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Dietary Anthocyanin-Rich Tart Cherry Extract Inhibits Intestinal Tumorigenesis in APC^{Min} Mice Fed Suboptimal Levels of Sulindac

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A promising approach for cancer chemoprevention might be a combination therapy utilizing dietary phytochemicals and anticarcinogenic pharmaceuticals at a suboptimal dosage to minimize any potential adverse side effects. To test this hypothesis, various dosages of anthocyanin-rich tart cherry extract were fed in combination with suboptimal levels of the nonsteroidal anti-inflammatory drug sulindac to APC^{Min} mice for 19 weeks. By the end of the feeding period, fewer mice that were fed the anthocyanin-rich extract in combination with sulindac lost more than 10% of body weight than mice fed sulindac alone. Mice that were fed anthocyanin-rich extract (at any dose) in combination with sulindac had fewer tumors and a smaller total tumor burden (total tumor area per mouse) in the small intestine when compared to mice fed sulindac alone. These results suggest that a dietary combination of tart cherry anthocyanins and sulindac is more protective against colon cancer than sulindac alone.

KEYWORDS: Anthocyanins; cancer; intestine; *Prunus cerasus*; mouse

INTRODUCTION

Anthocyanins are a group of bioactive phytochemicals that may aid in explaining the association between diet and colorectal cancer in humans. Anthocyanins have been demonstrated to inhibit selectively the growth of human colon cancer cell lines (*1*, *2*) and restrict intestinal tumorigenesis in rat and mice models for human colon cancer (*3*−*5*). Furthermore, anthocyanins constitute a significant part (12.5 mg/day/person) of the U.S. diet (*6*). Anthocyanins are widely distributed in the plant kingdom and are especially enriched in berries, grapes, and cherries (*6*, *7*). For example, tart cherries contain between 100 and 400 mg of anthocyanins/kg of fresh weight (*8*).

Anthocyanin-rich tart cherry extract has been shown to decrease proliferation of human colon cancer cell lines (*9*). Also, its antioxidant properties, as evidenced by decreased lipid peroxidation in vitro, and anti-inflammatory properties, as shown by decreased cyclooxygenase (COX) 1 and 2 enzyme activity in vitro, provide further support for the hypothesis that tart cherry anthocyanins might be protective against colon cancer (*8*, *10*). In a previous study conducted in our laboratory, anthocyanin-rich tart cherry extract added to the drinking water was associated with fewer and smaller tumors in the cecum of APC^{Min} mice (*9*).

APC^{Min} mice are a commonly used animal model to study the effect of dietary and pharmaceutical agents on colorectal cancer (*11*). APC^{Min} mice inherit a mutated copy of the adenomatous polyposis coli (APC) gene. These mice develop intestinal adenomatous polyps, especially in the small intestine, if the wild-type APC allele is lost by somatic mutation or loss of the APC gene (*12*). Similarly, humans with familial adenomatous polyposis have a mutated copy of the human homologue of the APC gene and develop multiple intestinal adenomas, some of which progress to adenocarcinomas. Furthermore, mutations in the APC gene are common in sporadic colorectal cancer (*13*) and are assumed to be an early, initiating event for sporadic and familial colorectal carcinogenesis (*14*). Thus, APC^{Min} mice are an appropriate model for the study of human colorectal carcinogenesis (*11*).

Sulindac, a potent nonsteroidal anti-inflammatory drug (NSAID) and inhibitor of COX-1 and COX-2 enzymes, has been shown to reverse the progression of adenomatous polyps in APC^{Min} mice and patients with familial adenomatous polyposis (*11*, *15*). However, higher, more effective dosages of sulindac and other NSAIDs have been associated also with increased risk for gastrointestinal bleeding and ulceration (*16*−*18*). To

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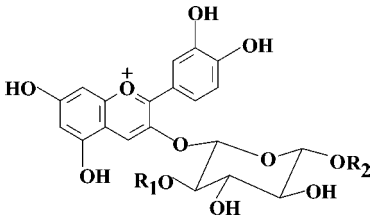

Figure 1. Structures of anthocyanins: 3-cyanidin 2″-*O*-β-D-glucopyranosyl-6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (R1 = glucose, R2 = rhamnose); 3-cyanidin 6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (R1 = H, R2 = rhamnose).

be acceptable by the public as a treatment and especially as a preventative, any approach needs to involve minimum risks. The use of sulindac at the effective dosages does not fulfill this criterion. Most dietary phytochemicals, including anthocyanins, have in comparison to pharmaceuticals a greatly reduced risk profile even when consumed at higher dosages (*19*). Therefore, the use of suboptimal dosages of sulindac in combination with dietary changes may provide a safer approach for cancer prevention (*17*, *20*). In addition, using a combination of diet and pharmaceuticals may be a more effective treatment than either treatment alone because alternative pathways that influence carcinogenesis could be inhibited (*21*).

The objectives of this study were to determine whether a combination of dietary anthocyanin-rich tart cherry extract and suboptimal levels of sulindac is more effective in inhibiting intestinal tumorigenesis in APC^{Min} mice than feeding sulindac alone and if the dietary dose of anthocyanin-rich extract influences this relationship.

MATERIALS AND METHODS

Isolation of Anthocyanin-Rich Extract from Tart Cherries. Anthocyanin-rich extract was prepared from tart cherries, *Prunus cerasus* L. cv. Balaton, according to procedures reported previously (*8*, *22*, *23*). Pitted and individually quick frozen (IQF) tart cherries were obtained from commercial growers (Traverse City, MI). The thawed fruits (1 kg) were blended with reverse osmosis (RO) water (500 mL) for 2 min using a commercial Waring blender. The puree was squeezed through layered cheesecloth, and the resulting filtrate was homogenized for 5 min in a Kinematica CH-6010 homogenizer (Roxdale, ON, Canada) and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000*g* for 20 min at 4 °C to obtain cherry supernatant (*8*).

