Pilot Study of Oral Anthocyanins for Colorectal Cancer Chemoprevention

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Abstract Naturally occurring anthocyanins possess colorectal cancer chemopreventive properties in rodent models. We investigated whether mirtocyan, an anthocyanin-rich standardized bilberry extract, causes pharmacodynamic changes consistent with chemopreventive efficacy and generates measurable levels of anthocyanins in blood, urine, and target tissue. Twenty-five colorectal cancer patients scheduled to undergo resection of primary tumor or liver metastases received mirtocyan 1.4, 2.8, or 5.6 grams (containing 0.5-2.0 grams anthocyanins) daily for 7 days before surgery. Bilberry anthocyanins were analyzed by high performance liquid chromatography (HPLC) with visible or mass spectrometric detection. Proliferation was determined by immunohistochemistry of Ki-67 in colorectal tumor. Concentrations of insulin-like growth factor (IGF)-I were measured in plasma. Mirtocyan anthocyanins and methyl and glucuronide metabolites were identified in plasma, colorectal tissue, and urine, but not in liver. Anthocyanin concentrations in plasma and urine were roughly dose-dependent, reaching ~179 ng/gram in tumor tissue at the highest dose. In tumor tissue from all patients on mirtocyan, proliferation was decreased by 7% compared with preintervention values. The low dose caused a small but nonsignificant reduction in circulating IGF-I concentrations. In conclusion, repeated administration of bilberry anthocyanins exerts pharmacodynamic effects and generates concentrations of anthocyanins in humans resembling those seen in *Apc*^{Min} mice, a model of FAP adenomas sensitive to the chemopreventive properties of anthocyanins. Studies of doses containing <0.5 gram bilberry anthocyanins are necessary to adjudge whether they may be appropriate for development as colorectal cancer chemopreventive agents.

Lack of toxicity is a pivotal requirement for agents to be potentially useful in cancer chemoprevention particularly considering the necessity for prolonged durations of administration. Intervention trials using drugs such as aspirin or celecoxib have been complicated by unwanted side effects (1, 2). There is an absolute requirement to discover and develop novel efficacious and safe alternatives to such drugs. Dietary phytochemicals exemplified by anthocyanins, bright blue- or red-colored polyphenols, which occur ubiquitously in vegetables and fruits, are attractive candidates for clinical evaluation, as they possess a good safety record. Studies in preclinical carcinogenesis models suggest that anthocyanins may prevent malignancies, notably in the gastrointestinal tract (3–8). However, many aspects of their clinical pharmacology, such as concentrations achievable in organs targeted for prevention of malignancy, are only poorly understood. Mechanistic properties of anthocyanins potentially related to chemoprevention encompass antiproliferation, apoptogenicity, and antioxidation (9). The insulin-like growth factor (IGF) signaling system, especially IGF-I and IGF binding protein-3 (IGFBP-3), which possess putative procarcinogenic and anticarcinogenic properties, respectively, has been implicated as a potential target of the polyphenols silibinin (10) and curcumin (11), two cancer chemopreventive phytochemicals with structural similarities to anthocyanins.

There is the growing realization in cancer chemoprevention agent development that mixtures—exemplified by dietary anthocyanins—may have distinct advantages over single agents, because of potential mechanistic synergy of mixture constituents and their decreased propensity to exert untoward effects when administered together at very low doses (12, 13). In the light of all these considerations, we conducted a clinical pilot study with mirtocyan, a standardized anthocyanin mixture extracted from bilberries, currently marketed as a nutraceutical with potential benefit in the treatment of vascular permeability, capillary vessel fragility, and ophthalmologic

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disorders.[5] Mirtocyan contains glycosides of the anthocyanidins cyanidin, delphinidin, malvidin, peonidin, and petunidin bound to glucose, galactose, or arabinose. The aim of this study was to generate pharmacodynamic and pharmacokinetic information to help plan the future clinical evaluation of anthocyanins in colorectal cancer chemoprevention studies. Colorectal cancer patients due to undergo surgical resection of the primary tumor or liver metastases received mirtocyan before surgery and provided tissue for assessment. This pilot study design, in which experimental cancer chemopreventive agents are administered to patients with established cancer, has been used, for example, with perillyl alcohol (14), curcumin (15), silibinin (16), and lycopene (17). Although issues germane to long-term safety and efficacy of such agents need evaluation in healthy humans and/or patients with preneoplasia, pilot studies in patients with cancer can be very useful as they allow early valuable insights into pharmacodynamic and pharmacokinetic properties. In general, information on tissue levels and pharmacodynamics would be difficult to accrue in healthy individuals or patients with preneoplasia. Using this approach, we wished to establish whether oral administration of mirtocyan generates measurable levels of anthocyanins in patients' blood, urine, colorectal, and liver tissue, and whether it causes inhibition of proliferation in colorectal tissue and affects circulating levels of IGF-I and IGFBP-3.

