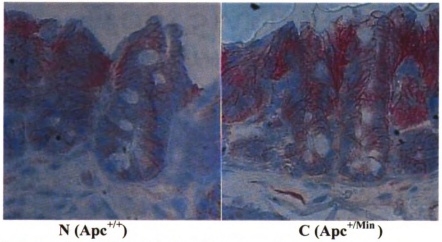
N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

(b) Colon



N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

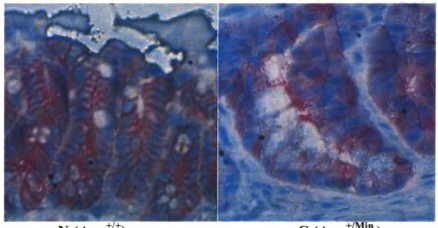
(c) Cecum



N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

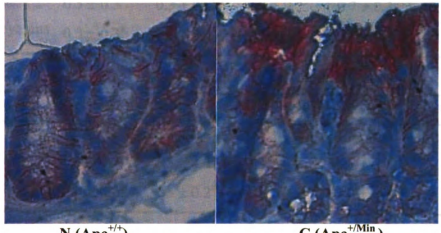
Figure 26. Expression of β-catenin in mice consuming sulindac

(a) Small Intestine



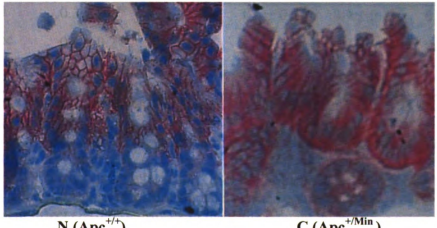
N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

(b) Colon



N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

(c) Cecum



N ($Apc^{+/+}$) C ($Apc^{+/Min}$)

Figure 27. Expression of β-catenin in mice consuming anthocyanins

Table 3. Average intensity scores of β-catenin expression in intestinal sections[1, 2]

A. Membrane staining in normal mice

Treatment	PSI[3]	MSI[3]	DSI	Colon	Cecum[4]
Control	1.8 ± 0.3^{abc}	1.5 ± 0.2^{b}	1.5 ± 0.4	1.7 ± 0.4	1.2 ± 0.2^{a}
Sulindac	1.5 ± 0.3^{ab}	1.3 ± 0.2^{ab}	2.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.3^{a}
Anthocyanins	1.5 ± 0.3^{a}	2.3 ± 0.2^{c}	2.0 ± 0.3	1.8 ± 0.3	2.0 ± 0.2^{b}

B. Cytosol staining in normal mice

Treatment	PSI	MSI	DSI	Colon	Cecum
Control	0.7 ± 0.3^{abc}	0.3 ± 0.2^{b}	0.5 ± 0.4	0.7 ± 0.4	0.7 ± 0.2^{a}
Sulindac	0.5 ± 0.3^{ab}	0.3 ± 0.2^{ab}	0.8 ± 0.3	0.7 ± 0.3	0.3 ± 0.3^{a}
Anthocyanins	0.3 ± 0.3^{a}	0.8 ± 0.2^{c}	0.5 ± 0.3	0.7 ± 0.3	0.8 ± 0.2^{b}

C. Membrane staining in Apc^{Min} mice

Treatment	PSI	MSI	DSI	Colon	Cecum
Control	1.5 ± 0.2^{ab}	0.8 ± 0.2^{a}	1.8 ± 0.2	1.8 ± 0.2	1.6 ± 0.2^{a}
Sulindac	1.7 ± 0.2^{bc}	1.5 ± 0.2^{b}	2.3 ± 0.2	2.1 ± 0.2	1.4 ± 0.2^{a}
Anthocyanins	2.0 ± 0.2^{c}	2.3 ± 0.2^{c}	2.3 ± 0.2	2.0 ± 0.2	2.1 ± 0.1^{b}

D. Cytosol staining in Apc^{Min} mice

Treatment	PSI	MSI	DSI	Colon	Cecum
Control	0.3 ± 0.2^{ab}	0.0 ± 0.2^{a}	0.5 ± 0.2	0.9 ± 0.2	0.6 ± 0.2^{a}
Sulindac	0.8 ± 0.2^{bc}	0.5 ± 0.2^{b}	0.7 ± 0.2	0.9 ± 0.2	0.3 ± 0.2^{a}
Anthocyanins	1.0 ± 0.2^{c}	1.3 ± 0.2^{c}	0.8 ± 0.2	1.4 ± 0.2	0.8 ± 0.2^{b}

[1]PSI; proximal small intestine, MSI; middle small intestine, DSI; distal small intestine.

[2]The intensity of β-catenin immunostaining was scored using a scale ranging from absent (0) to very high (4).

[3]Treatment x carrier status interaction ($P<0.05$). Means in the same column not sharing a common superscript are significantly different ($p<0.05$).

[4]Treatment effect ($P<0.05$). Means in the same column within each category not sharing a common superscript are significantly different ($p<0.05$).

Anthocyanins and Sulindac Do Not Influence Expression of mRNA of APC, β-catenin and Most of the Predicted Target Genes of Wnt/β-catenin Signaling. Expression of β-catenin and the predicted target genes of the Wnt signaling pathway was determined in small intestine, colon and cecum of Apc^{Min} mice using cDNA microarray analysis (Table 4). Anthocyanins and sulindac did not alter the expression of mRNA of APC and β-catenin in all three intestinal sections of Apc^{Min} mice. For the predicted target genes of the Wnt/β-catenin signaling, anthocyanins decreased the expression of cyclin D1 compared to sulindac in cecum (0.59 vs 0.79). Treatment did not influence the expression of mRNA for the other predicted target genes – MMP7, PPARδ and C-Myc – in all three tissue sections of Apc^{Min} mice.

cDNA Microarray Analysis Identified Several Clusters of Differentially Expressed Genes in Small intestine, Colon and Cecum of Apc^{Min} Mice. All gene identifications were based on BLAST scores greater than 100 as of May 1, 2002. There were 497 genes influenced by any treatment (F value for treatment effect < 0.05) in SI, 534 in Colon, and 501 in cecum (Fig. 28). None of the genes was influenced by treatment in all three intestinal sections. There were 8 genes influenced by treatment both in small intestine and colon (Table 5). Relative to controls, sulindac down-regulated 3 of these 8 genes in small intestine and four genes in colon. Sulindac up-regulated 2 genes in colon compared with controls. Relative to controls, anthocyanins up-regulated 2 of the 8 genes and down-regulated three genes in small intestine. Anthocyanins down-regulated three of the 8 genes in colon compared with controls. There was little overlap in the effects of sulindac and

anthocyanins on expression of these eight genes. In small intestine, both compounds down-regulated the expression of a single gene (unknown function). Both compounds down-regulated the expression of placental lactogen 2 in colon, but expression of this gene was up-regulated by anthocyanins and not influenced by sulindac in small intestine.

There were 12 genes influenced by treatment both in small intestine and cecum (Table 6). These include 6 genes coding for known proteins (voltage-dependent calcium channel beta-3 subunit, growth arrest specific protein 5, tubulin beta 3, collagen IV, kangai), 5 unknown and 1 hypothetical protein gene. Of these 12 genes, sulindac reduced expression of 3 genes in small intestine and none of the genes in the colon when compared to controls. None of these 12 genes were up-regulated by sulindac relative to control. In small intestine relative to controls, anthocyanins reduced the expression of calcium channel beta 3 subunit, growth arrest protein 5, tubulin beta 3, collagen IV and one unknown gene and increased the expression of alternative splicing factor, 1 unknown gene, and 1 hypothetical protein. Expression of these genes was similary affected by anthocyanins in cecum except for an increased expression of calcium channel subunit and decreased expression of kangai 1 (Table 6).

Twenty genes were differentially influenced by treatment in both cecum and colon. Among those genes changed both in colon and cecum, anthocyanins up-regulated 9 genes and down-regulated 3 genes compared to both controls and sulindac in cecum (Table 7). The up-regulated genes were nuclear antigen Sp100, serine/threonine-protein kinase 4, acid ceramidase 1, CD36 antigen-like 2, signal

transducing adaptor molecule 2, UMP-CMP kinase, and 3 unknown genes. The down- regulated genes relative to both control and sulindac were zinc finger protein RIN ZF, 1 no hit, and 1 hypothetical protein gene. In colon, sulindac influenced the expression of most of these 20 genes, whereas anthocyanins did not, compared to control. Relative to controls, sulindac up-regulated genes for zinc finger protein LIN ZF, MORF-related gene X, nuclear antigen Sp100, serine/threonin-protein kinase 4, acid ceramidase1, 1 no hit, and 7 unknown genes and down-regulated CD36 antigen-like 2, signal transducing adaptor molecule 2, UMP-CMP kinase, 1 hypothetical protein, and 1 no hit gene (Table 7).

Differentially Expressed Genes in Cecum of Apc^{Min} Mice. Cecal epithelium from anthocyanin-treated mice had significantly increased expression of 79 genes compared to mice fed the control and sulindac diets (Table 8). Anthocyanins increased the expression of the hemachromatosis (HFE) gene, ras-related C3 botulinum toxin substrate 1, and Ly6/Neurotoxin 1 protein genes more than 2-fold compared to both control and sulindac.

Anthocyanins also significantly decreased the expression of 44 genes compared to both control and sulindac (Table 9). The decrease in expression of 2 unknown genes by anthocyanins was more than 2-fold compared to control or sulindac.

Differentially Expressed Genes in Colon of Apc^{Min} Mice. Anthocyanins significantly increased the expression of 33 genes in colonic epithelium compared to both control and sulindac treated mice (Table 10). Anthocyanins significantly decreased the expression of 33 genes compared to both control and sulindac treated

mice (Table 11). The larger fold changes were an increase of expression for cytochrome b5, adolase C5 and 1 unknown gene (Table 10) and decrease for serum protein MSE55 (Table 11).

Differentially Expressed Genes in Small Intestine of Apc^{Min} Mice. In small intestine, 53 and 244 genes were up-regulated by sulindac (Table 12 and 13) and anthocyanins (Table 13 and data not shown), respectively, when compared to controls. Among these genes, 19 genes were up-regulated by sulindac and anthocyanins (Table 13). The genes that were increased in their expression in small intestine both by sulindac and anthocyanins include 9 known genes. Among genes influenced specifically by sulindac (Table 12), the expression of ATPase subunit 4 gene was increased by 3.36-fold in mice consuming sulindac compared to mice consuming the control diet. The expression of Tax interacting protein gene was increased most (1.5-fold) by sulindac and LIM domain binding 1 gene (1.79-fold) by anthocyanins (Table 13).

Sulindac and anthocyanins also down-regulated a large group of genes in the small intestine. 108 genes were down-regulated by sulindac (Table 14 and 15) and 47 genes were down-regulated both by sulindac and anthocyanins (Table 15) when compared to control. Genes that were down-regulated specifically by sulindac more than 1.5-fold were 1 no hit and 1 unknown gene. Adipocyte-specific protein 5 and novel cell death-regulatory protein GRIM19 were down-regulated by 1.49-fold and 1.44-fold, respectively (Table 14). Genes that were down-regulated more that 1.5-fold in their expression by both sulindac and anthocyanins were fatty acid binding protein 3, glutathione S-transferase and 2 unknown genes (Table 15).

Table 4. Expression of β-catenin and target genes of Wnt/β-catenin signaling in intestinal sections of Apc^{Min} mice[1,2]

Plate ID[3]	Gene Name	C	S	A	SEM[4]
		Small Intestine			
H3080G10	APC	0.75	0.69	0.77	0.08
H3080F09	APC	0.62	0.71	0.63	0.05
H3031E05	β-catenin	1.26	1.59	1.25	0.23
H3084D05	Cyclin D1	0.67	0.64	0.57	0.04
H3093H12	MMP7	0.69	0.71	0.80	0.04
H3155B12	PPARdelta	1.48	1.13	1.45	0.24
H3076D10	C-myc	1.26	1.27	1.17	0.12
H3089H11	C-myc	0.87	0.80	0.70	0.09
		Colon			
H3080G10	APC	0.64	0.76	0.72	0.07
H3080F09	APC	0.73	0.78	0.79	0.04
H3031E05	β-catenin	1.45	1.54	1.67	0.20
H3084D05	Cyclin D1	0.66	0.61	0.62	0.06
H3093H12	MMP7	0.84	0.83	0.79	0.05
H3155B12	PPARdelta	1.16	1.08	1.18	0.14
H3076D10	C-myc	1.24	0.97	1.00	0.11
H3089H11	C-myc	0.63	0.71	0.67	0.06
		Cecum			
H3080G10	APC	0.68	0.73	0.82	0.06
H3080F09	APC	0.72	0.69	0.68	0.03
H3031E05	β-catenin	1.48	1.70	1.67	0.31
H3084D05	Cyclin D1	0.69^{ab}	0.76^{b}	0.59^{a}	0.16
H3093H12	MMP7	0.88	0.85	0.70	0.12
H3155B12	PPARdelta	1.18	1.09	1.16	0.27
H3076D10	C-myc	1.01	0.93	0.90	0.14
H3089H11	C-myc	0.64	0.65	0.71	0.06

[1]Data represent mean intensity values of mRNA expression for the corresponding gene in the treatment groups, C(control), S(sulindac) and A(anthocyanins).
[2]Microarray slides have two spots for a few identical genes. APC and C-myc have two spots each and 2 intensity values from each spot were obtained.
[3]Plate ID: sequence number for NIA 15K mouse cDNA library set.
[4]SEM; standard error of mean.
[a,b,c]Means in the same row not sharing a common superscript are significantly different ($P<0.05$).

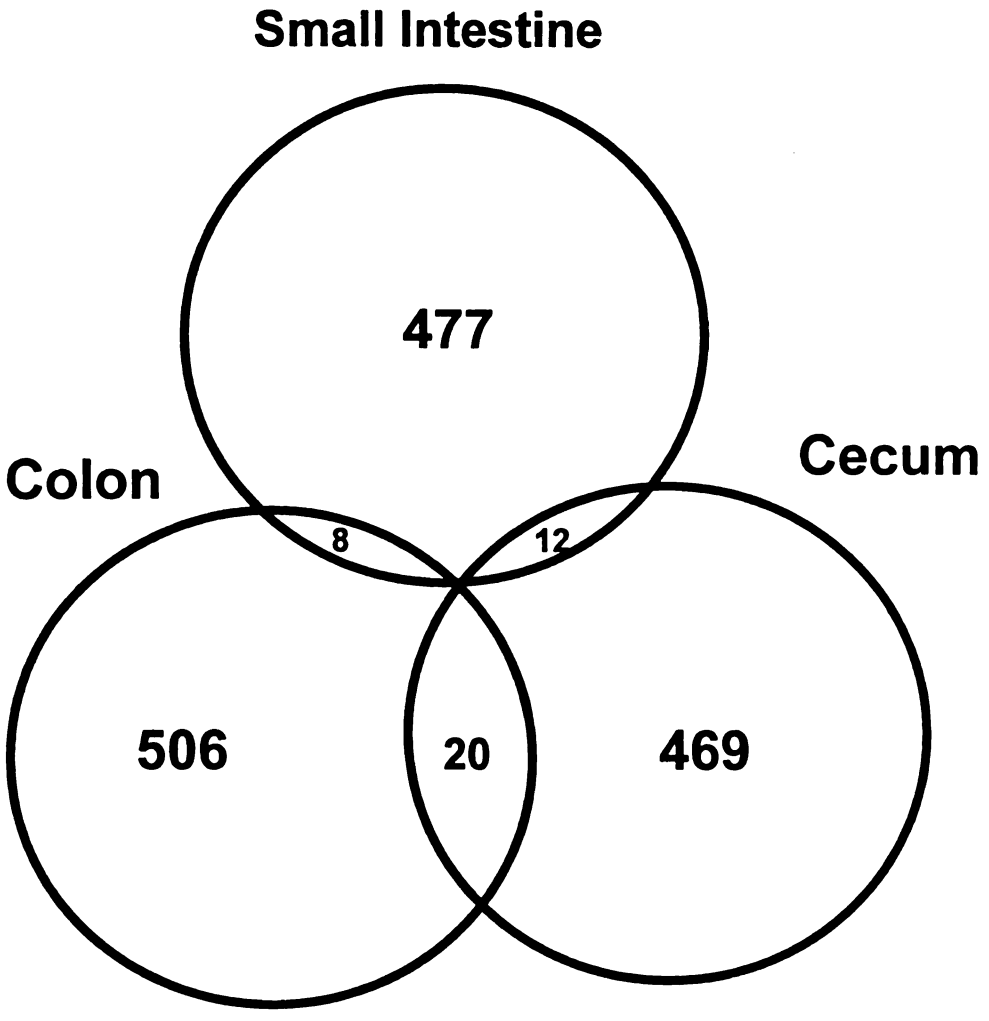


Figure 28. Numbers of genes influenced by any treatment ($P<0.05$) in intestinal sections of APC^{Min} mice. Numbers overlapping two sections were influenced by treatment in both tissues.