For the isolation of anthocyanins, 100 g of XAD-16 resin (1 kg, mesh size 20−60; Supelco, Bellefonte, PA) was soaked in methanol (MeOH; 1.5 L) for 10 min and filtered through a medium-porosity sintered funnel under vacuum. It was then washed with RO water (2 L × 3) to yield MeOH-free resin. Next, the resin was slurry packed using water in a glass column (33 × 6 cm, bed volume = 1.9 L; *22*), and the column was further washed with RO water (9 L) until the eluent reached a pH of 7. The tart cherry supernatant was then applied to the XAD-16 column and eluted with water (12 L) until the pH of the eluant was 7. The adsorbed anthocyanins were then eluted with methanol acidified with hydrochloric acid (HCl) (2.5 L, pH 3.0). The methanolic solution containing the anthocyanins was concentrated at 35 °C under vacuum to remove the MeOH, and the resulting aqueous solution was then lyophilized (*23*). This procedure was repeated until a sufficiently large quantity of anthocyanin powder was prepared for these experiments. The lyophilized anthocyanin-rich extract was stored at −80 °C until it was added to the diets. The anthocyanin composition of this preparation was determined using HPLC (*8*). The anthocyanin-rich extract used for the study contained approximately 50% (wt/wt) anthocyanins as a mixture of 65% of 3-cyanidin 2″-*O*-β-D-glucopyranosyl-6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside and 35% of 3-cyanidin 6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (**Figure 1**; *9*).

Table 1. Composition (in Weight Percent) of Diets

	anthocyanin diets				
ingredient	0	375	750	1500	3000
casein	22.12	22.12	22.12	22.12	22.12
L-cystine	0.33	0.33	0.33	0.33	0.33
cornstarch	31.707	31.670	31.632	31.557	31.407
dyetrose	10.57	10.57	10.57	10.57	10.57
sucrose	10	10	10	10	10
cellulose	5	5	5	5	5
soybean oil	15	15	15	15	15
AIN-93G mineral mix	3.87	3.87	3.87	3.87	3.87
AIN-93G vitamin mix	1.11	1.11	1.11	1.11	1.11
choline bitartrate	0.28	0.28	0.28	0.28	0.28
tert-butyl hydroquinone	0.003	0.003	0.003	0.003	0.003
sulindac	0.01	0.01	0.01	0.01	0.01
anthocyanin-rich extract	0	0.0375	0.075	0.15	0.30

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 animal research facilities overseen by the Michigan State University Laboratory Animal Resources Unit in the Food Science and Human Nutrition Department. Mice were housed in plastic cages in temperature (23 ± 2 °C) and humidity (40−60%) controlled rooms with a 12 h light−dark light cycle. All mice had continual access to food and water and were observed daily for health status. APC^{Min} mice were produced by mating C57BL/6J $APC^{Min/+}$ males with C57BL/6J $APC^{+/+}$ female mice from a breeding colony maintained at Michigan State University.

At 4−5 weeks of age, mice were weaned and randomly assigned to one of five different dietary treatments after stratification for gender. The five diets contained 0, 375, 750, 1500, or 3000 mg of anthocyanin-rich tart cherry extract/kg of diet, respectively (**Table 1**). The experimental diets were powdered, modified AIN-93G diets. In comparison to the standard AIN-93G diet (*24*), the fat content was increased by adding 15% (wt/wt) instead of 7% (wt/wt) soybean oil to the diet to better reflect the caloric contribution of dietary components in Western diets. The concentrations of essential nutrients were also increased by approximately 15% in comparison to the standard AIN-93G diet to compensate for any decrease in diet intake resulting from the increased energy density of the modified diet. All diets contained 100 mg of sulindac/kg of diet. The dietary dose of 100 mg of sulindac/kg of diet was based on previous research in our laboratory and chosen to achieve a partial inhibition in intestinal tumorigenesis. We have previously observed that administration of sulindac in drinking water (200 mg/L) restricts small intestinal tumorigenesis to the point that APC^{Min} mice can be maintained until over 1 year of age without significant weight losses.

Mice were tested for APC^{Min} carrier status (*12*) only after initiation of dietary treatments. Therefore, the number of APC^{Min} mice per treatment differed among diets (0 mg/kg, 16 females, 13 males; 375 mg/kg, 15 females, 12 males; 750 mg/kg, 16 females, 19 males; 1500 mg/kg, 13 females, 11 males; 3000 mg/kg, 14 females, 12 males). Body weights of mice were determined weekly throughout the study. Mice were sacrificed by CO_2 inhalation followed by exsanguination after 19 weeks of dietary treatment or when significant morbidity occurred. Significant morbidity was defined as the loss of >10% of a mouse's highest body weight or development of mammary tumors to a size that impaired movement. Nineteen weeks of dietary treatment was used as the endpoint for the remaining mice because at that time the number of mice that had lost >10% of their highest body weights exceeded 10% of the total number of mice used in the experiment (15 of 141 mice).

Measurement of Intestinal Tumors. After sacrifice, the small intestine, cecum, and colon and, if present, mammary tumors were excised from each mouse. The small intestine was further divided into three sections of equal length: proximal, medial, and distal small intestine. All intestinal sections were cut longitudinally and rinsed with lukewarm tap water to remove intestinal contents. The sections were briefly held in phosphate-buffered saline solution (pH 7.4) until pinned flat on cardboard and then were fixed for 1 day in 10% neutral-buffered

formalin solution (v/v; pH 7.4). Intestinal sections were stored at room temperature in 1% neutral buffered formalin solution (v/v) until further analysis.

To facilitate tumor identification and quantification, tissues were stained in phosphate-buffered saline solution (pH 7.4) containing 0.2% (w/v) methylene blue. Using a stereomicroscope and a measuring grid, tumor number and dimensions of individual tumors were determined for each intestinal section. All small intestinal tumors were sessile. The size of each small intestinal tumor was calculated by using the formula $\pi \times$ [(length $\times$ width)/4]. In the cecum and colon, only papillary tumors were counted. The size of tumors of the cecum and colon were calculated with the formula $\pi \times$ [(length $\times$ width $\times$ height)/6]. One cecal papillary tumor was observed in the whole study (in a female APC^{Min} mouse consuming the 0 mg/kg anthocyanin diet). For statistical analysis, this cecal tumor was added to the counts and sizes of the colonic section for that mouse. All tumor counting was performed by one person who was blind to the treatments.

Data Analysis. The data were analyzed using SAS version 8.2 (*25*). For body weight data, a mixed model procedure (PROC MIXED) was used. The fixed covariates were sex (male, female), diet (0, 375, 750, 1500, 3000 mg/kg anthocyanin-rich extract), week of treatment, sex by week of treatment interaction, and diet by week of treatment interaction. A first-order autoregressive variance−covariance matrix was used to account for correlations between samples of the same animal. A similar model was used for tumor number, average tumor area, and total tumor area in the small intestinal sections except that week of treatment was replaced by intestinal section (proximal, medium, and distal small intestine) and a completely unrestricted variance−covariance matrix was used to account for correlations between samples of the same animal. For average and total volume of papillary tumors in the large intestine (only mice with three-dimensional tumors were used) and for tumor number, average tumor area, and total tumor area in the complete small intestine, a general linear model (PROC GLM) was used. The fixed covariates were sex and diet. For the presence of papillary large intestinal tumors and for weight loss >10% of the highest body weight, a generalized linear model with binomial distribution and logistic link function (PROC GENMOD) was used. The covariates were sex and diet. A similar model was used for number of papillary large intestinal tumors except that a multinomial distribution and a cumulative logistic link function (PROC GENMOD) were used. To determine whether the dietary anthocyanin-rich extract influenced intestinal tumorigenesis, the average value of mice receiving any of the anthocyanin and sulindac treatments was compared to the average value of mice receiving sulindac alone by using a chi-square test in the ESTIMATE statement of PROC GENMOD or by using a *t* test in the ESTIMATE statement of PROC MIXED and PROC GLM, respectively. To determine whether there was a linear or quadratic dose−response relationship between dietary anthocyanin-rich extract and intestinal tumorigenesis, the average values of mice receiving different dosages of anthocyanin were compared using linear and quadratic statistical contrasts, respectively, in the statistical programs described in the previous sentence. Least-squares means ± SEM (standard error of the mean) are presented in **Figures 2−4** and **Table 2**. Significance was declared at $P \leq 0.05$, and trends toward significance were declared at $P \leq 0.10$.

RESULTS

Addition of dietary anthocyanin-rich extract had little influence on the weight gain of APC^{Min} mice fed sulindac at suboptimal levels (**Figure 2**). In the last weeks of the experiment, a smaller proportion of mice that were fed the anthocyanin-rich extract in combination with sulindac (9 of 112 mice; 2, 4, 2, and 1 receiving 375, 750, 1500, and 3000 mg/kg anthocyanin-rich extract, respectively) lost >10% of their body weight at the end of the feeding period when compared to mice fed sulindac alone (6 of 29 mice; $P = 0.04$). In addition, mice that were fed the anthocyanin-rich extract had or tended to have statistically significantly higher body weight when compared to mice fed sulindac alone (week 17, 0.768 ± 0.431 g, $P =$ 0.08; week 18, 0.833 ± 0.432 g, $P = 0.06$; week 19, 0.970 ± 0.435 g, $P = 0.03$; **Figure 2)**. No linear or quadratic relationships between dietary anthocyanin-rich extract content and body weight were observed (data not shown). Eleven of the 74 female APC^{Min} mice developed a mammary tumor during the experiment, but the risk of mammary tumor incidence was not influenced by diet (mammary tumors were detected in 1, 3, 5, 2, and 0 female mice receiving 0, 375, 750, 1500, and 3000 mg/kg anthocyanin extract, respectively).

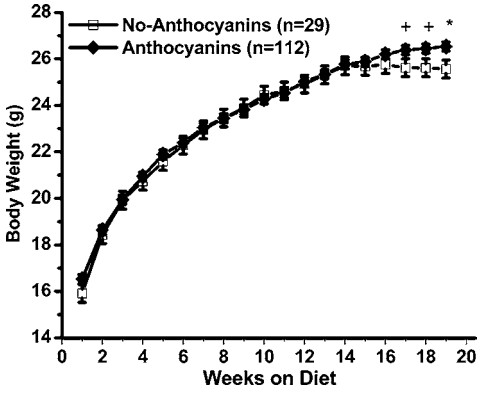

Figure 2. Influence of dietary anthocyanin-rich tart cherry extract on body weight of APC^{Min} mice fed suboptimal levels of sulindac (100 mg/kg of diet). Mice receiving different dietary anthocyanin dosages were pooled. Data represent least-squares mean ± SEM. Mice fed anthocyanins in combination with sulindac had or tended to have statistically significantly higher body weight in the last three weeks on the diet than mice fed sulindac alone.

Small intestinal tumor data demonstrated that a combination of dietary anthocyanin-rich extract and sulindac was more effective in decreasing tumorigenesis in the small intestine of APC^{Min} mice than feeding sulindac alone (**Figure 3**). On average, APC^{Min} mice fed anthocyanins and sulindac had a 20% smaller total tumor area (absolute difference = 10.25 ± 4.86 mm^2/mouse) in the small intestine than APC^{Min} mice fed sulindac alone ($P = 0.04$; **Figure 3A**). Differences in total tumor area are a product of differences in tumor number, average tumor area, or both. On average, APC^{Min} mice fed a combination of anthocyanin and sulindac had a 22% lower tumor number (−8.50 ± 3.51 tumors/mouse) in the small intestine than APC^{Min} mice fed sulindac alone ($P = 0.02$; **Figure 3B**). In comparison, the average tumor size of APC^{Min} mice was similar for mice fed anthocyanins in combination with sulindac and mice fed sulindac alone (absolute difference: 0.09 ± 0.11 mm^2/tumor; $P = 0.45$; **Figure 3C**). No statistically significant linear or quadratic associations between dietary anthocyanin dosage and tumor number or area were observed (**Figure 3**).