Table 5. Expression of 8 genes influenced by treatment both in small intestine and colon[1]

Plate ID[2]	Gene Name	C	S	A	SEM[3]
	Small Intestine				
H3046B04	placental lactogen 2	0.66^{a}	0.75^{a}	0.94^{b}	0.05
H3085E07	unknown	0.72^{a}	0.85^{ab}	0.91^{b}	0.04
H3022F05	3-phosphoglycerate dehydrogenase (Phgdh)	0.84^{ab}	0.78^{a}	1.06^{b}	0.06
H3084B06	F-box protein FBX15	0.80^{b}	0.65^{a}	0.88^{b}	0.03
H3045C02	ubiquitin-conjugating enzyme	0.69^{b}	0.54^{a}	0.71^{b}	0.02
H3003D11	lymphoid-specific transcription factor NFATc3	1.70^{b}	1.34^{ab}	0.71^{a}	0.20
H3090D11	unknown	0.89^{b}	0.70^{a}	0.71^{a}	0.04
H3015G01	no hit found	1.61^{b}	1.27^{ab}	1.16^{a}	0.10
	Colon				
H3046B04	placental lactogen 2	0.80^{c}	0.72^{b}	0.64^{a}	0.01
H3085E07	unknown	0.66^{a}	0.93^{b}	0.63^{a}	0.06
H3022F05	3-phosphoglycerate dehydrogenase (Phgdh)	0.75^{a}	0.88^{b}	0.82^{ab}	0.03
H3084B06	F-box protein FBX15	0.84^{b}	0.67^{b}	0.59^{a}	0.06
H3045C02	ubiquitin-conjugating enzyme	0.73^{b}	0.75^{b}	0.67^{a}	0.02
H3003D11	lymphoid-specific transcription factor NFATc3	1.92^{b}	1.32^{a}	1.71^{b}	0.09
H3090D11	unknown	0.92^{b}	0.71^{a}	0.92^{b}	0.04
H3015G01	no hit found	1.48^{b}	1.25^{a}	1.59^{b}	0.05

[1]Data represent mean intensity values of mRNA expression for the corresponding gene in the treatment groups, C(control), S(sulindac) and A(anthocyanins).
[2]Plate ID: sequence number for NIA 15K mouse cDNA library set
[3]SEM; standard error of mean.
[a,b,c]Means in the same row not sharing a common superscript are significantly different ($P<0.05$).

Table 6. Expression of 12 genes influenced by treatment both in small intestine and cecum[1]

Plate ID[2]	Gene Name	C	S	A	SEM[3]
	Small Intestine				
H3117A06	voltage-dependent calcium channel beta-3 subunit	0.75^{b}	0.59^{a}	0.64^{a}	0.02
H3113A12	growth arrest specific protein 5	1.07^{b}	0.88^{ab}	0.79^{a}	0.06
H3057B12	tubulin beta 3	1.44^{b}	1.27^{ab}	1.00^{a}	0.08
H3067F05	collagen IV	0.96^{b}	0.72^{a}	0.66^{a}	0.06
H3051G02	unknown	1.68^{b}	1.46^{ab}	1.07^{a}	0.13
H3154D02	kangai 1 (suppression of tumorigenicity 6)	0.82^{ab}	0.61^{a}	0.91^{b}	0.06
H3060C12	unknown	0.84^{b}	0.66^{a}	0.78^{b}	0.03
H3119E07	hypothetical	0.75^{a}	0.73^{a}	0.86^{b}	0.03
H3104H02	alternative splicing factor	0.79^{a}	0.81^{a}	0.98^{b}	0.02
H3033B06	unknown	0.74^{ab}	0.65^{a}	0.94^{b}	0.05
H3042A04	unknown	0.74^{a}	0.69^{a}	0.87^{b}	0.03
H3064D02	unknown	0.73^{ab}	0.63^{a}	0.80^{b}	0.03
	Cecum				
H3117A06	voltage-dependent calcium channel beta-3 subunit	0.62^{a}	0.61^{a}	0.75^{b}	0.03
H3113A12	growth arrest specific protein 5	1.78^{b}	1.55^{ab}	1.38^{a}	0.08
H3057B12	tubulin beta 3	3.16^{b}	2.40^{ab}	1.94^{a}	0.26
H3067F05	collagen IV	0.88^{b}	0.91^{b}	0.66^{a}	0.05
H3051G02	unknown	1.35^{b}	1.16^{ab}	0.81^{a}	0.12
H3154D02	kangai 1 (suppression of tumorigenicity 6)	0.84^{b}	0.70^{ab}	0.63^{a}	0.04
H3060C12	unknown	0.62^{a}	0.63^{a}	0.73^{b}	0.02
H3119E07	hypothetical	0.63^{a}	0.62^{a}	0.98^{b}	0.04
H3104H02	alternative splicing factor	1.55^{a}	1.29^{a}	1.04^{b}	0.08
H3033B06	unknown	0.68^{a}	0.67^{a}	0.91^{b}	0.05
H3042A04	unknown	0.77^{b}	0.76^{b}	0.62^{a}	0.04
H3064D02	unknown	0.61^{a}	0.59^{a}	0.73^{b}	0.02

[1]Data represent mean intensity values of mRNA expression for the corresponding gene in the treatment groups, C(control), S(sulindac) and A(anthocyanins).
[2]Plate ID: sequence number for NIA 15K mouse cDNA library set
[3]SEM; standard error of mean.
[a,b,c]Means in the same row not sharing a common superscript are significantly different ($P<0.05$).

Table 7. Expression of 20 genes influenced by treatment both in cecum and colon[1]

Plate ID[2]	Gene Name	C	S	A	SEM[3]
	Cecum				
H3112C06	no hit found	31.02^{b}	30.29^{b}	7.06^{a}	4.99
H3073B01	histocompatibility complex region containing the Q region of class I	0.76^{b}	0.69^{ab}	0.59^{a}	0.04
H3061A05	zinc finger protein RIN ZF (RINZF)	0.82^{b}	0.85^{b}	0.66^{a}	0.04
H3114G07	no hit found	1.25^{b}	1.04^{a}	1.01^{a}	0.05
H3117D10	hypothetical	0.93^{b}	0.87^{b}	0.78^{a}	0.03
H3088E03	unknown	0.85^{ab}	0.88^{b}	0.77^{a}	0.03
H3132A08	unknown	0.64^{ab}	0.70^{b}	0.59^{a}	0.02
H3148F01	unknown	0.69^{b}	0.60^{a}	0.66^{ab}	0.02
H3068E08	unknown	0.66^{a}	0.81^{b}	0.63^{a}	0.03
H3030E03	unknown	0.66^{a}	0.77^{b}	0.71^{ab}	0.02
H3078H05	MORF-related gene X	0.70^{ab}	0.65^{a}	0.83^{b}	0.04
H3052B09	nuclear antigen Sp100 (Sp100)	0.61^{a}	0.61^{a}	0.74^{b}	0.03
H3056H07	unknown	0.59^{a}	0.63^{a}	0.72^{b}	0.02
H3138A02	SERINE/THREONINE-PROTEIN KINASE 4 (P21-ACTIVATED KINASE 4)	0.58^{a}	0.61^{a}	0.74^{b}	0.02
H3092C04	acid ceramidase (Asah1)	0.57^{a}	0.64^{a}	0.78^{b}	0.04
H3156B09	unknown	0.63^{a}	0.63^{a}	0.90^{b}	0.03
H3111D12	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 2 (Cd36l2)	1.74^{a}	1.85^{a}	2.53^{b}	0.19
H3007H10	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	1.76^{a}	1.51^{a}	2.79^{b}	0.28
H3144A09	unknown	0.83^{a}	0.82^{a}	1.35^{b}	0.13
H3007F10	UMP-CMP kinase (UMP-CMPK)	1.51^{a}	1.62^{a}	2.69^{b}	0.16
	Colon				
H3112C06	no hit found	25.10^{b}	7.04^{a}	22.14^{b}	3.29
H3073B01	histocompatibility complex region containing the Q region of class I	0.69^{ab}	0.79^{b}	0.64^{a}	0.03
H3061A05	zinc finger protein RIN ZF (RINZF)	0.69^{a}	0.90^{b}	0.68^{a}	0.05
H3114G07	no hit found	1.08^{a}	1.61^{b}	1.20^{a}	0.06
H3117D10	hypothetical	0.97^{b}	0.75^{a}	0.79^{a}	0.04
H3088E03	unknown	0.71^{a}	0.85^{b}	0.75^{a}	0.02
H3132A08	unknown	0.62^{a}	0.79^{b}	0.68^{a}	0.03
H3148F01	unknown	0.62^{a}	0.72^{b}	0.68^{ab}	0.02
H3068E08	unknown	0.59^{a}	0.72^{b}	0.65^{a}	0.02

H3030E03	unknown	0.69^{a}	0.94^{b}	0.62^{a}	0.04
H3078H05	MORF-related gene X	0.70^{a}	0.88^{b}	0.68^{a}	0.05
H3052B09	nuclear antigen Sp100 (Sp100)	0.59^{a}	0.73^{b}	0.63^{ab}	0.03
H3056H07	unknown	0.66^{ab}	0.72^{b}	0.58^{a}	0.03
H3138A02	SERINE/THREONINE-PROTEIN KINASE 4 (P21-ACTIVATED KINASE 4)	0.65^{a}	0.85^{b}	0.66^{a}	0.05
H3092C04	acid ceramidase (Asah1)	0.63^{a}	0.82^{b}	0.62^{a}	0.04
H3156B09	unknown	0.81^{a}	0.89^{b}	0.81^{a}	0.02
H3111D12	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 2 (Cd36l2)	2.01^{b}	1.40^{a}	1.87^{b}	0.13
H3007H10	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	2.67^{b}	1.79^{a}	2.51^{b}	0.19
H3144A09	unknown	0.67^{a}	0.82^{b}	0.85^{b}	0.03
H3007F10	UMP-CMP kinase (UMP-CMPK)	2.92^{b}	1.76^{a}	2.86^{b}	0.27

[1]Data represent mean intensity values of mRNA expression for the corresponding gene in the treatment groups, C(control), S(sulindac) and A(anthocyanins).
[2]Plate ID: sequence number for NIA 15K mouse cDNA library set
[3]SEM; standard error of mean.
[a,b,c]Means in the same row not sharing a common superscript are significantly different ($P<0.05$).

Table 8. List of genes up-regulated by anthocyanins vs control and sulindac in cecum

Plate ID[1]	Gene Name[2]	A/C[3]	A/S[3]
H3144F08	hemochromatosis (HFE) gene	2.86	3.26
H3018C09	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac 1)	2.64	2.79
H3131F08	Ly6/neurotoxin 1 protein	2.33	3.38
H3004A09	unknown	1.79	1.61
H3042E10	unknown	1.75	1.60
H3001H08	adrenomedullin precursor	1.71	1.79
H3101B09	unknown	1.61	1.67
H3016E09	unknown	1.59	1.42
H3144C09	tetratricopeptide repeat domain	1.58	1.49
H3119E07	hypothetical	1.55	1.57
H3004B09	mitochondrial ribosomal protein S7	1.55	1.39
H3078D06	unknown	1.49	1.48
H3004F11	unknown	1.47	1.52
H3014H02	hypothetical	1.45	1.38
H3023D09	unknown	1.45	1.42
H3045D09	NEUROENDOCRINE DIFFERENTIATION FACTOR	1.45	1.35
H3051H08	initiate factor 5	1.44	1.55
H3061E10	unknown	1.44	1.35
H3104E01	unknown	1.44	1.46
H3146B05	unknown	1.44	1.25
H3035G12	unknown	1.44	1.45
H3012B09	nemo-like kinase	1.43	1.33
H3006D05	no hit found	1.41	1.31
H3023C01	hypothetical	1.41	1.39
H3014G07	astrocytic phosphoprotein	1.40	1.36
H3008G10	bladder cancer associated protein	1.39	1.44
H3008G05	translocase of inner mitochondrial membrane 8	1.39	1.36
H3001A02	secretin	1.37	1.41
H3094G12	unknown	1.35	1.26
H3082F05	unknown	1.34	1.36
H3144G09	unknown	1.33	1.26
H3047H09	unknown	1.32	1.17
H3033B06	no hit found	1.32	1.35
H3067G09	unknown	1.32	1.27
H3067G10	unknown	1.29	1.25
H3098E01	no hit found	1.29	1.25
H3052G06	plastin 2	1.29	1.25
H3064F09	chloride channel regulator Icln pseudogene	1.28	1.26
H3147F06	malate NADP oxidoreductase	1.28	1.23

H3081E07	Sjogren syndrome antigen A2	1.28	1.29
H3082A04	unknown	1.28	1.28
H3100F05	unknown	1.27	1.13
H3047C03	early onset breast cancer protein 2	1.27	1.16
H3134C08	unknown	1.26	1.13
H3098B02	unknown	1.26	1.22
H3060C06	unknown	1.26	1.22
H3035E03	hypothetical	1.25	1.20
H3039A12	unknown	1.25	1.26
H3134A09	unknown	1.25	1.23
H3055B07	unknown	1.24	1.24
H3027A07	translation repressor NAT1	1.24	1.16
H3060D05	beta-defensin 8	1.23	1.17
H3080E02	unknown	1.23	1.28
H3087C08	unknown	1.23	1.22
H3022D09	beta-glucoronidase	1.22	1.21
H3037A04	no hit found	1.21	1.17
H3134E02	solitary transfer RNA-Asp	1.21	1.12
H3052C08	coproporphyrinogen oxidase	1.21	1.17
H3014B04	unknown	1.21	1.21
H3082F09	unknown	1.21	1.23
H3117A06	voltage-dependent calcium channel beta-3 subunit	1.20	1.23
H3065G09	unknown	1.20	1.13
H3064D02	unknown	1.19	1.24
H3157B09	unknown	1.19	1.12
H3044E02	ribonucleotide reductase M2	1.19	1.23
H3045C06	unknown	1.18	1.13
H3060C12	unknown	1.18	1.16
H3084C03	hippocalcin-like 1	1.18	1.19
H3134H09	unknown	1.17	1.15
H3065E12	unknown	1.16	1.14
H3074E09	unknown	1.16	1.12
H3032A02	unknown	1.15	1.20
H3133H04	unknown	1.15	1.17
H3055A04	unknown	1.13	1.18
H3093G12	unknown	1.12	1.15
H3126A12	DNA POLYMERASE EPSILON P12 SUBUNIT	1.11	1.14
H3057E08	unknown	1.11	1.17
H3027D11	unknown	1.10	1.12
H3054F10	unknown	1.08	1.19

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set

[2]This list of genes includes those that were significantly up-regulated by anthocyanins compared to control and sulindac ($P<0.05$). Names written in capital

letters are for the respective homolog of the genes. The total number of genes that were up-regulated by anthocyanins is 79, which includes 44 unknown, 4 no hit found and 4 hypothetical protein genes.

[3]Ratio of intensity score: A/C; anthocyanins versus control, A/S; anthocyanins versus sulindac. Ratio represents fold change in gene expression in tissue of mice consuming anthocyanins when compared that of mice consuming control or sulindac.