The tumor data for the different small intestinal sections provided a more detailed picture of the relationship between dietary anthocyanin intake and decreased tumorigenesis in the small intestine in APC^{Min} mice that were fed sulindac at suboptimal levels (**Figure 4**). On average, APC^{Min} mice fed a combination of anthocyanins and sulindac had or tended to have a smaller total tumor area in the proximal third (−5.66 ± 2.99 mm^2/mouse; $P = 0.06$) and medial third (−4.73 ± 1.72 mm^2/mouse; $P = 0.007$) of the small intestine but not in the distal third (+0.15 ± 1.57 mm^2/mouse; $P = 0.92$) when compared to APC^{Min} mice fed sulindac alone (**Figure 4A**). The tumor number was affected similarly in the three small intestinal sections (**Figure 4B**). APC^{Min} mice fed anthocyanins in combination with sulindac had or tended to have a smaller total tumor number in the proximal (−3.28 ± 1.68 tumors/mouse; $P = 0.05$), medial

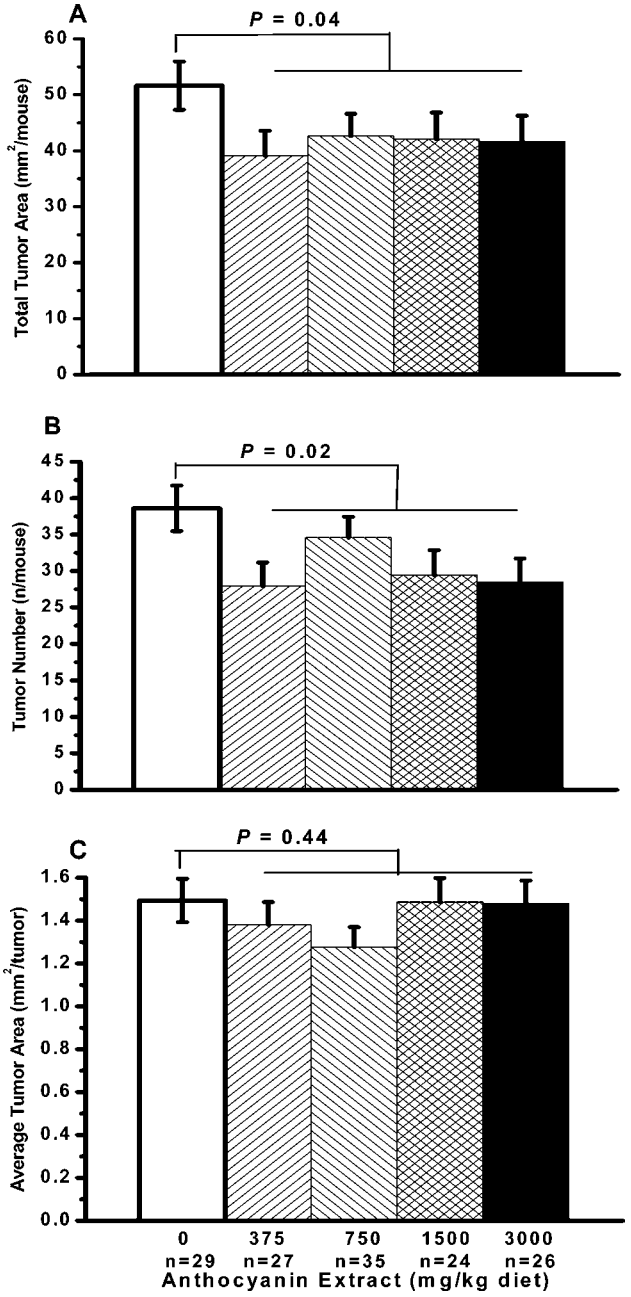

Figure 3. Influence of dietary anthocyanin-rich tart cherry extract on (**A**) total tumor area per mouse, (**B**) tumor number, and (**C**) average tumor area of the small intestine in APC^{Min} mice fed suboptimal levels of sulindac (100 mg/kg of diet). Data represent least-squares mean ± SEM.