Table 9. List of genes down-regulated by anthocyanins vs control and sulindac in cecum

Plate ID[1]	Gene Name[2]	C/A[3]	S/A[3]
H3119A02	unknown	2.27	1.77
H3146G09	unknown	2.01	2.35
H3065C02	GTP-binding protein NGB	1.78	1.42
H3119C02	olfactory receptor gene cluster	1.72	1.71
H3003G04	cell adhesion molecule nectin-3 alpha	1.63	1.55
H3104D01	beta-1,4-galactosyltransferase	1.57	1.45
H3039F01	LEUKOTRIENE B4 12-HYDROXYDEHYDROGENASE	1.55	1.51
H3117H05	OXYSTEROL BINDING PROTEIN-RELATED PROTEIN 3	1.55	1.43
H3072C07	unknown	1.45	1.28
H3157E05	zinc transporter 4	1.42	1.35
H3071E04	unknown	1.37	1.44
H3075A02	ornithine decarboxylase	1.36	1.33
H3134D02	intermediate filament proteins family	1.36	1.32
H3090H01	chromatin structural protein	1.34	1.33
H3094A05	hypothetical	1.34	1.28
H3035A05	no hit found	1.34	1.35
H3157D05	unknown	1.32	1.40
H3067F05	unknown	1.32	1.38
H3032F04	unknown	1.31	1.26
H3082F04	unknown	1.29	1.30
H3105F07	hypothetical	1.28	1.30
H3090D06	unknown	1.28	1.21
H3142G03	unknown	1.25	1.37
H3131E06	AS oncogene family	1.25	1.25
H3042A04	unknown	1.24	1.23
H3044D08	unknown	1.24	1.25
H3131E05	unknown	1.24	1.20
H3074C05	unknown	1.24	1.16
H3081D02	Bcl-2-related ovarian killer protein, apoptosis activator	1.23	1.26
H3096C02	no hit found	1.22	1.29
H3033A09	no hit found	1.21	1.33
H3067G06	no hit found	1.21	1.26
H3033C10	unknown	1.19	1.19
H3044D03	unknown	1.19	1.16
H3072C05	unknown	1.19	1.12
H3150H07	no hit found	1.18	1.14
H3105G09	aryl hydrocarbon receptor nuclear translocator-like	1.18	1.26
H3033D05	unknown	1.17	1.20
H3037C05	unknown	1.15	1.17

H3080G07 unknown	1.15	1.14
H3117H06 unknown	1.14	1.19
H3084A06 spindlin	1.14	1.18
H3126D02 unknown	1.14	1.12
H3136G08 no hit found	1.12	1.11

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set
[2]This list of genes includes those that were significantly down-regulated by anthocyanins compared to control and sulindac ($P<0.05$). The total number of genes that were down-regulated by anthocyanins is 44, which includes 24 unknown, 6 no hit found and 2 hypothetical protein genes.
[3]Ratio of intensity score: C/A; control versus anthocyanins, S/A; sulindac versus anthocyanins. Ratio represents fold change in gene expression in tissue of mice consuming anthocyanins when compared that of mice consuming control or sulindac.

Table 10. List of genes up-regulated by anthocyanins vs control and sulindac in colon

Plate ID[1]	Gene Name[2]	A/C[3]	A/S[3]
H3029A08	cytochrome b5	1.73	1.36
H3063C01	unknown	1.53	1.54
H3051C03	aldolase C	1.52	1.48
H3009C07	heat shock protein	1.49	1.32
H3071C08	E(y)2 PROTEIN, CG10347 PROTEIN	1.48	1.37
H3069H09	unknown	1.44	1.31
H3008E10	no hit found	1.41	1.50
H3095E02	unknown	1.40	1.22
H3008C06	unknown	1.38	1.34
H3003G03	45S pre rRNA	1.37	1.36
H3075E12	TRANSPORTIN-SR	1.36	1.32
H3125G09	unknown	1.34	1.25
H3036G07	epithelial cell transmembrane protein antigen precursor	1.33	1.43
H3131A08	TRANSLOCON-ASSOCIATED PROTEIN, SIGNAL SEQUENCE RECEPTOR BETA SUBUNIT)	1.32	1.18
H3074A08	TRANSFORMING PROTEIN RHOA (RhoA GTPase)	1.32	1.35
H3051E03	unknown	1.31	1.48
H3042E08	interleukin 1 receptor-associated kinase (Il1rak)	1.30	1.35
H3003D06	inhibitor of growth family member 1 (ING1)	1.29	1.27
H3094G06	unknown	1.27	1.37
H3108A08	BIMP1 (apoptosis regulator)	1.27	1.34
H3136F07	guanine monphosphate synthetase (GMPS)	1.25	1.13
H3153H04	CALCINEURIN B	1.25	1.28
H3058C10	coagulation factor II receptor-like 3 (F2RL3)	1.23	1.34
H3010D11	unknown	1.23	1.55
H3113G05	unknown	1.21	1.16
H3122A12	S-adenosylmethionine decarboxylase	1.21	1.10
H3032A06	butyrophilin-like 2	1.20	1.28
H3041G12	CELL CYCLE PROGRESSION RESTORATION GENE 3	1.15	1.11
H3044F03	unknown	1.14	1.14
H3050C12	unknown	1.14	1.10
H3008B10	ATPase, H+ transporting, lysosomal I (Atp6i)	1.14	1.26
H3008G04	GATA binding protein 2	1.13	1.23
H3006F08	unknown	1.13	1.34

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set
[2]This list of genes includes those that were significantly up-regulated by anthocyanins compared to control and sulindac ($P<0.05$). The total number of genes that were up-regulated by anthocyanins is 33, which includes 10 unknown genes and 1 no hit gene.

[3]Ratio of intensity score: A/C; anthocyanins versus control, A/S; anthocyanins versus sulindac. Ratio represents fold change in gene expression in tissue of mice consuming anthocyanins when compared that of mice consuming control or sulindac.

Table 11. List of genes down-regulated by anthocyanins vs control and sulindac in colon

Plate ID[1]	Gene Name[2]	C/A[3]	S/A[3]
H3130F06	SERUM PROTEIN MSE55	1.59	1.43
H3086B08	unknown	1.39	1.24
H3073E04	initiation factor 5	1.36	1.34
H3089F08	unknown	1.33	1.24
H3074H01	unknown	1.31	1.28
H3040D03	PSD-95/SAP90-associated protein-4	1.30	1.35
H3123D04	unknown	1.29	1.29
H3089D05	hypothetical	1.28	1.44
H3026H05	unknown	1.27	1.36
H3053A06	inositol 1,4,5-trisphosphate 3-kinase B (ITPKB)	1.26	1.19
H3028H05	KIAA0938 protein (KIAA0938)	1.26	1.39
H3046B04	placental lactogen 2	1.25	1.14
H3105D11	PHOSPHOLYSINE PHOSPHOHISTIDINE INORGANIC PYROPHOSPHATE PHOSPHATASE	1.25	1.18
H3086H05	Deleted in colorectal cancer(Dcc)	1.24	1.34
H3026D05	N-myristoyltransferase 1	1.22	1.21
H3033F06	neuronal apoptosis inhibitory protein 7 (Naip7)	1.21	1.15
H3017A07	A kinase (PRKA) anchor protein (yotiao) 9 (AKAP9)	1.21	1.20
H3068H04	ribonuclease/angiogenin inhibitor 2 (Rnh2)	1.21	1.19
H3106B07	orphan nuclear receptor (NR2E1)	1.20	1.20
H3090H08	KIAA0676 PROTEIN	1.20	1.19
H3128F02	hypothetical	1.19	1.26
H3045H05	unknown	1.19	1.30
H3068F08	protein tyrosine phosphatase, non-receptor type 21	1.19	1.25
H3096F11	HNK-1 sulfotransferase	1.18	1.22
H3078H10	ADP-ribosylation factor 1	1.18	1.27
H3112F03	degenerative spermatocyte	1.18	1.21
H3056D05	no hit found	1.15	1.14
H3100D11	no hit found	1.14	1.09
H3134C09	Rhesus blood group protein (Rh)	1.14	1.33
H3068B02	hypothetical FYVE-FINGER-CONTAINING	1.13	1.18
H3074F09	RAB5 EFFECTOR PROTEIN RABENOSYN-5	1.13	1.14
H3128B11	putative oncogene (HLC14-06-P)	1.13	1.07
H3045C02	ubiquitin-conjugating enzyme	1.09	1.11

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set
[2]This list of genes includes those that were significantly down-regulated by anthocyanins compared to control and sulindac ($P<0.05$). The total number of genes

that were down-regulated by anthocyanins is 33, which includes 6 unknown, 2 no hit and 3 hypothetical protein genes.
[2]Ratio of intensity score: C/A; control versus anthocyanins, S/A; sulindac versus anthocyanins. Ratio represents fold change in gene expression in tissue of mice consuming anthocyanins when compared that of mice consuming control or sulindac.

Table 12. List of genes up-regulated by sulindac vs control in small intestine

Plate ID[1]	Gene Name[2]	S/C[3]
H3111B05	no hit found	3.53
H3139D09	ATPase subunit 6	3.36
H3119B08	no hit found	2.43
H3016H08	no hit found	2.15
H3112H02	no hit found	2.06
H3020A01	no hit found	1.95
H3155H07	unknown	1.85
H3117H08	actin related protein 2/3 complex, subunit 4	1.77
H3009D08	unknown	1.72
H3109B12	putative nuclear protein	1.57
H3069D02	hypothetical	1.56
H3117C01	KRUPPEL-RELATED ZINC FINGER PROTEIN	1.55
H3114G09	no hit found	1.54
H3118A01	no hit found	1.52
H3058E07	microtubule-associated protein 4	1.42
H3139D12	cytoplasmic dynein intermediate chain 2	1.40
H3076E07	unknown	1.39
H3158G06	erythroid differentiation regulator	1.37
H3085E11	unknown	1.33
H3130A07	unknown	1.32
H3132G06	hypothetical	1.28
H3068C02	unknown	1.27
H3032A01	translocase of inner mitochondrial membrane	1.26
H3085C07	zinc finger protein	1.26
H3117E10	unknown	1.23
H3150H11	hypothetical	1.22
H3139C06	unknown	1.22
H3047G07	unknown	1.22
H3138C07	unknown	1.21
H3041A07	no hit found	1.19
H3155A07	unknown	1.18
H3046A02	unknown	1.18
H3040C12	unknown	1.15
H3001D07	unknown	1.12

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set
[2]The list of genes includes those that were significantly up-regulated by sulindac compared to control ($P<0.05$). Names written capital letters are for the respective homolog of the genes. The total number of genes that were up-regulated by sulindac is 53, which includes 19 co-regulated with anthocyanins (Table 13), 16 unknown, 6 no hit and 3 hypothetical protein genes.

[3]Ratio of intensity score; S/C: sulindac versus control. Ratio represents fold change in gene expression in tissue of mice consuming sulidac when compared that of mice consuming control.

Table 13. List of genes up-regulated by sulindac and anthocyanins in small intestine

Plate ID[1]	Gene Name[2]	S/C[3]	A/C[3]
H3019H02	TAX INTERACTION PROTEIN 1	1.45	1.46
H3023H01	no hit found	1.36	1.38
H3155E09	hypothetical	1.36	1.39
H3102C12	ADP-ribosylation-like factor 6 interacting protein	1.30	1.22
H3125C12	guanine nucleotide binding protein, alpha inhibiting 3	1.29	1.20
H3145A05	hypothetical	1.27	1.29
H3149A09*	mas proto-oncogene,insulin-like growth factor type 2 receptor (Igf2r), L41ps, and Au76 peusogenes	1.26	1.13
H3149H02	unknown	1.26	1.17
H3066E10	no hit found	1.23	1.25
H3060E03	no hit found	1.23	1.29
H3077H06	LIM domain binding 1	1.21	1.79
H3026G06	tumor rejection antigen gp96	1.20	1.18
H3148H07	hypothetical	1.19	1.35
H3128B04	liver-specific bHLH-Zip transcription factor	1.19	1.22
H3108A01	interferon-stimulated protein	1.16	1.29
H3063G07	unknown	1.16	1.40
H3039G11	unknown	1.13	1.13
H3109B07	unknown	1.13	1.10
H3155C06	heat stable antigen	1.12	1.22

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set

[2]The list of genes includes those that were significantly up-regulated by both sulindac and anthocyanins compared to control ($P<0.05$).

Names written capital letters are for the respective homolog of the genes. The total number of genes that were up-regulated by both sulindac and anthocyanins is 19, which includes 4 unknown, 3 no hit and 3 hypothetical protein genes.

[3]Ratio of intensity score; S/C: sulindac versus control, A/C: anthocyanins versus control. Ratio represents fold change in gene expression in tissue of mice consuming sulidac or anthocyanins when compared that of mice consuming control.

*The true identity of the H3149A09 gene cannot be identified at this time and could be one of the four.

Table 14. List of genes down-regulated by sulindac vs control in small intestine

Plate ID[1]	Gene Name[2]	C/S[3]
H3071D01	no hit found	1.68
H3086A12	unknown	1.54
H3154B11	adipocyte-specific protein 5	1.49
H3146F03	hypothetical	1.46
H3123H07	novel cell death-regulatory protein GRIM19	1.44
H3107D06	unknown	1.41
H3065F02	unknown	1.40
H3033F02	no hit found	1.38
H3134E01	hemoglobin, beta adult major chain	1.36
H3122H02	unknown	1.36
H3122F10	putative TH1 protein	1.36
H3142F09	hypothetical	1.34
H3087F06	dynein	1.34
H3108F07	alpha-L-iduronidase	1.33
H3049B04	no hit found	1.32
H3136F06	unknown	1.32
H3132G02	hypothetical	1.31
H3096H01	no hit found	1.30
H3138B03	unknown	1.30
H3156C08	metaxin	1.30
H3033D08	unknown	1.30
H3108D06	unknown	1.29
H3020F09	hypothetical	1.29
H3045C02	ubiquitin-conjugating enzyme	1.28
H3060C12	unknown	1.28
H3025G06	hypothetical	1.27
H3126H02	no hit found	1.27
H3045H04	nonmuscle heavy chain myosin	1.27
H3060D01	CCR4-NOT transcription complex, subunit 7	1.27
H3065B01	hypothetical	1.27
H3078G08	unknown	1.26
H3035D04	GM2 ganglioside activator protein	1.25
H3042B06	unknown	1.25
H3042F12	serine (or cysteine) proteinase inhibitor	1.25
H3033A02	no hit found	1.25
H3108F08	programmed cell death 10	1.24
H3081F03	hypothetical	1.24
H3084B06	F-box protein	1.23
H3074C07	unknown	1.22
H3140F02	unknown	1.22

H3072C12	unknown	1.21
H3015E11	PDZ and carboxyl terminal LIM domain 1	1.21
H3099H06	unknown	1.20
H3044D04	unknown	1.20
H3112B06	unknown	1.20
H3020H08	unknown	1.20
H3065B11	unknown	1.20
H3057D02	RhoGDI-1	1.19
H3016F02	unknown	1.18
H3047G01	unknown	1.18
H3101F01	hypothetical	1.18
H3104D02	unknown	1.17
H3060F12	hypothetical	1.16
H3122A08	unknown	1.16
H3096F03	unknown	1.16
H3109F02	DREV protein	1.15
H3113F11	unknown	1.14
H3100G04	no hit found	1.14
H3064B06	no hit found	1.13
H3077A12	hypothetical	1.11
H3104E04	no hit found	1.07

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set
[2]The list of genes includes those that were significantly down-regulated by sulindac compared to control ($P<0.05$). The total number of genes that were down-regulated by sulindac is 108, which includes 47 genes co-regulated with anthocyanins (Table 15), 25 unknown, 9 no hit and 10 hypothetical protein genes.
[3]Ratio of intensity score; C/S: sulindac versus control. Ratio represents fold change in gene expression in tissue of mice consuming sulidac when compared that of mice consuming control.

Table 15. List of genes down-regulated by sulindac and anthocyanins in small intestine

Plate ID[1]	Gene Name[2]	C/S[3]	C/A[3]
H3104D07	fatty acid binding protein 3	2.34	1.78
H3113B12	no hit found	1.97	3.30
H3156A10	unknown	1.84	2.16
2038	glutathione S transferase	1.67	1.69
H3007A01	unknown	1.66	1.56
H3091A01	hypothetical	1.65	1.39
H3105B07	hypothetical	1.52	1.38
H3124H12	no hit found	1.49	3.02
H3010H09	unknown	1.43	1.49
H3018G10	unknown	1.42	1.39
H3004B03	no hit found	1.37	1.55
H3064F03	hypothetical	1.37	1.28
H3114G02	unknown	1.37	1.33
H3039E10	serine/threonine kinase	1.36	1.26
H3078F01	unknown	1.36	1.33
H3051E02	unknown	1.35	1.37
H3100F10	unknown	1.33	1.27
H3067F05	unknown	1.32	1.44
H3127G08	hypothetical	1.31	1.36
H3098E02	no hit found	1.30	1.26
H3100E10	hypothetical	1.30	1.36
H3143A07	unknown	1.28	1.46
H3097E12	unknown	1.28	1.20
H3084F07	unknown	1.28	1.39
H3072E11	unknown	1.27	1.25
H3090D11	unknown	1.27	1.26
H3024F08	protein kinase, interferon inducible double stranded RNA dependent activator	1.27	1.28
H3117A06	voltage-dependent calcium channel beta-3 subunit	1.26	1.17
H3142G12	hypothetical	1.26	1.13
H3057C12	hypothetical	1.26	1.35
H3088B01	hypothetical	1.26	1.26
H3008C03	cytotoxic granule-associated RNA binding protein	1.25	1.28
H3047D07	nuclear receptor subfamily 5, group A, member 2	1.25	1.24
H3033D06	no hit found	1.24	1.31
H3142C06	hypothetical	1.23	1.13
H3090F07	unknown	1.23	1.14
H3100H05	unknown	1.22	1.30
H3119B07	unknown	1.21	1.23

H3126F07 unknown	1.21	1.19
H3052A08 unknown	1.19	1.26
H3065C06 unknown	1.15	1.12
H3035G10 unknown	1.15	1.19
H3132G08 unknown	1.14	1.12
H3067B05 unknown	1.14	1.21
H3127G05 palmitoyl-protein thioesterase	1.13	1.17
H3032D10 no hit found	1.12	1.11
H3134H05 unknown	1.11	1.19

[1]Plate identification number: sequence number for NIA 15K mouse cDNA library set. Plate ID 2038 is not in the NIA 15K mouse cDNA library and added by Dr. John Lapres' lab in Michigan State University.