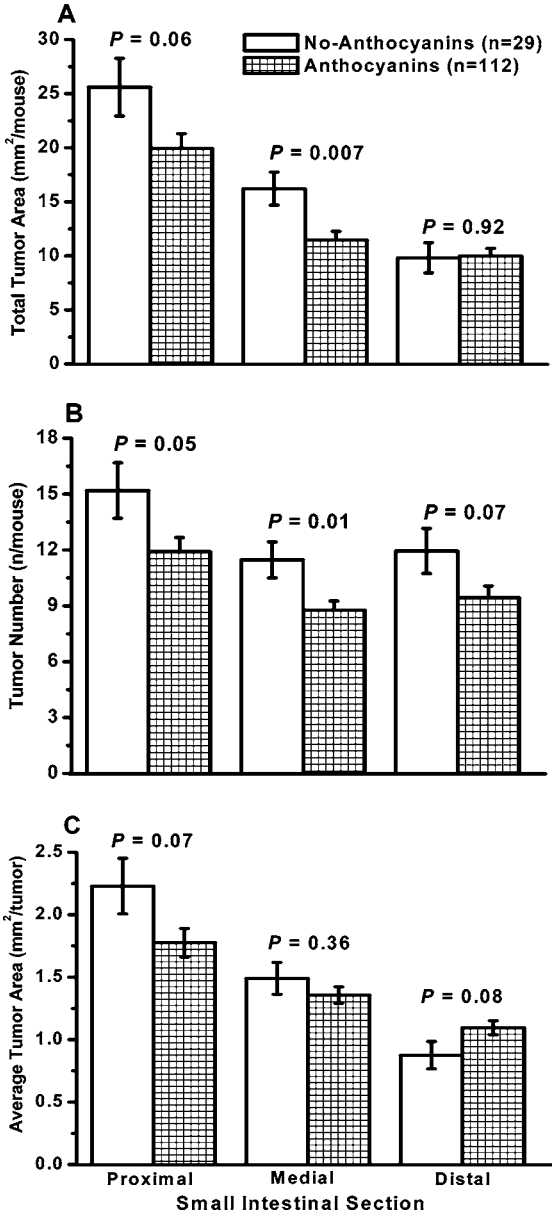

Figure 4. Influence of dietary anthocyanin-rich tart cherry extract on (**A**) total tumor area per mouse, (**B**) tumor number, and (**C**) average tumor area for different small intestinal sections in APC^{Min} mice fed suboptimal levels of sulindac (100 mg/kg of diet). Mice receiving different dietary anthocyanin dosages were pooled. Data represent least-squares mean ± SEM.

(−2.71 ± 1.09 tumors/mouse; $P = 0.01$), and distal third (−2.51 ± 1.36 tumors/mouse; $P = 0.07$) of the small intestine in comparison to APC^{Min} mice fed sulindac alone (**Figure 4B**). In comparison, the average tumor area was affected differently in the three small intestinal sections (**Figure 4C**). APC^{Min} mice fed a combination of anthocyanins and sulindac tended to have a smaller average tumor area in the proximal third (−0.45 ± 0.25 mm^2/tumor; $P = 0.07$) and a larger average tumor area in the distal third (+0.22 ± 0.12 mm^2/tumor; $P = 0.08$) of the small intestine when compared to APC^{Min} mice fed sulindac alone (**Figure 4C**). No statistically significant linear or quadratic associations between dietary anthocyanin dosage and tumor number or area were observed in any of the three intestinal sections (data not shown).

The relationship between dietary anthocyanin intake and intestinal tumorigenesis in APC^{Min} mice fed sulindac at suboptimal levels differed between the small intestine (**Figure 3**) and the large intestine (**Table 2**). On average, APC^{Min} mice fed anthocyanins in combination with sulindac had a similar prevalence, number, and volume of papillary tumors in the large intestine as APC^{Min} mice fed sulindac alone (**Table 2**). Differences in prevalence, number, and total volume of papillary tumors of mice were detected between diets of different anthocyanin content. APC^{Min} mice receiving the lower two dietary dosages of crude anthocyanin extracts had or tended to have a lower prevalence ($P = 0.07$) and number of papillary tumors ($P = 0.04$) and a lower total tumor volume per tumor-bearing mouse ($P = 0.08$) when compared to APC^{Min} mice consuming the higher two dosages of anthocyanins. More

Table 2. Influence of Dietary Anthocyanin-Rich Tart Cherry Extract on Prevalence, Number, and Volume of Papillary Tumors in the Large Intestine of APC^{Min} Mice Fed Suboptimal Levels of Sulindac (100 mg/kg of Diet)[a]

	papillary tumors in large intestine			
	prevalence (mice/total mice)	number (*n*/mouse)	total volume (mm^3/mouse)	mean volume (mm^3/tumor)
diet (mg of crude anthocyanin extract/kg of diet)				
0	13/29	0.62 ± 0.15	7.77 ± 2.25	6.46 ± 1.23
375	7/27	0.33 ± 0.12	5.37 ± 3.05	4.17 ± 1.67
750	12/35	0.40 ± 0.83	7.05 ± 2.35	6.15 ± 1.29
1500	13/24	0.83 ± 0.19	8.94 ± 2.24	5.97 ± 1.23
3000	10/26	0.62 ± 0.22	12.74 ± 2.56	7.07 ± 1.40
stat contrast		probability values		
presence	0.45	0.54	0.77	0.67
linear	0.32	0.26	0.05	0.24
quadratic	0.06	0.04	0.88	0.67

[a] Data represent mean ± SEM. The data for total and mean tumor volume refer only to mice having at least one papillary tumor. The statistical contrast "presence" compares the average value of the mice receiving anthocyanin to the average value of mice receiving no anthocyanin. The statistical contrasts "linear" and "quadratic" test for a linear or quadratic dose–response relationship between dietary crude anthocyanin extract dose and average tumor values.

specifically, APC^{Min} mice fed 1500 mg of anthocyanin-rich extract/kg of diet had or tended to have in comparison to APC^{Min} mice receiving the lower two dosages a greater prevalence ($P = 0.04$ for 375 mg/kg dosage; $P = 0.10$ for 750 mg/kg dosage; $P = 0.03$ for both dosages combined) and number of papillary tumors ($P = 0.03$ for 375 mg/kg dosage; $P = 0.04$ for 750 mg/kg dosage; $P = 0.01$ for both dosages combined). APC^{Min} mice fed 3000 mg of anthocyanin-rich extract/kg of diet tended ($P = 0.07$) to have a greater total tumor volume when compared to APC^{Min} mice receiving 375 mg/kg of diet. When tumor volume data were $\log_e$-transformed, the P value of this comparison was significant ($P = 0.04$).

DISCUSSION

The aim of this study was to test whether a combination of dietary tart cherry anthocyanin-rich extract and sulindac is more cancer-preventive than sulindac alone. There was a moderate, but significant, decrease in small intestinal tumor burden in APC^{Min} mice that were fed tart cherry anthocyanin-rich extract in addition to sulindac that can be attributed primarily to a significant decrease in tumor number (**Figure 3**). Previous papers have reported that APC^{Min} mice fed an anthocyanin-rich blueberry extract had fewer small intestinal tumors (*3*) and that a combination of ursodeoxycholate with a suboptimal level of sulindac was more effective in decreasing small intestinal tumor number than feeding sulindac alone (*17*). As a second line of evidence, it has been shown in carcinogen-induced rat models, using colonic aberrant crypt foci (ACF) as biological markers for colon cancer, that dietary anthocyanins and anthocyanin-rich extracts or foods inhibit colonic ACF formation (*4*, *5*, *26*, *27*) and that a combination treatment of sulindac and epigallocatechin gallate, a flavanol similar in structure to anthocyanins, was more effective in decreasing ACF development than either treatment alone (*20*).