[2]This list of genes includes those that were significantly down-regulated by both sulindac and anthocyanins compared to control ($P<0.05$). The total number of genes that were down-regulated by both sulindac and anthocyanins is 47, which includes 24 unknown, 6 no hit and 9 hypothetical protein genes.

[3]Ratio of intensity score; C/S: sulindac versus control, C/A: anthocyanins versus control. Ratio represents fold change in gene expression in tissue of mice consuming sulidac or anthocyanins when compared that of mice consuming control.

DISCUSSION

In this study, we confirmed our previous results in which we found that anthocyanins and cyanidin suppressed tumor development in cecum of Apc^{Min} mice. In the previous experiments, the compounds (anthocyanins, cyanidin and sulindac) were administered in drinking water to a relatively small number (n=10) of mice per treatment group for 10 weeks. In this study, the extent to which dietary anthocyanins and sulindac inhibited intestinal tumor development was determined in a larger number of Apc^{Min} mice (n=25) when fed compounds in the diet for 8 weeks. This approach increased the stability of the administered anthocyanins, increased power of the experiment by increasing the number of replicates, and reduced the morbidity of mice by shortening the promotion period.

In this experiment, dietary anthocyanins significantly reduced adenoma number in all three intestinal sections. Cecal adenoma burden (volume) also was reduced and small intestinal adenoma mean diameter was intermediate in mice consuming anthocyanins. Sulindac reduced adenoma number and mean diameter in small intestine, but increased cecal adenoma number when compared to controls.

Koide et al. (1996) first reported that feeding red rice prolonged survival of Balb/C mice inoculated i.p. with Meth/A lymphoma cells and that hydrolyzed anthocyanins decreased the growth of HCT-15 cancer cells. Later, they found that sugar-bonded bioflavonoids extracted from red soybeans and red beans prolonged survival of Balb/C mice bearing tumor Meth/A cells and that anthocyanin fractions extracted from red soybeans inhibited the growth of HCT-15 cells (Koide et al., 1997). Pool-Zobol et al. (1999) have shown that anthocyanins reduced H_2O_2-

induced DNA strand breaks but did not prevent endogenous generation of oxidized DNA bases in HT 29 clone 19A human colon cancer cells. Recently, it has been reported that purple corn anthocyanins suppressed the promotion of colon tumors caused by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats initiated with dimethyl hydrazine. However, these researchers did not test the potential of purple corn color anthocyanins to directly suppress tumor development (Hagiwara et al., 2001).

The second objective of this study was to determine the influence of dietary anthocyanins and sulindac on expression and cellular localization of β-catenin in the intestine of Apc^{Min} mice. Contrary to our expectations, immunostaning of β-catenin in intestinal tissues demonstrated that sulindac generally did not influence the expression of β-catenin protein, whereas anthocyanins increased β-catenin expression in some intestinal regions compared to control or sulindac. Intracellular distribution of β-catenin was primarily in the membrane in all mice. These results are contradictory to the findings of other investigators. Mutanen et al. (2000) reported that rye bran diet prevented tumor formation in Apc^{Min} mice and decreased the cytosolic β-catenin level of intestinal epithelial cells. Cellular distribution of β-catenin also was modified by sphingolipid consumption (Schmelz et al., 2001). Sphingolipid feeding reduced adenoma numbers in all regions of the intestine in Apc^{Min} mice and sphingosine reduced cytosolic and nuclear β-catenin expression (Schmelz et al, 2001).

Gene expression results from the cDNA microarray analysis showed that expression of the mRNA transcripts for β-catenin and APC were not altered by

treatment, suggesting that transcriptional regulation did not cause the increase in β-catenin protein expression in the intestine of Apc^{Min} mice consuming anthocyanins. Other possible explanations for the increase in β-catenin expression by anthocyanins would be increased stability of β-catenin mRNA, decreased degradation of β-catenin, or increased translation. Additional experiments are necessary to address these possibilities.

APC mutation is permissive for aberrant accumulation of cytoplasmic β-catenin, which then translocates to the nucleus and binds Tcf/Lef transcription factors and causes increased transcriptional activation of genes in the Wnt/β-catenin signaling pathway. Known target genes of this signaling include PPARδ, C-Myc, Cyclin D1 and MMP7. These genes have been found to be overexpressed in human colonic adenocarcinomas and in many human colonic tumor cell lines (He et al. 1999; Zhang et al., 1997; Crawford et al., 1999; Tetsu and McCormick, 2000; Newell et al., 1994). Our interest in testing the potential of tart cherry anthocyanins and cyanidin to inhibit tumor development in Apc^{Min} mice stemmed from the observation that these compounds inhibit the activities of COX enzymes (Wang et al., 1999). McEntee et al. (1999) reported that NSAID-induced regression of intestinal adenomas is associated with reduced levels of β-catenin. NSAIDs also have been shown to inhibit PPARδ activity in HCT116 and SW480 cell lines in a dose-dependent manner (He et al., 1999). Therefore, we hypothesized that anthocyanins reduce tumor development in Apc^{Min} mice in a manner similar to sulindac by suppressing Wnt/β-catenin signaling.

In contrast to our initial hypothesis, our results indicate that neither anthocyanins nor sulindac influenced the expression of mRNA of β-catenin and most of the predicted target genes of the Wnt/β-catenin signaling. Anthocyanin treatment modestly reduced the expression of cyclin D1 compared to sulindac in cecum but not in the other intestinal tissues. The expression of the other predicted target genes – MMP7, PPARδ and C-Myc – were not altered by anthocyanins or sulindac in all intestinal sections of Apc^{Min} mice. These results suggest that the mechanisms responsible for reductions in intestinal tumor development by sulindac or anthocyanins likely are not associated with inhibition of Wnt/β-catenin signaling. We consider it likely that tumor suppression associated with these compounds is via mechanisms other than normalized Wnt/β-catenin signaling.

Although most hypotheses for explaining the anticarcinogenic effects of NSAIDs have focused on their common property to inhibit COX enzymes, the mechanism of the anticarcinogenic effect of these drugs has not been fully established. Little information on potential mechanisms whereby anthocyanins may suppress intestinal tumor development is available. Anthocyanins have been shown to inhibit the activity of epithermal growth factor receptor (EGFR) kinase. Meiers et al. (2001) demonstrated that two anthocyanins, cyanidin and delphinidin, are potent inhibitors of the EGFR kinase. Administration of inhibitors to the EGFR kinase also has been shown to reduce intestinal adenoma development in Apc^{Min} mice (Torrance et al., 2000).

A total of 1,492 genes were influenced by dietary treatment in any of the intestinal sections – small intestine, ceum or colon. A relatively small subset of

these gene effects overlapped across any two intestinal regions, and no single gene was influenced by treatment in all regions. To begin narrowing this list of gene changes to a shorter list of genes that merit future investigation, we have used two strategies. First, we identified genes influenced by treatment in more than one intestinal region. Second, we focused on gene changes that corresponded to the observed tumor numbers. For example, we are particularly interested in genes up- or down-regulated specifically by anthocyanins relative to sulindac and control treatments in the cecum or colon.

Although gene changes are ranked in the tables by fold change, it is important to consider that an arbitrary selection of a fold change value as significant (i.e. 2-fold) is probably not appropriate for two reasons. First, all gene changes reported in this experiment are based on statistical analysis of variance. Unlike many microarray experiments, in this study we have used true replication and valid statistical approaches to identify genes significantly influenced by treatment. Secondly, it is well established that the intestinal epithelium is a mixed cell population comprised by proliferating cells, terminally differentiated cells, and cells undergoing programmed cell death. Any changes in gene expression that occurred specifically in one of these cell types would be diluted by expression in the other epithelial cell types.

We identified several clusters of differentially expressed genes in cecum, colon and small intestine of Apc^{Min} mice using cDNA mcroarray analysis. Tumor inhibition by anthocyanins was most prominent in cecum. For this reason, we focused on genes changed specifically by anthocyanins relative to control and

sulindac in the cecum (Table 8). Although hemochromatosis gene and Ly6/Neurotoxin protein gene were influenced to the greatest extent by anthocyanins, there is very little information available on their functions or potential role in tumor development. Ras-related botulinum C3 substrate 1 also was up-regulated by anthocyanins. Clostridium botulinum C3 represents a major family of the bacterial toxins that transfer the ADP-ribose moiety of NAD to specific amino acids in acceptor proteins to modify key biological activities in eukaryotic cells, including protein synthesis, differentiation, transformation, and intracellular signaling (Han et al., 2001). C3-like transferases have been shown to ADP-ribosylate the small guanosine triphosphate (GTP)- binding protein family, Rho, resulting in functional inactivation of Rho (Wilde et al., 2001).

Among the many genes altered by anthocyanins, we are particularly interested in those genes that potentially are involved in cellular differentiation (neuroendocrine differentiation factor), DNA synthesis and repair (ribonucleotide reductase M2), and regulation of apoptosis (translocase of inner mitochondrial membrane 8). Ribonucleotide reductases catalyze the production of the deoxyribonucleotides required for DNA replication and repair. Yamaguchi et al. (2001) demonstrated that a newly identified ribonucleotide reductase, p53R2, is directly regulated by p53 for supplying nucleotides to repair damaged DNA. Translocases of the inner mitochondrial membrane mediate transport of mitochondrial proteins from the cytosol into the mitochondria in conjunction with translocases of the outer mitochondrial membrane (Halestrap et al., 2000). Translocases of inner mitochondrial membrane also influence mitochondrial

membrane permeability, which is a critical step of apoptotic pathways (Crompton, 2000). Brenner et al. (2000) demonstrated that adenine nucleotide translocator (ANT), the most abundant inner mitochondrial membrane protein, interacted with Bax, a proapoptotic member of the Bcl-2 family to yield an efficient composite channel. This opening of the mitochondrial permeability transition pore is thought to be involved in apoptosis.

Altered intracellular signaling by increased expression of initiation factor 5, serine/threonine kinases (tetratricopeptide repeat domain, nemo-like kinase) and suppressor of mitogen-activated protein (MAP) kinase (astrocytic phophoprotein) also merit further investigation. Initiation factor 5 plays a role in translation initiation following recognition of the start codon (Das and Maitra, 2000). It has been shown that initiation factor 5 is required for hydrolysis of GTPase and acts as a GTPase-activator protein (Paulin et al., 2001). Nemo-like kinase is a serine-threonine protein kinase that connects MAP kinase and Wnt/β-catenin signaling pathways in *Caenorhabditis elegans, Xenopus laevis and Drosophila melanogaster* (Ishitani et al., 1999; Rocheleau et al, 1999; Verheyen et al., 2001). Ishitani et al. (1999) demonstrated that activation of TAK1-MAP kinase (a kinase activated by transforming growth factor-beta) stimulated nemo-like kinase activity and downregulated Wnt signaling by inhibiting the interaction of the β-catenin-TCF complex with DNA. Astrocytic phosphoproteins are targets of numerous extracellular signals received by astrocytes. A major astrocytic phosphoprotein, PEA-15, has been identified as a protein kinase C substrate (Estelles et al., 1996). Formstecher et al. (2001) report that PEA-15 blocked epithelial receptor kinase

(ERK)-dependent transcription and proliferation by binding ERKs and preventing their localization in the nucleus. Genetic deletion of PEA-15 also resulted in increased ERK nuclear localization with consequent increased cFos transcription and cell proliferation. No functional information is available on bladder cancer associated protein.

Genes that were down-regulated in their expression by anthocyanins relative to control and sulindac include genes (Table 9) coding for G-protein coupled receptors (GTP-binding protein NGB, olfactory receptor cluster), cell adhesion proteins (nectin-3 alpha, beta-1,4-galactosyltransferase), enzymes involved in cell proliferation (ornithine decarboxylase), an oncogenic protein (AS oncogene family), an apoptosis-related protein (Bcl-2-related ovarian killer protein), chromatin structural proteins (intermediate filament proteins family, chromatin structural protein), and a nucloesome assembly protein (spindlin). G-protein coupled receptors are cell surface receptors that are coupled to heterotrimeric G-proteins (GTP-binding proteins). They have binding sites in the extracellular domain for glycoprotein hormones. Down-regulated expression of these G-protein coupled receptors may indicate a reduction in intracellular signaling necessary for tumor growth. Beta-1,4-galactosyltranferase 1 is the key enzyme transferring galactose to the terminal N-acetylglucosamine (GlcNAc) of glycoconjugates (Furukawa and Sato, 1999). It also serves as a cell adhesion molecule by recognizing and binding to terminal GlcNAc of glycoconjugates on the adjacent cell surface and matrix. Reduced expression of ornithine decarboxylase is also an important finding regarding the potential cellular actions of anthocyanins. Ornithine decarboxylase is a rate-limiting enzyme for

synthesis of polyamines that ultimately regulate DNA synthesis (Williams-Adams et al., 1972). It is well known that intracellular polyamine concentrations and ornithine decarboxylase activity are both increased in colorectal cancer tissue and in premalignant polyps (Wallace and Caslake, 2001).

In colon, anthocyanin feeding reduced adenoma numbers compared to both control and sulindac treated mice. Anthocyanins also tended to reduce colonic adenoma volume compared to controls. Anthocyanins specifically increased the expression (Table 10) of genes coding for proteins involved in signal transduction (heat shock protein, transforming protein Rho A, interleukin 1 receptor-associated kinase, calcineurin B), transcription (GATA binding protein 2), mRNA splicing (transportin-SR), translation (45S pre rRNA), apoptosis control (BIMP1, inhibitor of growth family 1) and cell cycle control (cell cycle progression restoration 3). Heat shock proteins are considered to be molecular chaperones which play a universal role in maintaining cellular homeostasis (Liu and Steinacker, 2001). Rho genes encoding small GTP-binding proteins have been implicated in actin organization and the interaction of the cytoskeleton with intracellular membranes (Lackie and Dow, 1999). It has been reported that signaling and transforming properties of G protein coupled receptors cause aberrant growth of cells via activation of Rho family small GTPases (Whitehead et al., 2001). Interleukin 1 receptor-associated kinase (IRAK-1) is essential for pro-inflammatory cytokine interleukin 1-beta signaling and interleukin 1 stimulation of nuclear factor κB (NF-κB), inducing an increase in the expression of many genes with roles in immunity and inflammation (Baldwin, 1996; Cooke et al., 2001). Calcineurin is a calmodulin-stimulated protein phosphatase and has a pivotal

role in the calcium/calmodulin-calcineurin dependent pathway (Aramburu et al., 2000). Calcineurin, or PP2B, is a highly conserved, Ca^{2+} calmodulin-dependent phosphoserine/phosphothreonine-specific phosphatase and regulates many processes including NMDA signaling, Na^{+}/K^{+} ATPase function, cardiac development and hypertrophy, T-cell activation, and angiogenesis (Cyert, 2001). GATA-binding proteins are a subfamily of zinc finger transcription factors with six members (GATA-1-6) that interact with the GATA deoxyribonucleic acid (DNA) sequence. GATA sequences are found in the regulatory regions of many genes including those encoding T-helper 2-like cytokines, receptors, adhesion molecules and enzymes (Caramori et al., 2001). The GATA family of transcription factors also regulates tissue-specific patterns of gene expression during development by functioning as transcriptional effectors of the small GTPase RhoA (Charron et al., 2001). 45 S rRNA is structurally homologous to mammalian 7 S RNA and plays an essential role in targeting proteins containing a particular signal peptide to the secretory apparatus by forming signal recognition-like particle. It also binds independently to protein elongation factor G (EF-G), and functions in the translation process (Nakamura et al., 2001). Transportin-SR proteins are serine/arginine-rich (SR) protein import receptors. SR proteins are a group of abundant arginine/serine (RS)-rich proteins and essential pre-mRNA splicing factors that are localized in the nucleus (Kataoka et al., 1999). The RS domain of these proteins serves as a nuclear localization signal and transportin-SR binds specifically and directly to the RS domains of several SR proteins (Lai et al., 2001). Inhibitor of growth family member 1(ING1) is a novel candidate tumor suppressor gene which is involved in the regulation of apoptosis (Ito

et al., 2002). ING1 (p33) is thought to interact with the p53 signaling pathway and regulate cellular growth (Ito et al., 2002). McAllister-Lucas et al. (2001) characterized BIMP1, a new signaling protein that binds Bcl10 and activates NF-κB. They found that BIMP1-mediated NF-kB activation required Bcl10 and IκB kinases. The involvement of BIMP1 in the regulation of apoptosis is not clear. Interestingly, expression of S-adenosylmethionine decarboxylase (SAMDC) was increased by anthocyanins. SAMDC is involved in polyamine synthesis with ornithine decarboxylase, implicating potential effects on cell proliferation in regulating colonic tumor growth (Milovica et al., 2001). The detailed functional information is not available for cell cycle progression restoration 3.

Anthocyanins caused down-regulation (Table 11) of genes coding for proteins involved in signal transduction (inositol 1,4,5-triphosphate 3-kinase B, phospholysine phosphohistidine inorganic phyrophosphate phosphatase, A kinase anchor protein (yotiao) 9, orphan nuclear receptor (NR2E1), protein tyrosine phosphatase non-receptor type 21, ADP-ribosylation factor [GTP-binding proteins]), tumor suppression (deleted in colon cancer (DCC)), translational and post-translational modification (N-myristoyltransferase 1), apoptosis (neuronal apoptosis inhibitory protein 7), cell proliferation control (ribonuclease/angiogenin inhibitor 2) and a putative oncogene. Inositol 1,4,5-triphosphate 3-kinase mediates cellular signaling by phosphorylating the Ca^{2+}-mobilizing second messenger inositol 1,4,5-triphosphate to yield the putative second messenger inositol 1,3,4,5-tetrakis phosphate (IP4). A kinase anchor protein (yotiao), a N-methyl-D-aspartate (NMDA) receptor-associated protein, has been found to bind cAMP-dependent protein kinase

II (Feliciello et al., 1999). Yotiao has also been found to bind the type I protein phosphatase (PP1) and attaches PP1 and cAMP-dependent protein kinase to NMDA receptors (Westphal et al., 1999). The downstream effect of the NMDA receptors related to yotiao protein is not clear. We found that anthocyanins decreased expression of the DCC gene, an unexpected observation since inactivation of DCC on chromosome 18 is known to be associated with colonic tumorigenesis and metastasis in humans (Vogelstein and Kinzler, 1993). Down-regulation of DCC by anthocyanins merits further study. N-myristoyltransferase catalyzes the co-translational and/or post-translational transfer of myristate to the amino terminal glycine residue of a number of important proteins. Maurer-Stroh et al. (2002) reported that N-myristoyltransferase catalyzes myristylation of kinases, phosphatases, proteasomal regulatory subunit 4, kinase interacting proteins and homologues of mitochondrial translocase.

We focused on the gene changes caused by sulindac and, to a lesser extent, anthocyanins in small intestine because these compounds reduced small intestinal tumor development. Sulindac alone up-regulated genes for ATPase 6 subunit, nuclear protein (putative nuclear protein), DNA-binding proteins (kruppel-related zinc finger protein, zinc finger protein), microtubule-asscoiated proteins (microtubule-associated protein 4, cytoplasmic dyenin intermediate chain 2), and regulation of apoptosis (translocase of inner mitochondrial membrane) (Table 12). ATPase is an enzyme which yields ADP and inorganic phosphate by releasing the terminal phosphate from ATP. Sequence variants of the ATPase 6 gene are thought to play a role in mitochondrial DNA maintenance and integrity in yeast. Maximo et

al. (2001) reported that germline polymorphisms of the ATPase 6 gene are associated with mitochondrial DNA common deletion mutation, which increases thyroid tumorigenesis. Cytoplasmic dynein is a microtubule associated protein with ATPase activity involved in microtubule associated movement.

The small intestinal genes up-regulated by both sulindac and anthocyanins (Table 13) include ones for adhesion (tax interaction protein 1), GTP-binding protein (ADP-ribosylation-like factor 6 interacting protein), GTP-binding inhibitor (GTP-binding protein alpha inhibiting 3), immune response (tumor rejection antigen gp96), and developmental decision (LIM domain binding). Tax protein has been identified as an axonal surface glycoprotein involved in cell adhesion. ADP-ribosylation factor is a ubiquitous GTP-binding protein. ADP-ribosylation is a form of post-translational modification of protein structure involving the transfer to protein of the ADP-ribosyl moiety of NAD (nicotinamide adenine dinucleotide). ADP-ribosylation is believed to play a part in normal cellular regulation. Tumor rejection antigen gp96 is a resident endoplasmic reticulum chaperone protein that directs peptides into the immune response pathway and assists in protein folding (Linderoth et al., 2000). Although the mechanism remains unclear, tumor rejection antigen gp96 can activate the immune system to slow or stop the progression of tumors by escorting tumor-derived immunogenic peptides into the endogenous antigen presentation pathway of antigen presenting cells (Reed and Nicchitta, 2000).

Among a total of 161 genes altered by sulindac in their small intestinal expression, there were more down-regulated genes (108) (Table 14 and 15) than up-regulated genes (53). Cell death regulation genes such as novel cell death-regulatory

protein GRIM19 and programmed cell death 10 were found to be down-regulated. Their exact function in cell death regulation has not been identified. Protein ubiquination and degradation may have been altered by down-regulation of ubiquitin-conjugating enzyme and F-box protein. Ubiquination has been identified as an important mechanism controling a diversity of cellular regulatory processes, including ubiquination-mediated protein turnover, protein targeting, and cell fate decisions. Clark et al. (1989) identified a developmentally essential gene, UbcB encoding ubiquiting conjugating enzyme, involved in substrate recognition in protein ubiquination. F-box protein is an adapter protein that is involved in associating proteins with the ubiquitin-driven proteolytic system. The F-box is a motif found in a wide variety of proteins including many cell cycle regulatory proteins, though various F-box proteins probably also play a part in regulation of transcription, signal transduction and development. Spiegelman et al. (2002) reported regulation of F-box proteins by the Wnt/β-catenin signaling pathway. Activation of the Wnt/β-catenin signal transduction pathway elevates F-box protein βTrCP levels, whereas it reduces expression and activity of another closely related F-box protein, HOS. DNA helicase hFDH has been identified as an F-box protein (Kim et al., 2002). Down-regulation of DNA helicase hFDH influences regulation of DNA metabolism. Transcription and intracellular signal transduction are also influenced by altering chemokine receptor (CCR-NOT transcription complex), serine proteinase inhibitor, and PDZ and carboxyl terminal LIM domain 1.

Genes that were down-regulated both by anthocyanins and sulindac (Table 15) included fatty acid biding protein (FABP) 3 and glutathione S transferase. FABP

is thought play a role in enterocyte fatty acid trafficking by binding long chain fatty acids and facilitating exchange of fatty acids between intracellular membranes. Liver FABP is highly expressed in the both the liver and small intestine and its expression is likely up-regulated by PPARδ activity in small intestine (Poirier et al., 2001). Poirier et al., (2001) demonstrated that the PPARδ/PPARα agonist GW2433 up-regulated the gene encoding liver FABP in the intestine of PPARα-null mice. Our results would indicate that anthocyanins and sulindac may alter the expression of genes that are downstream of PPARδ signaling but not PPARδ expression itself. Glutathione S transferase (GST) is a major phase II detoxification enzyme that catalyzes the conjugation of electrophilic substrates to glutathione. Although a protective role of the glutathione biotransformation in colorectal carcinogenesis has been suggested, studies that compared GST polymorphisms in relation to colon cancer risk are not conclusive with respect to an increased or decreased risk associated with a particular genotype (Strange and Fryer, 1999; Wormhoudt et al., 1999; Grubben et al., 2001).

We were also interested in studying genes that were similarly influenced by treatment in different intestinal sections to enable us to identify patterns of gene changes that may be globally related to tumor development in the intestine. It was somewhat surprising that relatively few genes were similarly influenced by treatment in the different intestinal sections. However, the profiles of adenoma development and effects of treatment on tumor development were different throughout the intestine. No single gene was influenced by treatment in all three intestinal sections (small intestine, colon and cecum). The expression patterns of genes influenced by

treatment in more than one region of the intestine often were not consistent in the various regions. Our observations suggest that the mechanisms controlling tumor promotion in Apc^{Min} mice likely differ in the various regions of the intestine. This hypothesis is supported by the distinct morphology of tumors in the small intestine versus the cecum and colon.

In summary, we have demonstrated that dietary anthocyanins inhibit tumor development in small intestine, colon and cecum of Apc^{Min} mice. Sulindac primarily inhibited small intestinal tumor development of Apc^{Min} mice. Tumor inhibition by anthocyanins and sulindac does not appear to be associated with reductions in cytoplasmic β-catenin expression or reduced activity of the Wnt/β-catenin signaling pathway. cDNA microarray results showed that most of the predicted target genes of Wnt/β-catenin signaling were not influenced by anthocyanins or sulindac, suggesting the presence of downstream compensatory mechanisms for tumor inhibition by these compounds. We identified several genes that were differentially expressed in mice exposed to the three dietary treatments in intestinal sections. Genes influenced by anthocyanins and sulindac are involved in a wide variety of cellular and molecular processes such as DNA synthesis and repair, transcription, mRNA processing, protein interactions and turnover, cell proliferation and differentiation, cell cycle control, and programmed cell death (apoptosis). The patterns of gene expression changes suggest that alterations of cellular and molecular processes by anthocyanins and sulindac likely are different in Apc^{Min} mice. Additional confirmatory analysis of these gene expression results will be necessary to further establish potential mechanisms whereby anthocyanins and sulindac influence tumor development.

CHAPTER VI

OVERALL SUMMARY AND CONCLUSIONS

Anthocyanins, a member of the flavonoid family of phytochemicals, are widely distributed in plants including beans, fruits, and vegetables (Harborne and Grayer, 1988). We hypothesized that tart cherry anthocyanins would suppress intestinal tumorigenesis because they were previously found to inhibit the activities of COX enzymes (Wang et al., 1999b). Compounds that inhibit COX 2 activity have been demonstrated to protect against colon cancer development in animal models and reduce the risk of colon cancer in humans (Giovannucci et al., 1995; Labayle et al., 1991). However, little research has been conducted to study the potential of anthocyanins to inhibit colon cancer development.

The first objective of this research was to determine the effects of tart cherry anthocyanins on the development of intestinal adenomas of Apc^{Min} mice. Apc^{Min} mice are a mutant mouse lineage predisposed to multiple intestinal neoplasia due to a mutation in the murine homolog of the APC gene (Su et al., 1992). The primary phenotype of Apc^{Min} mice is the development of multiple adenomas throughout the small intestine, cecum and colon. Effective use of Apc^{Min} mice in this research required an assessment of their normal process of tumor development. This was necessary to establish appropriate tumor endpoints for subsequent studies. Therefore, we first examined the time of development and morphology of intestinal adenomas in Apc^{Min} mice to obtain baseline data on tumor development.

The first experiment was conducted to determine the influence of age and sex on the numbers and sizes of small intestinal, cecal and colonic tumors in Apc^{Min} mice. Apc^{Min} mice were weaned at 28 days of age and fed Teklad 8640 rodent diet

throughout the experiment. Mice were sacrificed at 30, 45, 60, 75, 90 and 120 days of age. As expected, the numbers and sizes of adenomas in the small intestine increased with age. The numbers of adenomas in the cecum and colon did not increase significantly with age, but the volume of colonic tumors increased significantly after 75 days of age. Sex did not influence the numbers of small intestinal, cecal or colonic tumors. Male mice had greater body weights, colon lengths and colon tumor volumes than female mice. Tumors were already present in 30 day-old mice. All small intestinal tumors were sessile (flat), whereas most cecal and colonic tumors were polypoid. These results provided important information that intestinal tumors develop early in life and progress rapidly in size as Apc^{Min} mice exceed 90 days of age.

We next examined the effects of tart cherry diets, anthocyanins, and their aglycone, cyanidin, on multiplicity and size of intestinal adenomas in Apc^{Min} mice. During a 10 week experimental period, Apc^{Min} mice (n=10/treatment) were exposed to the following treatments; 1) control diet (modified AIN 93G diet containing 220 g/kg protein, 150 g/kg soybean oil and 50 g/kg cellulose), 2) control diet + 800 mg/L anthocyanins in the drinking water, 3) control diet + 200 mg/L cyanidin in the drinking water, 4) control diet + 200 mg/L sulindac in the drinking water, and 5) modified control diet containing 200 g/kg freeze-dried pitted tart cherries. Apc^{Min} mice consuming anthocyanins, cyanidin, and tart cherries had significantly fewer numbers and smaller size of tumors in the cecum. Mice consuming sulindac had smaller small intestinal tumors, whereas mice consuming tart cherries had bigger

small intestinal tumors in average diameter. The total numbers and burden of colonic tumors were not significantly influenced by treatment.

We also determined the potential of tart cherry anthocyanins, their aglycone, cyanidin, and their degradation products to directly inhibit growth of the human colon cancer cell lines HT 29 and HCT 116. Both anthocyanins and cyanidin reduced the growth of human colon cancer cells. The concentrations of anthocyanins and cyanidin required to cause a 50% inhibition of cell growth was 780 μM and 63 μM for HT 29, respectively and 285 μM and 85 μM for HCT 116, respectively. Anthocyanin degradation products did not influence cell growth. These results indicated that cyanidin directly inhibits the growth of colon cancer cells.

Based on these results, we conducted a second feeding study with Apc^{Min} mice to determine the extent to which dietary anthocyanins and sulindac 1) inhibit intestinal tumorigenesis, 2) influence expression and cellular localization of β-catenin, and 3) influence expression of target genes of the Wnt/β-catenin signaling pathway as well as global gene expression profiles. The dietary treatments were; 1) control diet (modified American Institute of Nutrition 93G diet containing 220 g/kg protein, 150 g/kg soybean oil and 50 g/kg cellulose), 2) control diet + 200 ppm sulindac, 3) control diet + 750 ppm anthocyanins. The concentrations of sulindac (200 ppm) and anthocyanins (750 ppm) were based on efficacious concentrations used in the previous experiment, but were administered in the diet in this study to increase their stability.

The results of this second study demonstrated that dietary anthocyanins significantly reduced adenoma development in all three intestinal sections – small

intestine, cecum and colon. Mice consuming anthocyanins had less cecal adenoma burden (volume) compared to mice consuming either sulindac or the control diet. Relative to controls, sulindac-consuming mice had fewer and smaller adenomas in small intestine, but larger cecal adenomas.

Sulindac did not influence the expression of β-catenin protein in intestinal epithelium. Anthocyanins increased β-catenin levels in the middle small intestine and cecum compared to control or sulindac. Intracellular distribution of β-catenin was primarily in the cell membrane of mice consuming all treatments. cDNA microarray analysis showed that the expression of mRNA for APC, β-catenin, and most of the target genes of Wnt/β-catenin signaling were not influenced by treatment. Collectively, these observations indicate that the mechanisms responsible for tumor inhibition by sulindac in small intestine and by anthocyanins in all three intestinal sections do not directly involve inhibition of the Wnt/β-catenin signaling pathway.

cDNA microarray analysis was used to screen for global profiles of gene expression that were influenced by anthocyanins and sulindac. A total of 1,492 genes were influenced by treatment in any of the intestinal regions. Relatively few genes were influenced by treatment in any two of the three intestinal sites – small intestine, cecum or colon. No single gene was influenced by treatment in all three regions. Genes influenced by anthocyanins and sulindac are involved in a wide variety of cellular and molecular processes such as DNA synthesis and repair, transcription, mRNA processing, protein interactions and turnover, cell proliferation and differentiation, cell cycle control, and programmed cell death. Among the many genes whose expression was influenced by treatment, we were particularly interested

in the following effects. Anthocyanins and sulindac up-regulated translocase of inner mitochondrial membrane 8 (regulation of apoptosis) in cecum and small intestine, respectively. Anthocyanins up-regulated ribonucleotide reductase M2 (DNA synthesis and repair) and nemo-like kinase, astrocytic phosphoprotein (signal transduction) whereas anthocyanins down-regulated ornithine decarboxylase (cell proliferation).