The data reported herein suggest that the cancer-protective effect of dietary anthocyanin-rich extract might not be uniform throughout the small intestine (**Figure 4**). Whereas tumor number was uniformly inhibited by anthocyanin feeding throughout the small intestine (**Figure 4B**), average tumor size tended to be smaller in the proximal third and larger in the distal third of the small intestine of mice consuming anthocyanins relative to those consuming sulindac alone (**Figure 4C**). As a result, tumor burden was decreased only in the proximal two-thirds of the small intestine in APC^{Min} mice receiving anthocyanin-rich extracts (**Figure 4A**). It is not uncommon that dietary and pharmaceutical agents provide cancer protection only to parts of the small intestine (*11*). Changes in gastrointestinal pH, microflora, passage rate, and protein expression patterns and changes of concentrations of cancer-protective agents between intestinal sections due to their metabolism and absorption have been proposed to explain why bioactive compounds have a protective effect only in parts of the small intestine (*11*). For example, higher concentrations of sulindac are needed to achieve the same cancer-protective effect in the proximal part of the small intestine than in the distal part (*17*). One reason is that sulindac has to be metabolized into pharmacologically active sulfide and sulfone derivatives to be cancer protective (*28*). This study suggests that, when co-administered, anthocyanins might complement the action of sulindac primarily by inhibiting intestinal carcinogenesis in the proximal parts of the small intestine. The differential effect of anthocyanins coincides with their absorption pattern in the gastrointestinal tract (*29*).

Multiple potential mechanisms have been proposed for the protective effect of anthocyanins against carcinogenesis. The antimutagenic and antioxidative properties of anthocyanins could prevent cancer initiation, the anti-inflammatory and antiproliferative properties could prevent cancer promotion, and the antimetastatic and proapoptotic properties could prevent cancer progression (*3*, *19*, *30*). There might be concerns whether such potential mechanisms are applicable to the results of this study because anthocyanin-rich tart cherry extract rather than pure anthocyanins were fed to APC^{Min} mice; however, an anthocyanin-rich tart cherry extract, very similar in composition to the one used in this study, has been shown in vitro to have potent antioxidative and anti-inflammatory properties as evidenced by inhibiting activities of prostaglandin endoperoxide H synthases I and II or COX-I and -II enzymes (*8*, *10*). Furthermore, the results of Wang et al. (*10*) suggested that a mixture of anthocyanins might be more effective than pure anthocyanins to inhibit prostaglandin endoperoxide H synthase I and II enzyme activity. Therefore, feeding anthocyanin-rich extract might be more beneficial than feeding pure anthocyanins. In addition, feeding fruit juice extract more closely approximates human dietary intake patterns than does feeding pure anthocyanins.

Assuming that the mice consumed on average 7.5 g/day of diet, approximately 2.8 mg/day of anthocyanin-rich extract would be consumed by mice consuming the diet containing 375 mg/kg of anthocyanin-rich extract. Converting the consumption data of a 25 mg mouse to that of a 70 kg adult human (on the basis of $kg^{0.75}$), this would be equivalent to 1.08 kg of anthocyanin-rich extract, which would be uncommon for a human diet. However, it should be considered that dosages of anthocyanin-rich extract lower than those used in this study might be protective against cancer development because the lowest dosage used in this study (375 mg of anthocyanin-rich extract/kg of diet) was effective in inhibiting intestinal adenoma development. Further studies are needed to determine the minimal dosage of tart cherry anthocyanin-rich extract required to achieve a protective effect against intestinal adenoma development in this model.

A dose−response relationship, which is one of the criteria for evaluating potential causal relationships, was not detected in this study (**Figure 3**). However, a dose−response relationship is not necessary for demonstrating potential causal relationships.

If anthocyanins exert their cancer-protective effect by interacting with cell receptors and the lowest dosage of anthocyanin-rich extract used in this study exceeds the saturation level of the receptor, a dose−response relationship cannot be detected. It has been shown in cancer cell lines that anthocyanins interact with receptors involved in carcinogenesis such as the epidermal growth-factor receptor (*31*). The consistency of the protective-effect of anthocyanin-rich extract across the four dosages (**Figure 3**) is also in concordance with the saturation model and suggests that anthocyanins might exert their cancer-protective effects in vivo via a receptor-mediated mechanism and that at a concentration range lower than that used in this study a dose−response relationship between anthocyanin extract and number of tumors might be detected.

The magnitude and, hence, the relative importance of the protective effect of anthocyanin-rich tart cherry extract on intestinal tumorigenesis are unclear. The protective effect of anthocyanin-rich extract was statistically significant only after pooling the results for all anthocyanin-containing diets, which suggest a small to moderate effect. The relatively high *P* values for an approximate 20% decrease in small intestinal tumor burden and number indicate also that there was considerable variation between animals for tumor data. One explanation for the high interindividual variability is that the mice were sacrificed at a time point during which considerable morbidity due to small intestinal adenoma development was occurring. This is reflected in the significant proportion of mice that lost >10% of their body weight in this study. Tumor number and size increase exponentially with age; therefore, the variability in small intestinal tumor number between mice will increase also as the mice are getting older and more morbid and larger numbers of mice are needed to detect significant differences. Another point of consideration for evaluating the magnitude of the cancer-protective effect of anthocyanin-rich extract is that, in a study conducted concurrent to this research and utilizing siblings to these mice and the same base diet, feeding anthocyanin-rich extract alone at 1500 mg/kg of diet (without sulindac) was not associated with decreased tumor burden and size in APC^{Min} mice (Bobe, unpublished results). Similar negative results of anthocyanins on small intestinal adenoma development were observed when the same anthocyanin-rich extract was administered to APC^{Min} mice in drinking water (*9*).