In summary, a series of experiments was conducted to determine the effects of anthocyanins on intestinal tumor development in Apc^{Min} mice. Our most important findings were 1) characterization of the early and rapid development and progression of intestinal tumors in Apc^{Min} mice, 2) demonstration that anthocyanins and cyanidin inhibited tumor development in Apc^{Min} mice, 3) tumor inhibition by anthocyanins throughout the intestine of Apc^{Min} mice, whereas sulindac only inhibited small intestinal tumors, 4) evidence that tumor inhibition in Apc^{Min} mice by anthocyanins and sulindac is not likely to be mediated through inhibition of the Wnt/β-catenin growth signaling pathway, and 5) identification of several genes that may be related to reduced tumor development by anthocyanins and sulindac.

Future experiments to further elucidate the effect of anthocyanins on intestinal development should include 1) confirmatory analysis using quantitative PCR or Northern blot analysis is needed to quantitate the changes in expression of genes identified by microarray analysis as being influenced by anthocyanins and/or sulindac, 2) cellular expression of signaling proteins and other markers associated with anthocyanin treatment by immunohistochemistry, 3) studies using other colon cancer models to confirm that anthocyanins inhibit colon cancer development, 4)

efficacy studies with compounds that are structurally related to anthocyanins but have greater stability.

APPENDICES

Table A1. Carrier status, body weights, and tissue lengths of mice from experiment I.

Animal ID	Sex	Age	Status[a]	Bwt (g)	SI length (mm)[b]	Colon length (mm)
1	M	30	C	14.0	303	64
2	M	30	C	14.9	317	75
3	F	30	C	12.9	305	79
4	F	30	C	16.8	309	71
5	M	30	C	14.9	316	79
6	F	30	C	10.0	289	59
7	F	30	C	14.3	290	55
8	F	30	C	15.0	288	66
9	M	30	N	16.5	288	66
10	F	30	N	15.3	329	64
11	M	30	N	16.3	320	68
12	F	30	N	16.3	318	70
13	M	30	N	19.7	350	75
14	F	30	N	14.8	314	63
15	F	30	N	14.0	309	49
16	F	30	N	15.0	350	72
17	M	30	N	10.2	265	53
18	M	30	N	10.5	252	64
19	M	30	N	11.6	285	64
20	M	30	N	17.5	334	82
21	M	30	N	14.4	311	76
22	M	30	N	13.9	304	76
23	M	45	C	19.0	342	83
24	F	45	C	18.2	334	80
25	M	45	C	16.7	320	74
26	M	45	C	20.8	320	81
27	M	45	C	18.2	300	80
28	F	45	C	15.8	295	71
29	M	45	C	19.4	333	83
30	F	45	C	16.9	310	73
31	M	45	C	19.0	325	79
32	M	45	C	18.5	335	67
33	M	45	N	20.5	310	72
34	M	45	N	20.5	342	81
35	F	45	N	17.0	302	74
36	M	45	N	21.5	309	85
37	M	45	N	21.0	330	85
38	F	45	N	16.0	325	70
39	M	45	N	18.9	339	74
40	M	60	C	19.5	303	69

41	M	60	C	24.3	338	85
42	M	60	C	21.3	333	94
43	F	60	C	17.5	324	69
44	F	60	C	18.9	346	80
45	M	60	C	20.7	340	81
46	F	60	C	18.3	322	70
47	F	60	C	18.8	345	80
48	M	60	C	21.1	335	70
49	M	60	N	21.9	333	80
50	M	60	N	23.9	380	97
51	F	60	N	19.7	309	75
52	M	60	N	22.6	325	76
53	F	60	N	19.5	370	78
54	F	60	N	18.4	340	75
55	F	60	N	18.9	349	82
56	M	60	N	23.5	365	89
57	M	75	C	23.1	362	84
58	F	75	C	19.1	357	78
59	M	75	C	21.2	330	81
60	M	75	C	21.5	356	85
61	F	75	C	19.4	330	80
62	M	75	C	20.7	370	79
63	M	75	C	21.9	330	85
64	F	75	C	18.5	339	80
65	F	75	C	19.0	347	77
66	M	75	N	24.5	340	75
67	M	75	N	20.2	355	78
68	M	75	N	24.5	370	85
69	F	75	N	19.0	327	72
70	M	75	N	24.3	374	80
71	M	75	N	24.8	366	78
72	F	75	N	20.3	360	78
73	F	75	N	20.6	350	82
74	F	75	N	18.3	353	73
75	F	75	N	19.7	343	82
76	F	75	N	21.2	356	83
77	F	75	N	17.8	325	83
78	M	75	N	21.3	336	90
79	M	90	C	25.4	360	81
80	F	90	C	19.2	330	80
81	F	90	C	20.5	345	72
82	F	90	C	21.1	362	75
83	M	90	C	23.2	318	83

84	F	90	C	18.9	337	71
85	F	90	C	19.3	354	75
86	M	90	C	22.2	350	87
87	F	90	C	17.5	300	71
88	M	90	C	21.4	317	89
89	F	90	N	20.4	356	76
90	M	90	N	24.7	379	98
91	M	90	N	25.9	360	55
92	F	90	N	21.7	356	62
93	M	90	N	27.6	391	89
94	F	90	N	20.0	361	83
95	M	90	N	25.2	360	70
96	F	90	N	22.8	373	75
97	M	90	N	27.5	370	75
98	M	90	N	25.1	340	63
99	M	90	N	23.4	356	87
100	M	90	N	25.1	370	80
101	F	90	N	21.1	330	81
102	F	120	C	21.6	366	81
103	F	120	C	21.6	380	70
104	M	120	C	27.3	376	70
105	F	120	C	23.5	365	85
106	M	120	C	22.6	394	76
107	M	120	C	26.7	374	100
108	M	120	C	24.0	405	95
109	F	120	C	19.2	367	86
110	M	120	N	27.0	390	85
111	M	120	N	25.6	371	83
112	F	120	N	23.0	365	70
113	F	120	N	23.1	396	77
114	F	120	N	21.2	382	83
115	F	120	N	22.1	370	84
116	M	120	N	27.3	367	97
117	M	120	N	24.8	389	88
118	F	120	N	21.3	381	80
119	M	120	N	26.4	368	85
120	M	120	N	26.5	374	95
121	F	120	N	21.5	386	85
122	M	120	N	26.8	360	90
123	M	120	N	26.1	375	90
124	F	120	N	20.3	380	86

[a]Carrier Status: C; carrier ($Apc^{+/Min}$), N; normal ($Apc^{+/+}$)
[b]Small intestine

Table A2. Numbers and sizes of adenomas in Apc^{Min} mice from experiment I.

	Adenoma Number						Diameter (mm)	Volume[c] (mm^3)	
ID[a]	SI[b]	PSI[b]	MSI[b]	DSI[b]	Cecum	Colon	SI[b]	Cecum	Colon
1	6	3	0	3	2	0	5	3	0
2	8	3	3	2	0	1	8.5	0	1
3	13	6	3	4	0	3	10.5	0	1.5
4	10	3	4	3	1	1	11	3	9
5	11	2	5	4	0	3	8	0	2.5
6	13	4	5	4	2	2	13.5	4	2.8
7	16	5	5	6	0	1	18	0	0.8
8	42	7	16	19	0	0	25.5	0	0
23	25	2	10	13	1	3	21	1.5	2
24	27	6	11	10	1	2	20.5	1	5
25	25	6	12	7	1	2	22	2	3
26	35	7	12	16	1	0	29.5	1	0
27	26	3	13	10	1	3	21.5	1.5	2
28	34	9	13	12	1	0	28.5	1	0
29	31	3	16	12	2	2	24.5	2.5	3.5
30	46	11	21	14	3	2	42.5	3.5	1
31	65	10	26	29	0	1	49	0	1
32	46	2	27	17	2	2	31	2.5	2
40	10	4	2	4	0	2	13	0	1
41	9	3	5	1	0	1	11.8	0	0.5
42	24	3	5	16	4	1	24.5	4	0.5
43	25	4	7	14	0	1	21.5	0	1
44	25	4	8	13	0	1	28.8	0	0.5
45	27	3	10	14	0	0	29	0	0
46	34	5	14	15	3	1	33	4	0.5
47	108	9	21	78	2	2	80	1.5	5
48	101	14	34	53	2	4	91	2	4.5
57	29	4	10	15	1	1	29.5	1.5	1
58	25	6	13	6	1	3	23	1	2.5
59	37	2	13	22	0	2	37	0	2
60	50	8	15	27	1	1	48	2	1
61	43	5	16	22	2	2	40.5	3.5	1
62	78	5	22	51	2	2	67.5	3	2.5
63	58	7	23	28	2	4	61.5	4.5	3
64	53	9	25	19	0	2	50.5	0	5
65	90	38	38	14	1	3	96.5	1.5	2
79	10	4	2	4	0	2	13	0	1
80	41	9	13	19	1	4	49	1	5

81	29	6	14	9	0	2	30	0	5.5
82	37	4	15	18	2	0	39.5	3.5	0
83	60	12	16	32	4	5	52	5	4
84	100	11	30	59	1	1	104.5	2	6
85	113	16	32	65	2	1	131	4.5	40
86	95	6	40	49	7	14	121	9	93.5
87	145	17	45	83	3	2	147.5	3.5	16.5
88	163	19	62	82	3	9	231	4	50.5
102	35	11	7	17	0	1	58.5	0	6
103	43	9	11	23	2	1	83	1.75	2.5
104	44	7	17	20	2	0	75.5	4.5	0
105	68	4	23	41	1	1	109	8	72
106	92	5	26	61	1	3	185.5	4	105
107	71	6	29	36	1	1	111	2	90
108	96	10	33	53	3	5	212.5	14	140
109	126	7	48	71	0	5	236	0	33.5

[a] Animal ID. Note that Normal ($Apc^{+/+}$) mice were excluded.

[b] SI; small intestine, PSI; proximal small intestine, MSI; middle small intestine, DSI; distal small intestine

[c] Values for adenoma volume of cecum and colon represent values calculated by width x length x height of adenomas. These values were transformed by $\pi/6$ to convert them to spherical tumor volumes in the final analysis.

Table A3. Weekly body weights (g) of Apc^{Min} mice from the experiment II.

ID[a]	Sex	Initial[b]	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10
Con 1	M	18.9	22.3	23.5	25.7	28.1	29.2	27.1	29.9	30.4	28.2	26.8
Con 2	M	17.9	20.8	22.3	23.5	25.2	26.6	29.4	28.8	28.4	28.2	26.8
Con 3	F	12.8	15.9	17.2	18.4	18	20	20.5	20	21.7	22.1	20.9
Con 4	F	16.1	17.2	18.3	19.4	20.1	20.4	20.6	22.1	22.5	22.1	22.8
Con 5	F	16.9	17.5	18.3	18.6	18.8	19.1	18.7	18.1	17.4	15.8	14.9
Con 6	F	16.4	18.4	18.8	20.1	20.2	20.7	21.2	20.8	21.1	21.6	20.8
Con 7	M	19.5	21.9	23.1	24.5	26.1	27	27.2	26.4	24.7	24.7	21.2
Con 8	M	15.6	20.8	22.6	23.8	23.6	26.5	26	26.8	26.8	25.5	24.9
Con 9	M	14.8	20.5	22.4	24.7	26.7	29.8	28.9	30.5	30.7	29.6	29.1
Ant 1	M	19.5	23.5	24.9	25.6	24.2	29.5	31.1	32.2	33.7	35	36
Ant 2	M	19.1	21.7	23.3	25.1	26.2	28.3	28.8	30.2	31.6	32.4	33.7
Ant 3	F	16.2	18.1	18.9	19.6	20.2	20.6	20.4	17.8	16	NA	NA
Ant 4	M	18.9	21.6	22.7	23.8	23.3	22.1	20.8	19.2	18.6	17.6	18.6
Ant 5	F	15.5	17.2	19.2	20.4	20	21.4	21.4	21.8	19.8	17.1	15.3
Ant 6	M	19.9	21.7	23.7	24.3	25.2	26.7	27.9	29.2	29.5	31.7	32.4
Ant 7	F	15.6	17.2	19.1	19.4	20	21.2	21.8	22.5	22.6	22.7	22.9
Ant 8	F	15.2	17.5	19.3	19.9	20.8	21.7	22	22	21	19.5	18.1
Cya 1	M	18	21.2	22.1	22.4	23.7	23.9	23.8	22.9	22.8	22.2	20.5
Cya 2	F	17.2	19.5	20.3	21.5	23.3	22	23.2	27	26.5	23.8	26.2
Cya 3	F	15.4	17.2	18.3	18.9	20.2	19.2	20.1	20.1	20.2	19.3	18
Cya 4	M	18	20.7	21.8	22.6	22.5	23.1	22.5	21.6	18.9	18.9	19.5
Cya 5	F	16.7	19	20.1	21.2	21.5	22.1	22.9	23.5	22.8	22.8	24
Cya 6	F	16.6	16.8	19	20.4	20.5	21.1	22	22.4	22.6	22.5	22.5
Cya 7	F	16.3	17.6	19.4	19.8	20.7	21.3	22.3	21.9	22.4	23	22.5
Sul 1	M	16	19.8	21.3	22.3	22.8	23.5	25	25.5	25.9	26.4	27.1
Sul 2	F	16.2	17.6	19.1	20	21	20	21.1	22	23.1	22.6	22.8
Sul 3	F	16.2	17.2	18.6	20.8	20.8	20.6	22.5	22.6	23.3	22.9	22.1
Sul 4	M	16.5	19.9	21.4	21.8	22.4	23.4	23.6	24.4	25.4	22.1	25.3
Sul 5	F	15.7	17.1	18.3	19.5	20.7	21.2	21.9	21.7	22.1	25.3	22.3
Sul 6	M	18	20.6	22.2	23.1	23.8	25	25.5	26.5	26	24.8	25.2
Sul 7	F	17	17.8	19.4	19.7	20.7	21.4	21.3	21.6	22.1	22.2	22.4
Sul 8	F	15.3	17.5	19.3	19.7	19.8	20.2	21.5	23.4	23.9	25.8	26.7
Sul 9	F	13.2	16.6	17.2	18.2	19.8	19.5	20.6	22.6	22.4	22.3	22.7
Sul 10	M	19	22.6	24.3	25.9	27.5	28.1	29	31.2	31.9	32.9	33.7
Che 1	M	17.5	20.3	22.2	23.2	23.3	24.2	24	26.1	26.4	26	24.5
Che 2	F	13.9	17.9	19.9	19	20.7	21.5	22.2	23	22.3	22.9	23.5
Che 3	F	15.9	17.5	18.6	19.9	19.9	17.3	16.7	15.2	15.7	15	NA
Che 4	M	19.3	21.3	22.2	22.5	23	22.4	21	17.7	19.7	14.3	NA
Che 5	M	18.9	21.1	22.1	22.1	22.9	22.7	23.4	23.5	19.5	NA	NA
Che 6	M	19.4	20.5	20.7	22.4	23	23.5	23.6	24.4	24.4	23.9	18.9

Che 7	M	19.4	20.6	21.2	21.7	22.3	23	23.3	23.9	23.7	23.2	19.2
Che 8	F	17.4	17.7	19.5	20.4	21.4	21.6	21	182	16.8	17.2	NA
Che 9	F	13.9	17.3	19.2	20.1	21.8	20.5	20.3	21.8	21.3	21	25.3
Che 10	F	14	17.5	18.5	19.6	20.3	22	22.3	20.4	22.5	20.8	NA

[a]Animal ID: Con; control, Ant; anthocyanins, Cya; cyanidin, Sul; sulindac, Che; 20% Cherry diet.

[b]Initial; body weight at the beginning of the experiment, week 10; body weight at the end of the experiment.

NA: Data not available due to death of animal

Table A4. Tissue lengths and numbers and sizes of adenomas in Apc^{Min} mice from experiment II.

	Length (mm)		Adenoma Number						Diameter (mm^2)	Volume (mm^3)	
ID[a]	SI[b]	Colon	SI[b]	PSI[b]	MSI[b]	DSI[b]	Cecum	Colon	SI[b]	Cecum	Colon
Con 1	363	93	44	10	20	14	2	4	60.5	11.5	4
Con 2	365	86	41	12	16	13	1	6	55	9	5.5
Con 3	356	84	33	9	13	11	5	2	45	4.5	1
Con 4	365	75	2	2	0	0	1	3	2	0.5	3
Con 5	352	79	157	29	57	71	3	5	217.5	4	3
Con 6	390	85	91	16	13	62	3	3	129.5	11	6.5
Con 7	390	88	47	15	16	16	2	3	69.5	2	3
Con 8	363	78	42	14	14	14	3	3	71	8	3
Con 9	370	87	48	15	22	11	1	4	68	2	12
Ant 1	370	98	10	4	4	2	0	6	13.5	0	5
Ant 2	370	90	12	5	5	2	0	2	12	0	2
Ant 3	325	56	147	27	54	66	2	5	164.5	5.5	30
Ant 4	380	87	131	38	32	61	0	4	155.5	0	5.5
Ant 5	375	82	129	34	51	44	0	3	175	0	11
Ant 6	375	95	2	2	0	0	2	2	2.5	4	3
Ant 7	366	63	54	17	20	17	0	1	97	0	1
Ant 8	370	72	121	26	45	50	1	2	198	2	3
Cya 1	377	84	52	25	6	21	1	5	67.5	4	17.5
Cya 2	390	84	96	20	44	32	1	4	122.5	1.5	4.5
Cya 3	350	80	7	5	2	0	0	3	6	0	2.5
Cya 4	375	83	70	3	34	33	0	2	104.5	0	2
Cya 5	367	88	35	31	2	2	0	2	42	0	2
Cya 6	360	75	47	7	15	25	2	4	54.5	2	5
Cya 7	370	82	52	18	7	27	0	6	75	0	13.5
Sul 1	372	95	32	6	14	12	3	7	43.5	7	20.5
Sul 2	360	90	0	0	0	0	10	8	0	15	6.5
Sul 3	366	85	8	5	3	0	9	3	6.5	12.5	6
Sul 4	384	85	50	8	20	22	4	6	40.5	8	6.5
Sul 5	374	83	19	5	9	5	2	7	16.5	2	6
Sul 6	380	78	92	12	30	50	3	2	88.5	4	2
Sul 7	386	88	17	7	4	6	3	9	13.5	5.5	7
Sul 8	366	98	15	7	3	5	2	3	13	2.5	2.5
Sul 9	371	80	8	4	4	0	3	5	7	17.5	4
Sul 10	360	92	6	2	4	0	1	3	6	2.5	3
Che 1	405	65	21	2	10	9	1	4	44	2	35
Che 2	350	80	10	3	2	5	0	5	10	0	4
Che 3	356	85	86	10	31	45	1	0	155	24	0

Che 4	375	82	63	21	21	21	0	2	111	0	2
Che 5	375	82	43	9	11	23	2	1	83	1.75	2.5
Che 6	336	80	69	9	21	39	1	8	120	4.5	43
Che 7	390	80	27	5	10	12	0	2	62	0	58
Che 8	440	80	80	19	27	34	1	1	142	2	3
Che 9	375	90	5	3	2	0	0	7	6	0	8
Che 10	365	92	12	5	4	3	0	3	11.5	0	4

[a]Animal ID: Con; control, Ant; anthocyanins, Cya; cyanidin, Sul; sulindac, Che; Cherry diet.

[b]SI; small intestine, PSI; proximal small intestine, MSI; middle small intestine, DSI; distal small intestine

[d]Values for adenoma volume of cecum and colon represent values calculated by width x length x height of adenomas. These values were transformed by $\pi/6$ to convert them to spherical tumor volumes in the final analysis.

Table A5. Sex, carrier status, and tissue lengths of mice from experiment III.

ID[a]	Sex	Carrier Status[b]	SI length (mm)[c]	Colon length (mm)
Con 1	F	N	340	65
Con 2	F	C	310	70
Con 3	M	C	354	80
Con 4	M	N	270	67
Con 5	M	C	350	75
Con 6	M	N	300	65
Con 7	F	C	330	65
Con 8	F	N	250	51
Con 9	F	C	262	57
Con 10	F	N	260	50
Con 12	M	C	240	59
Con 13	M	N	307	55
Con 14	F	N	330	60
Con 15	F	C	224	76
Con 16	M	C	330	81
Con 17	M	N	320	73
Con 18	F	C	305	55
Con 19	F	N	295	65
Con 20	F	N	305	76
Con 21	M	C	321	65
Con 22	F	N	260	56
Con 23	F	C	230	54
Con 24	F	N	288	58
Con 25	M	C	315	70
Con 26	M	N	330	70
Con 27	M	N	285	70
Con 28	F	N	300	62
Con 29	M	C	270	67
Con 30	M	C	292	69
Con 31	M	N	316	79
Con 32	M	N	323	73
Con 33	F	N	321	65
Con 34	M	N	336	69
Con 35	F	C	299	65
Con 36	F	C	305	66

Con 37	F	C	336	71
Con 38	F	N	336	71
Con 39	F	C	286	69
Con 40	M	C	296	70
Con 41	M	N	302	70
Con 42	M	N	345	76
Con 43	M	N	305	72
Con 44	M	C	327	75
Con 45	M	N	311	71
Con 46	M	N	326	76
Con 47	M	N	311	73
Con 48	F	N	302	64
Con 49	F	C	305	68
Con 50	M	N	303	71
Ant 1	M	N	338	70
Ant 2	M	C	372	95
Ant 3	F	C	360	90
Ant 4	M	C	384	85
Ant 5	M	N	290	60
Ant 6	M	N	320	75
Ant 7	F	C	315	65
Ant 8	F	N	315	65
Ant 9	M	C	325	63
Ant 10	M	N	261	62
Ant 11	M	C	252	55
Ant 12	F	N	260	61
Ant 13	F	N	313	59
Ant 14	F	C	293	68
Ant 15	F	C	259	63
Ant 16	F	C	330	62
Ant 17	M	N	297	71
Ant 18	M	N	317	77
Ant 19	M	C	305	77
Ant 20	M	C	320	78
Ant 21	M	N	290	65
Ant 22	M	C	290	70
Ant 23	F	N	305	50
Ant 24	F	C	290	55

Ant 25	M	N	290	69
Ant 26	M	C	273	60
Ant 27	F	C	247	57
Ant 28	F	N	265	66
Ant 29	M	C	311	74
Ant 30	M	N	307	70
Ant 31	M	C	309	67
Ant 32	M	C	314	71
Ant 33	F	N	318	61
Ant 34	F	N	315	72
Ant 35	F	N	314	63
Ant 36	F	N	307	61
Ant 37	F	C	290	69
Ant 39	M	C	310	75
Ant 40	M	N	322	76
Ant 41	M	C	320	81
Ant 42	M	C	294	73
Ant 43	M	C	300	68
Ant 44	M	C	300	67
Ant 45	F	C	278	68
Ant 46	F	N	321	67
Ant 47	F	C	298	70
Ant 48	F	C	319	73
Ant 49	F	C	295	59
Ant 50	M	N	311	61
Sul 1	M	C	345	90
Sul 2	M	C	310	78
Sul 3	M	N	310	70
Sul 4	M	C	300	64
Sul 5	M	C	330	67
Sul 6	F	C	375	82
Sul 7	F	N	300	60
Sul 8	F	N	305	65
Sul 9	F	N	330	75
Sul 10	F	C	273	61
Sul 11	M	C	273	61
Sul 12	M	C	247	58
Sul 13	F	N	311	75

Sul 14	F	N	320	62
Sul 15	F	C	235	67
Sul 16	M	C	346	67
Sul 17	M	N	330	75
Sul 18	F	C	325	73
Sul 19	F	N	328	72
Sul 20	M	C	346	75
Sul 22	M	C	305	65
Sul 23	M	C	338	77
Sul 24	F	N	303	59
Sul 25	F	C	305	65
Sul 26	M	N	293	69
Sul 27	F	C	297	67
Sul 28	F	N	298	62
Sul 29	M	C	337	71
Sul 30	M	C	331	74
Sul 31	F	N	328	67
Sul 32	F	C	305	70
Sul 33	F	N	316	65
Sul 34	F	N	297	70
Sul 35	F	C	319	64
Sul 36	M	C	328	70
Sul 37	F	C	296	62
Sul 38	M	N	311	65
Sul 39	F	C	319	67
Sul 40	M	N	315	71
Sul 41	M	N	325	72
Sul 42	F	C	300	64
Sul 43	M	C	315	69
Sul 45	M	C	326	74
Sul 46	M	C	331	67
Sul 47	M	C	306	68
Sul 48	F	N	311	62
Sul 49	F	C	291	63
Sul 50	F	N	294	60

[a]Animal ID: Con; control, Ant; anthocyanins, Sul; sulindac
[b]Carrier status: C; carrier ($Apc^{+/Min}$), N; normal ($Apc^{+/+}$)
[c]Small intestine

Table A6. Weekly body weights (g) of mice from experiment III.

ID[a]	Initial[b]	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
Con 1	13.9	17.9	19.8	20.5	21.8	23.5	24 .6	25.1	24
Con 2	14.5	16.5	17.1	18.3	19.2	20.3	20.7	21.3	22
Con 3	14.1	19.4	22	23.8	24.5	25.2	26.1	26.5	27.5
Con 4	13.1	17.9	19.9	21.3	22	23.7	24.2	25	26.4
Con 5	14	19.7	22.2	24	25	25.8	26.4	27.7	27.9
Con 6	14.8	19.1	20.9	22.7	22.9	23.9	24.7	25.4	25.3
Con 7	13.1	17.6	19	20.5	21.4	21.4	21.2	21.3	20.5
Con 8	13.7	17.7	16.9	18.7	18.4	20.7	21.8	22.6	23.3
Con 9	13.2	17.5	17.3	19.2	20.1	21.4	22.9	24.7	25.5
Con 10	13.4	17.6	16.4	18	18.3	20.6	21.9	22.1	22.5
Con 12	14.4	19.8	21.6	22.7	23.9	26.9	27.5	27.7	28.1
Con 13	15	20.6	23.9	26.3	26.5	27.3	28.2	28.8	28.6
Con 14	16.9	18.3	19.9	20.3	22.8	23.5	23.8	24.5	25.5
Con 15	16.9	18	19.1	20.9	21.8	23.4	23.6	24.3	26.8
Con 16	18.4	22.4	24	25.4	26.9	28	28.3	29.8	29.3
Con 17	19.2	22.3	23.9	25.2	26	26.8	27.2	27.8	28.1
Con 18	15.3	17.4	18.9	18.8	20	20.4	21.5	22.5	22.4
Con 19	13.1	17.5	18.1	19.5	21.5	21.8	23.8	25.8	26.6
Con 20	15	18.1	19.4	19.9	21.5	21.5	23.8	25	25.4
Con 21	13.7	20	22.6	23.8	25.1	26.2	27.6	28.7	29.9
Con 22	12.6	17.8	18.2	19.5	20.5	21.1	22.9	22.4	22.7
Con 23	11.1	17.7	17.3	18	18.8	20.2	20.6	20.9	21.2
Con 24	12.9	18.2	18.9	20.2	21.8	21.9	23.1	23.1	23.5
Con 25	14.6	19.4	21.7	23.4	24.7	25.2	26.8	27.7	29.1
Con 26	16.9	21.3	24.9	26.8	28	28.3	30.1	31.6	33
Con 27	13.7	19.3	21.1	22.9	23.8	24.4	25.5	26.2	27.3
Con 28	13.3	19.4	20	23.1	25.4	26.3	28.2	28.6	29
Con 29	12.5	18.6	20.2	21.9	22.9	23.1	23.5	24.3	24
Con 30	12.5	19.3	20.9	22.7	23.5	23.8	24.1	25	24.6
Con 31	15.3	21.7	22.8	24.4	24.9	25.7	27	27.9	28.3
Con 32	14.4	21.5	24.2	25.4	26.8	27.8	28.5	29.7	30.1
Con 33	13.7	18.9	18.7	19.7	20.4	21.1	22.4	22.7	23.3
Con 34	14.6	19.8	22.2	23.3	25	25.5	26.5	28	28.6
Con 35	12.5	16.7	18	18.4	19	19.7	20.7	21	20.7
Con 36	12.7	16.7	17.2	18.3	18.6	20.5	21.2	21.5	20.6

Con 37	15.2	18	19	18.9	20.7	22.4	23.9	24.3	24.7
Con 38	14	19.8	20.1	21.3	23	23.8	26	28.3	27.3
Con 39	11.4	15.6	16.5	17	18.2	19.7	20.1	20.5	20.9
Con 40	13.8	19.2	20.4	21.3	23	23.5	24.2	24.8	24.5
Con 41	13.1	19.5	21	21.8	22.5	23.6	24.2	24.6	24.2
Con 42	18	18.5	21.7	24.4	27.2	29.4	31.2	32.5	34.8
Con 43	15.7	19.4	21.7	24.4	26.6	27.7	28	28.2	29.6
Con 44	15.4	19.4	22.8	25.4	27.9	30	30.4	29.9	30.5
Con 45	13.7	15.3	20.4	20.5	22.6	24.4	25.1	26.6	28.7
Con 46	14.1	15.2	20.4	21.5	23.4	25.4	26.4	27.2	28
Con 47	13.2	13.9	19.2	21	23.4	25.4	26.5	27.4	27.7
Con 48	13.8	16.8	18.6	18.9	19.9	20.6	21.9	21.9	22.5
Con 49	13.8	16.9	16.9	18.1	19.3	19.9	20.2	20.1	20.6
Con 50	14.7	19.5	21.1	23	23.6	24.8	25.7	26.2	23.6
Ant 1	19.4	23.1	25.6	26.7	27.8	29.4	29	30.7	31.1
Ant 2	15.4	18.2	18.7	20.1	20.4	21.8	22.3	23.1	23.6
Ant 3	15.7	18.8	19	20.3	20.9	23.6	23.6	26.1	26.2
Ant 4	9.4	14.1	19.2	21	22.7	24.3	25	26	26.6
Ant 5	9.3	14.2	20	21.1	22.9	24.1	24.3	25.5	26.2
Ant 6	9.8	14.6	2.5	21.5	22.9	24.4	24.2	25.1	25.7
Ant 7	9.1	12.8	17	17.2	17.7	19	19.2	19.8	23.9
Ant 8	9.2	12.5	16.6	18.2	18.3	19.9	20.1	20.9	21.2
Ant 9	14.4	20.3	22.9	24.1	24.7	26.2	26.4	27.1	27.2
Ant 10	16	20.6	23.5	25.5	26.9	28.2	29.8	30.3	30.2
Ant 11	11.8	18.2	21.5	24.7	26.6	28	29.6	29.9	31.1
Ant 12	13.3	17.7	16.8	19.3	19.4	21.1	22.8	22.1	22.3
Ant 13	13.8	16.9	16.9	18.8	18.5	21.4	22.5	21.2	22.6
Ant 14	11.9	16.6	17.3	19.5	18.4	20.2	21.9	23	24.1
Ant 15	10.5	14.6	16	16.2	16.2	17.6	19.3	19.2	19.6
Ant 16	16.7	18.2	21.1	21.7	24.5	25.9	27.3	27.7	28.2
Ant 17	19.6	22	23.6	24.4	24.6	25.3	25.9	27.4	27.5
Ant 18	17.2	21.4	23.1	23.6	24.8	25.9	26.9	28.7	28.6
Ant 19	15.7	19.8	21.2	22	22.6	23.1	23.2	24	24.2
Ant 20	18.6	22.2	24.1	25.2	27	27.3	27.3	27.8	24.9
Ant 21	17.7	21.7	24.3	25.9	27.2	27.8	28	29	29.6
Ant 22	17.4	21.3	23.4	24.5	25.9	26.7	27.2	28.6	17.9
Ant 23	14.6	18.7	20.2	21.3	21.4	21.3	22.6	23.6	23.5
Ant 24	14.6	17.9	19.2	19.4	19.8	21.1	21.4	21.8	21.9