The protective influence of dietary anthocyanin-rich tart cherry extract was reflected in a decreased morbidity of APC^{Min} mice (**Figure 2**). Using weight loss as a proxy for morbidity, a significantly smaller proportion of APC^{Min} mice receiving the combination of anthocyanin extract and sulindac lost >10% of their maximum body weight at the end of the study period than those receiving sulindac alone (8 vs 21%). Similarly, a smaller proportion of female APC^{Min} mice lost >10% of their maximum body weight at the end of the study period than male APC^{Min} mice (3 of 74 female mice versus 12 of 67 male mice; $P = 0.04$). As previously reported by Kang et al. (*9*), weight loss in mice is associated with a greater number of large-sized small intestinal tumors. Supporting this relationship, the average tumor size in the proximal third of the small intestine in APC^{Min} mice that lost >10% of their maximum body weight at the end of the study period was larger than in the healthier APC^{Min} mice (2.76 ± 0.26 vs 1.72 ± 0.12 mm^2/tumor). This relationship can be explained by the fact that large-sized tumors perforate the intestinal wall, as was also observed in this study, which leads to anemia, malabsorption, discomfort, and, consequently, weight loss of the mice.

It is noteworthy that using a combination of dietary anthocyanin-rich extract and sulindac significantly extended the lifespan of APC^{Min} mice. Approximately 10% of APC^{Min} mice receiving a combination of dietary anthocyanin-rich extract and sulindac lost >10% of their peak body weight by 24 weeks of age. In comparison, this degree of morbidity was observed after 14 weeks of age when the same base diet (minus sulindac) was fed to siblings to the mice used in this experiment (results not shown). Safety and tolerability are concerns when high dosages of sulindac are ingested: Jacoby et al. (*17*) reported increased mortality rates caused by bleeding, perforated gastrointestinal ulcers, or both when greater dietary dosages of sulindac were administered (0% at 75 mg/kg of diet, 17% at 150 mg/kg diet; 83% at 500 mg/kg of diet). Similar effects have been reported for humans (*16*, *32*). In this study, no adverse health effects of dietary sulindac were observed when it was fed at 100 mg/kg of diet, which is supported by the growth chart data (**Figure 2**). There is a competing risk profile for sulindac treatment because higher dosages of sulindac are more protective against small intestinal tumor development but also are associated with higher mortality rates (*17*). APC^{Min} mice tolerated doses of up to 3000 mg of anthocyanin-rich extract/kg of diet for 19 weeks well and did not show any adverse effects such as decreased weight gain (**Figure 2**), morbidity, or mortality. The only visible change associated with feeding increasing amounts of anthocyanin-rich extract was that the urine and feces darkened, which indicated that the extract was absorbed. Anthocyanin-rich berry extract has been shown also to be safe when given at equally high dosages (*33*). Although more accurate tests to determine the tolerability and safety of anthocyanin-rich tart cherry extract are lacking, the results reported herein suggest a low risk profile for dietary anthocyanin-rich tart cherry extract. Therefore, using a combination of dietary anthocyanin-rich extract and suboptimal dosages of sulindac might provide the same cancer-protective effects as observed with higher dosages of sulindac alone without the increased rates of gastrointestinal bleeding and, therefore, might be a promising approach for colon cancer prevention in high-risk groups, such as FAP carriers.

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LITERATURE CITED

(1) Malik, M.; Zhao, C.; Schoene, N.; Guisti, M.; Moyer, M.; Magnuson, B. Anthocyanin-rich extract from *Aronia meloncarpa* E. induces a cell cycle block in colon cancer but not normal colonic cells. *Nutr. Cancer* **2003**, *46*, 186−196.

(2) Zhao, C.; Guisti, M.; Malik, M.; Moyer, M.; Magnuson, D. Effects of commercial anthocyanin-rich extracts on colonic cell growth. *J. Agric. Food Chem.* **2004**, *52*, 6122−6128.

(3) Cooke, D.; Steward, W.; Gescher, A.; Marczylo, T. Anthocyans from fruits and vegetables—does bright colour signal cancer chemopreventive activity. *Eur. J. Cancer* **2005**, *41*, 1931−1940.

(4) Hagiwara, A.; Miyashita, K.; Nakanishi, T.; Sano, M.; Tamano, S.; Kadota, T.; Koda, T.; Nakamura, M.; Imaida, K.; Nobuyuki, I.; Shirai, T. Pronounced inhibition by a natural anthocyanin, purple corn color of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP)-associated colorectal carcinogenesis in male F344 rats pretreated with 1,2-dimethylhydrazine. *Cancer Lett.* **2001**, *171*, 17−29.

(5) Hagiwara, A.; Yoshino, H.; Ichihara, T.; Kawabe, M.; Tamano, S.; Koda, T.; Nakamura, M.; Imaida, K.; Nobuyuki, I.; Shirai, T. Prevention by natural food anthocyanins, purple sweet potato color and red cabbage color, of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-associated colorectal carcinogenesis in rats initiated with 1,2-dimethylhydrazine. *J. Toxicol. Sci.* **2002**, *27*, 57−68.

(6) Wu, X.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069−4075.

(7) Prior, R. Absorption and metabolism of anthocyanins: potential health effects. In *Phytochemical: Mechanism of Action*; Meskin, M., Bidlack, W., Davies, A., et al., Eds.; CRC Press: Boca Raton, FL, 2004; pp 1−19.

(8) Seeram, M.; Momin, R.; Nair, M.; Bourquin, L. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine* **2001**, *8*, 362−369.