Ant 25	13.6	20.5	22.7	24.3	25.6	26	26	27	27.6
Ant 26	10.6	17.3	19.6	21.5	22.7	23	24	24.8	26.3
Ant 27	11.5	16.1	17.9	19	19.8	20.1	21	21.5	23.6
Ant 28	12.1	16.7	17.4	18.7	19.1	20	20	21.7	22.2
Ant 29	16.9	22.2	23.4	25.1	26.3	26.5	26.9	28.8	29.5
Ant 30	13.8	18	20.6	22.9	24.3	25	25.9	26.9	27.7
Ant 31	14.4	19.4	22.2	24.6	25.6	26.4	27.3	28.4	28.7
Ant 32	14.3	19.5	22	24.1	24.6	24.9	25.9	27.2	27.5
Ant 33	13.1	16.7	18.5	19.4	19.8	20.9	21	22.1	22.6
Ant 34	13.4	17.6	18.8	19.1	20.2	21.3	23	22.8	21.9
Ant 35	14	17.3	17.6	18.1	18.5	20	21.5	22.1	21.9
Ant 36	13.9	18.8	18.3	19.9	22	22	22.6	24.5	25
Ant 37	14.1	18.5	19.2	19	21.3	23.2	23.6	25.3	26.3
Ant 39	13.5	20.2	22.3	23.2	25.2	26.4	26.6	27.2	27.9
Ant 40	15.4	21.2	23.4	24.7	25.9	27.3	28.5	28.4	28.8
Ant 41	14.7	20.8	23.2	25.2	26.9	27.4	28.5	27.9	28.4
Ant 42	10.3	14.8	19.3	21.4	22.8	23.8	24.3	25	26.1
Ant 43	9.7	14.4	19.3	21.6	24.1	25.4	26.5	27.1	28
Ant 44	10	15	19.4	21.5	23.5	24.6	25.5	26.2	26.9
Ant 45	8.3	9.5	13.2	14.9	15.4	16.5	17.1	18.2	19.9
Ant 46	13	14.1	17.7	18.8	20.6	20.7	21.5	23.4	24.5
Ant 47	14	17.4	18.6	18.3	20.2	21.9	21.4	22.3	22.2
Ant 48	13	16.9	17.3	19.9	19.5	20.8	21.1	23.3	21.8
Ant 49	13.2	16.2	17.5	18.4	19	20.2	21.1	21.6	20.6
Ant 50	13.8	19.8	20.6	21.9	23.7	25.1	25.9	27.1	26.5
Sul 1	18	20.9	21.8	24.1	25.2	26.5	27.3	27	27.2
Sul 2	18.1	20.5	22	24	24.6	26.9	27	27.4	27.2
Sul 3	18.2	20.2	22.2	23.4	24.3	26.1	26	26.2	25.9
Sul 4	9.1	12.8	17.4	19.3	19.5	21.2	21.5	22.5	23.1
Sul 5	9.9	14.9	18.6	19.9	20.8	21.9	22.3	23.5	23.4
Sul 6	8.8	12.5	16.6	17.1	17.8	18.9	19.8	19.9	19.9
Sul 7	8.9	12.4	16.6	16.5	16.5	17.6	18.1	19.1	18.7
Sul 8	9.4	13.2	17.7	18	18.5	19.6	20.5	20.8	21.6
Sul 9	13.3	17.4	17	19.2	19.6	21.1	21.6	22.4	22.3
Sul 10	11.9	16.7	17.1	18.3	18.9	20	21	21.4	23.1
Sul 11	12.6	17.9	18.9	20.2	21	22.3	23.2	23.7	23.6
Sul 12	12.4	17.8	18.8	20.2	20.4	21.6	22.2	22.9	22.8
Sul 13	13.5	16.2	16.8	17.7	18.1	19.9	21.3	22.4	22.2

Sul 14	11.8	16.4	17.5	19.3	19.4	21.3	22	21.9	22.4
Sul 15	16.4	16.7	18.8	19.7	21	21.8	22.4	23.2	24.5
Sul 16	18.8	21.9	24.4	25.8	26.5	27.3	28.1	28.8	29
Sul 17	15.9	18.6	20.9	23.1	24.5	26.4	26.9	27.4	27.2
Sul 18	15.2	17.5	18.6	20.8	21.7	22.4	23	24.6	25.4
Sul 19	14.5	17.6	18.7	20.8	22.2	22.6	23.4	23.5	24.5
Sul 20	15.1	17.7	21	23	24	24.6	25.3	26.5	26.7
Sul 22	15.3	20.8	22.4	22.9	24.5	23.5	24.5	24.5	24.6
Sul 23	13.8	20.5	23	24.6	26.3	26.3	27.3	27.7	27.6
Sul 24	12.1	15.2	16.8	17.6	18.2	18.8	19.5	19.7	19.9
Sul 25	11.6	16	18.2	18.3	19	19.7	20.3	20.9	21.2
Sul 26	12.1	18.7	20.8	22.8	23.2	24.1	24.4	25	25.3
Sul 27	11	16	16.3	17.7	19.1	18.5	18.9	19.5	20
Sul 28	11.5	17.4	18.1	19.5	21	20.3	20.9	20	22.1
Sul 29	14	19.9	21.6	22.4	23.2	23.8	24.3	24.8	24.4
Sul 30	13.8	19.2	21.1	22.8	23.9	25.3	25.8	26.8	26.1
Sul 31	15.2	19.3	19.6	21.4	21.8	23.9	25.4	25.7	26.7
Sul 32	12.8	16.6	17.9	18.7	18.7	19.1	20.6	22.2	23.1
Sul 33	12.7	16.7	17.1	17.7	18.2	18.5	18.8	20.3	19.9
Sul 34	11.1	16.6	17.3	17.8	18.2	19.3	21.1	20.8	20.1
Sul 35	13	17.6	17.7	19	19.7	19.8	21.4	21.9	21.9
Sul 36	16.9	20.2	21	22.5	23.4	24	24.9	25.3	25.3
Sul 37	12.1	15.1	17	17.5	18.5	19	19.3	19.4	19.5
Sul 38	14.4	18.4	19.8	20.4	21.7	22.1	22.8	23.2	22.7
Sul 39	13.1	17.1	17.2	18.9	19.7	20.9	21.1	21.7	21.9
Sul 40	12.8	18	19.5	21	21.8	22.5	23	24.3	25.3
Sul 41	13.8	18.8	21.1	22.5	23.7	24.6	25.7	26.1	26.5
Sul 42	11.8	14.9	17.1	18.8	21.2	22	23	23.4	24.1
Sul 43	9.4	14.3	18.3	19.1	20.3	21.6	22.4	22.8	23.4
Sul 45	11.7	13.6	16.9	17.5	18.4	19.1	20.2	21.3	21.3
Sul 46	16.2	21	22.2	23.9	24.7	25.2	26.8	27.2	28
Sul 47	12.4	15.7	16.8	17.5	18.9	20.6	21.5	20.6	21.8
Sul 48	14.6	17.1	18.5	19.4	21.6	22.1	24	23.9	23.8
Sul 49	12.6	13.8	14.8	16.2	17.6	18.2	18.8	19.2	19.5
Sul 50	13.7	16.9	17.1	18.1	19.3	20	20.3	20.7	20.8

[a]Animal ID: Con; control, Ant; anthocyanins, Sul; sulindac

[b]Initial; body weight at the beginning of the experiment, Week 8; body weight at the end of the experiment.

Table A7. Numbers and sizes of adenomas in Apc^{Min} mice from experiment III.

	Adenoma Number			Diameter (mm)		Volume[c] (mm^3)		
ID[a]	Total	SI[b]	MSI[b]	DSI[b]	Cecum	Colon	SI[b]	Cecum	Colon
Con2	18	11	7	1	2	21.5	12	32	
Con3	12	12	0	0	0	10.5	0	0	
Con 5	9	6	3	1	1	15	0.25	24	
Con 7	39	22	17	2	1	50.5	7	6	
Con 9	8	5	3	1	2	9	1	1.5	
Con 12	8	4	4	2	5	11	2	34	
Con 15	20	12	8	1	1	25	0.5	0.5	
Ccon 16	41	14	27	1	3	66	4	40	
Con 18	23	11	12	0	0	31.5	0	0	
Con 21	25	17	8	1	3	31	9	3	
Con 23	36	19	17	0	0	45	0	0	
Con 25	24	14	10	1	3	30.5	1	2.5	
Con 29	17	6	11	0	2	21.5	0	2	
Con 30	28	15	13	1	1	37.5	1	24	
Con 35	52	23	29	1	1	66.5	1.5	1	
Con 36	56	30	26	1	1	65	4	27	
Con 37	14	10	4	1	6	17	2	5	
Con 39	15	6	9	0	5	18	0	5	
Con 40	19	8	11	2	4	22	3	4	
Con 44	56	31	24	2	2	84.5	8	7	
Con 49	40	21	19	2	2	48	2	2	
Ant 2	8	1	7	0	0	9	0	0	
Ant 3	6	0	6	1	0	9	0.25	0	
Ant 4	44	20	22	4	6	32.5	8	6.5	
Ant 7	9	4	5	0	0	11.5	0	0	
Ant 9	3	3	0	0	1	4	0	12	
Ant 11	9	4	5	2	0	10.5	1	0	
Ant 14	21	8	13	0	0	26	0	0	
Ant 15	13	8	5	0	1	21	0	6	
Ant 16	21	5	16	0	1	21.5	0	12	
Ant 19	12	7	5	1	0	9	1	0	
Ant 20	20	12	8	1	1	25.5	1	9	
Ant 22	5	3	2	0	1	4.5	0	9	
Ant 24	16	7	9	1	0	17	1	0	
Ant 26	25	7	18	1	1	27.5	1	4	
Ant 27	18	6	12	0	0	17	0	0	
Ant 29	14	7	7	0	1	20	0	1	
Ant 31	25	7	18	0	0	25	0	0	

Ant 32	18	15	3	0	1	17.5	0	1
Ant 37	6	4	2	0	0	7.5	0	0
Ant 39	13	5	8	0	0	25	0	0
Ant 41	23	12	11	1	2	27	4	34
Ant 42	6	1	5	1	1	7	4	4
Ant 43	7	4	3	0	0	8	0	0
Ant 44	11	4	7	0	2	15	0	10
Ant 45	34	16	18	1	2	42	9	30
Ant 47	18	9	9	1	1	11	2	2
Ant 48	13	7	6	2	0	12.5	2.5	0
Ant 49	41	18	23	0	2	39.5	0	1.5
Sul1	5	3	2	5	1	4.5	1.25	12
Sul 2	5	2	3	1	1	5.5	0.5	16
Sul 4	9	5	4	1	2	13.5	6	4
Sul 5	0	0	0	1	1	0	2	0.5
Sul 6	3	1	2	0	0	6	0	0
Sul 10	7	2	5	3	4	6.5	8.5	2.75
Sul 11	1	0	1	2	3	1	10	1.75
Sul 12	10	6	4	2	2	11.5	8	12
Sul 15	10	7	3	2	1	6.5	1.5	0.5
Sul 16	0	0	0	0	1	0	0	16
Sul 18	6	3	3	1	0	5.5	2	0
Sul 20	7	3	4	1	2	5.5	20	9.5
Sul 22	5	3	2	1	2	4	9	18.5
Sul 23	7	5	2	3	0	5.5	4	0
Sul 25	9	4	5	2	1	7	10	6
Sul 27	16	6	10	1	1	16	12	0.5
Sul 29	6	3	3	1	1	5.5	12	18
Sul 30	12	4	8	1	4	12.5	12	3.5
Sul 32	4	2	2	7	1	5.5	20	1
Sul 35	28	18	10	1	1	23.5	9	9
Sul 36	7	3	4	1	2	7	12	2
Sul 37	8	4	4	2	3	10	10	11
Sul 39	7	3	4	0	3	9.5	0	3.5
Sul 42	3	2	1	3	1	5.5	9	2
Sul 43	4	2	2	1	2	3	20	2
Sul 45	25	16	7	1	2	19.5	16	10
Sul 46	29	13	16	4	3	21	20	9
Sul 47	10	6	4	3	1	9.5	12	4
Sul 49	8	4	4	7	1	11	16.5	1

[a] Animal ID. Note that Normal ($Apc^{+/+}$) mice were excluded.

[b] SI; small intestine, PSI; proximal small intestine, MSI; middle small intestine, DSI; distal small intestine

[c]Values for adenoma volume of cecum and colon represent values calculated by width x length x height of adenomas. These values were transformed by $\pi/6$ to convert them to spherical tumor volumes in the final analysis.

Table A8. Intensity scores of β-catenin expression in small intestine of mice from experiment III.

ID[a]	Carrier Status[b]	Proximal Small Intestine			
		Top[c]		Bottom[c]	
		Membrane	Cytoplasm	Membrane	Cytoplasm
Con 40	C	2	1	1	0
Con 44	C	2	1	1	0
Con 49	C	2	0	1	0
Con 42	N	2	1	1	1
Con 50	N	3	1	1	0
Sul 43	C	2	1	2	0
Sul 46	C	2	1	1	1
Sul 47	C	2	1	1	1
Sul40	N	1	0	1	0
Sul 50	N	3	1	1	1
Ant 45	C	3	1	2	1
Ant 48	C	2	1	1	0
Ant 49	C	3	2	1	1
Ant 40	N	1	0	1	0
Ant 50	N	3	1	1	0
		Middle Small Intestine			
Con 40	C	0	0	0	0
Con 44	C	1	0	1	0
Con 49	C	2	0	1	0
Con 42	N	2	0	1	0
Con 50	N	2	1	1	0
Sul 43	C	2	1	1	0
Sul 46	C	2	1	1	0
Sul 47	C	2	1	1	0
Sul 40	N	1	0	1	0
Sul 50	N	2	1	1	0
Ant 45	C	3	2	2	1
Ant 48	C	3	2	2	1
Ant 49	C	2	1	2	1
Ant 40	N	2	1	2	0
Ant 50	N	3	2	2	0

			Distal Small Intestine		
Con 40	C	3	1	1	0
Con 44	C	2	1	2	0
Con 49	C	2	1	1	0
Con 42	N	NA	NA	NA	NA
Con 50	N	2	1	1	0
Sul 43	C	3	1	2	0
Sul 46	C	2	1	1	0
Sul 47	C	3	2	2	0
Sul 40	N	3	1	3	1
Sul 50	N	2	1	1	0
Ant 45	C	3	2	2	0
Ant 48	C	3	1	1	0
Ant 49	C	4	2	1	0
Ant 40	N	2	1	1	0
Ant 50	N	3	1	2	0

[a]Animal ID: Con; control, Ant; anthocyanins, Sul; sulindac
[b]Carrier status: C; carrier ($Apc^{+/Min}$), N; normal ($Apc^{+/+}$)
[c]Top; villus region in the top of the small intestine, Bottom; crypt region in the bottom of the small intestine.

Table A9. Intensity scores of β-catenin expression in colon of mice from experiment III.

ID[a]	Status[b]	Top[c]		Middle[c]		Bottom[c]	
		M	C	M	C	M	C
Con 40	C	2	1	1	0	1	0
Con 44	C	3	2	1	0	1	1
Con 49	C	3	2	2	1	2	1
Con 42	N	NA	NA	NA	NA	NA	NA
Con 50	N	3	2	1	0	1	0
Sul 43	C	3	1	2	1	2	1
Sul 46	C	3	1	2	0	2	0
Sul 47	C	3	2	1	1	1	1
Sul 40	N	2	1	0	0	0	0
Sul 50	N	3	1	1	1	1	1
Ant 45	C	2	1	0	1	1	1
Ant 48	C	4	2	2	2	2	1
Ant 49	C	4	3	1	1	2	1
Ant 40	N	2	1	1	0	1	0
Ant 50	N	3	1	2	1	2	1

[a]Animal ID: Con; control, Ant; anthocyanins, Sul; sulindac
[b]Carrier status: C; carrier ($Apc^{+/Min}$), N; normal ($Apc^{+/+}$)
[c]Top; top one third of the crypts, Middle; middle one third of the crypts, Bottom; bottom one third of the crypts in the colon, M; membrane, C; cytoplasm

Table A10. Intensity scores of β-catenin expression in cecum of mice from experiment III.

ID[a]	Status[b]	Top[c]		Middle[c]		Bottom[c]	
		M	C	M	C	M	C
Con 40	C	3	1	1	0	1	0
Con 44	C	3	2	0	0	1	0
Con 49	C	4	2	1	0	0	0
Con 42	N	2	1	0	0	0	0
Con 50	N	3	2	2	1	0	0
Sul 43	C	3	1	2	0	1	0
Sul 46	C	2	1	0	0	0	0
Sul 47	C	3	1	1	0	1	0
Sul 40	N	NA	NA	NA	NA	NA	Na
Sul 50	N	3	1	1	0	0	0
Ant 45	C	3	2	2	0	2	0
Ant 48	C	3	2	1	0	1	1
Ant 49	C	4	2	2	0	1	0
Ant 40	N	4	1	2	0	1	0
Ant 50	N	3	2	2	1	0	0

[a]Animal ID: Con; control, Ant; anthocyanins, Sul; sulindac
[b]Carrier status: C; carrier ($Apc^{+/Min}$), N; normal ($Apc^{+/+}$)
[c]Top; top one third of the crypts, Middle; middle one third of the crypts, Bottom; bottom one third of the crypts in the cecum, M; membrane, C; cytoplasm

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