(9) Kang, S. Y.; Seeram, N.; Nair, M.; Bourquin, L. Tart cherry anthocyanins inhibit tumor development in APC^{Min} mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* **2003**, *194*, 13−19.

(10) Wang, H.; Nair, M.; Strasburg, G.; Change, Y. C.; Booren, A.; Gray, J.; DeWitt, D. Antioxidant and anti-inflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, *62*, 294−296.

(11) Corpet, D.; Pierre, F. Point: from animal models to prevention of colon cancer. Systematic review of chemoprevention in Min mice and choice of the model system. *Cancer Epidemiol. Biomarkers Prev.* **2003**, *12*, 391−400.

(12) Su, L.; Kinzler, K.; Vogelstein, B.; Preisinger, A.; Moser, A.; Luongo, C.; Gould, K.; Dove, W. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* **1992**, *256*, 668−70.

(13) Lüchtenborg, M.; Weijenberg, M.; de Goeij, A.; Wark, P.; Brink, M.; Roemen, G.; Lentjes, M.; de Bruijn, A.; Goldbohm, R.; van't Veer, P.; van den Brandt, P. Meat and fish consumption, *APC* gene mutations and hMLH1 expression in colon and rectal cancer: a prospective cohort study (The Netherlands). *Cancer Causes Control* **2005**, *16*, 1041−1054.

(14) Fearon, E.; Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* **1990**, *61*, 759−767.

(15) Asano, T.; McLeod, R. Nonsteroidal anti-inflammatory drugs (NSAID) and aspirin for preventing colorectal adenomas and carcinomas (review). *Cochrane Database Syst. Rev.* **2004**, *1*, 1−28.

(16) Bertoni, G.; Sassatelli, R.; Bedogni, G.; Nigrisoli, E. Sulindac-associated ulcerative pouchitis in familial adenomatous polyposis. *Am. J. Gastroenterol.* **1996**, *11*, 2431−2432.

(17) Jacoby, R.; Cole, C.; Hawk, E.; Lubet, R. Ursodeoxycholate/sulindac combination treatment effectively prevents intestinal adenomas in a mouse model of polyposis. *Gastroenterology* **2004**, *127*, 838−844.

(18) Wolfe, M.; Lichtenstein, D.; Sing, G. Gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs. *N. Engl. J. Med.* **1999**, *340*, 1888−1899.

(19) Hou, D. X. Potential mechanism of cancer chemoprevention by anthocyanins. *Curr. Mol. Med.* **2003**, *3*, 149−159.

(20) Ohishi, T.; Kishimoto, Y.; Miura, N.; Shiota, G.; Kohri, T.; Hara, Y.; Hasegawa, J.; Isemura, M. Synergistic effects of (−)-epigallocatechin gallate with sulindac against colon carcinogenesis of rats treated with azoxymethane. *Cancer Lett.* **2002**, *177*, 49−56.

(21) Schwartz, B.; Birk, Y.; Raz, A.; Mader, Z. Nutritional−pharmacological combinations. A novel approach to reducing colon cancer incidence. *Eur. J. Nutr.* **2004**, *43*, 221−229.

(22) Chandra, A.; Nair, M.; Iezzoni, A. Isolation and stabilization of anthocyanins from tart cherries. *J. Agric. Food Chem.* **1993**, *41*, 1062−1065.

(23) Wang, H.; Nair, M.; Iezzoni, A.; Strasburg, G.; Booren, A.; Gray, J. Quantification and characterization of anthocyanins in Balaton tart cherries. *J. Agric. Food Chem.* **1997**, *45*, 2556−2560.

(24) Reeves, P.; Nielsen, F.; Fahey, Jr., G. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **1993**, *123*, 1939−1951.

(25) SAS release 8; SAS Institute: Cary, NC, 2001.

(26) Harris, G.; Gupta, A.; Nines, R.; Kresty, L.; Habib, S.; Frankel, W.; LaPerle, K.; Gallaher, D.; Schwartz, S.; Stoner, G. Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydrozy-2′-deoxyguanosine levels in the Fischer 344 rat. *Nutr. Cancer* **2001**, *40*, 125−133.

(27) Magnuson, B.; Lala, G.; Kwon, Y.; Kwon, Y.; Yu, T.; Friedman, J.; Obele, C.; Malik, M. Anthocyanin-rich extracts inhibit growth of human colon cancer cells and azoxymethane-induced colon aberrant crypts in rats: implications for colon cancer chemoprevention. *Cancer Epidemiol. Biomarkers Prev.* **2003**, *12*, 1323S−1324S.

(28) Babbar, N.; Ignatenko, N.; Casero, R.; Gerner, E. Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J. Biol. Chem.* **2003**, *276*, 47762−47775.

(29) Matuschek, M.; Hendriks, W.; McGie, T.; Reynolds, G. The jejunum is the main site of absorption for anthocyanins in mice. *J. Nutr. Biochem.* **2006**, *17*, 31−36.

(30) Galvano, F.; La Fauci, L.; Lazzarino, G.; Fogliano, V.; Ritieni, A.; Ciappellano, S.; Battistini, N.; Tavazzi, B.; Galvano, G. Cyanidins: metabolism and biological properties. *J. Nutr. Biochem.* **2004**, *15*, 2−11.

(31) Meiers, S.; Kemény, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. The anthocyanins cyandin and delphinidin are potent inhibitors of the epidermal growth factor receptor. *J. Agric. Food Chem.* **2001**, *49*, 958−962.

(32) Ladenheim, J.; Garcia, G.; Titzer, D.; Herzenberg, H.; Lavori, P.; Edsos, R.; Omary, B. Effect of sulindac on sporadic colonic polyps. *Gastroenterology* **1995**, *108*, 1083−1087.

(33) Morazzoni, P.; Bombardelli, E. *Vacciumium myrtillus* L. *Fitoterapia* **1996**, *67*, 3−29.

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