Materials and Methods

Patients and procurement of blood and tissues

The study was approved by the Leicestershire Northamptonshire & Rutland Research Ethics Committee. Fifteen patients with histologically confirmed colorectal adenocarcinomas and 10 patients with confirmed colorectal liver metastases, all amenable to surgical resection, were recruited into the trial between October 2006 and May 2008 and gave written informed consent. The study was pilot in nature, it was not blinded, and it was designed around the patients' routine care. Subjects were selected based on a consecutive colorectal cancer patient population and randomized with respect to dose level. Patient characteristics are shown in Table 1. Peripheral blood was collected in heparinized tubes before intervention and 1 h after the penultimate mirtocyan dose, portal blood at surgery. Blood was centrifuged to generate plasma, which was kept on ice until storage. Patients were recruited into the study after their diagnosis of cancer. This means that they had their endoscopy, in which cancer tissue but not normal tissue is taken routinely to enable diagnosis, before recruitment. Retrospective consent was obtained from them to use biopsies. Therefore presurgery biopsy samples of normal colorectal tissue could not be obtained. Colectomy and liver surgery occurred 6.0 ± 2.7 h and 4.7 ± 1.2 h, respectively, after the last mirtocyan dose. Samples (~1 gram) of tissue were taken from resection specimens, in the case of normal colorectal tissue from sites 5 and 10 cm proximal and distal, respectively, to the tumor. Utmost care was taken to perform tissue processing as rapidly as possible, within minutes after tissues has been resected. Surgical tissue samples did not show histologic evidence of necrosis when reviewed by a pathologist. Tissue samples were snap frozen (liquid nitrogen). Biomatrices were stored at −80° C for up to 2 mo. Preliminary HPLC analysis established that bilberry anthocyanins are stable under these conditions in tissues and plasma.

[5] http://www.mirtoselect.com

Intervention and dose

Mirtocyan (formerly mirtoselect), a standardized extract of bilberries (supplied by Indena S.p.A.), was prepared by an industrial proprietary process ensuring constant and reproducible anthocyanin composition (36%, w/w). Predominant anthocyanin constituents are delphinidin-3-galactoside, delphinidin-3-glucoside, and delphinidin-3-arabinoside, and cyanidin-3-galactoside and cyanidin-3-glucoside (Indena datasheet). Other anthocyanins in mirtocyan are cyanidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside, and malvidin-3-arabinoside. Mirtocyan also contains other polyphenols (phenolic acids, flavonols, proanthocyanidins; ~18%), carbohydrates and aliphatic organic alcohols (~29%), fats (~0.04%), nitrogen compounds (~1%), ash (~0.7%), with the remaining 15% undefined. Mirtocyan was formulated in gelatin capsules (0.47 gram per capsule; Nova Pharmaceuticals) and administered thrice a day for 7 d at total daily doses of 1.4, 2.8, or 5.6 grams. These doses span the dietary dose of mirtocyan in Apc^{Min} mice (~450 mg/kg mouse = ~2.6 g per 80 kg human extrapolated by dose-surface area comparison; see ref. 18), which reduced adenoma number by 30% (8). The last third of the daily dose was administered in the early morning before surgery, when the patients were fasting.

Analysis of anthocyanins by HPLC-VIS and -MS/MS

Anthocyanins exist in a pH-dependent dynamic equilibrium of at least four tautomers (19). Only one, the flavylium cation (Fig. 1) predominating at pH of <2, is colored. Acidification of biomatrix extracts transformed colorless tautomers into flavylium ions detectable by HPLC with visible spectroscopy (VIS).

Biomatrices were thawed, and plasma was centrifuged. Colorectal tumor and normal tissue samples (150 mg) were homogenized (PBS, 1:10 w/v) and centrifuged (5, 000 × *g*, 20 min); the residue was rehomogenized and recentrifuged. Aliquots of plasma supernatant (2 mL), urine (1 mL), or colorectal tissue supernatant were subjected to solid phase extraction as described before (7). In the case of liver, tissue was homogenized with PBS (1:2.5 w/v) containing formic acid (2%). The homogenate was mixed with acetone/formic acid (9:1), and the mixture was centrifuged. The supernatant was dried (nitrogen), and the residue was reconstituted in water/formic acid. Solid phase eluate (for plasma, urine, colorectal tissue) and liver extract were analyzed by HPLC with detection by VIS (510 nm) or tandem mass spectrometry (MS/MS), the latter with selected reaction monitoring (SRM), operated in the positive ion mode, as described and validated previously (20). Anthocyanin concentrations were estimated semiquantitatively using a standard curve for authentic cyanidin-3-glucoside, based on the simplifying assumption that all detected anthocyanins possess similar molar absorption coefficients. The molecular weight of cyanidin-3-glucoside was applied to all anthocyanins measured; therefore, results are approximate values prefaced by the ~ sign.

Pharmacodynamic analyses

Colorectal tumor sections, obtained before and after the consumption of mirtocyan, were stained for Ki-67 (mouse anti-human monoclonal antibody; Dako) or cleaved caspase-3 (cleaved caspase-3 Asp 175 polyclonal antibody; New England Biolabs). Tissue-antibody reaction was visualized by a commercial kit (Dako). Representative fields were selected in the biopsies and from superficial regions of the resected tumor specimens. The total number of epithelial cells and the number of positively staining epithelial cells were counted in six, adjacent high power fields (magnification, ×400; Leitz Orthoplan microscope, Leica DC 300 camera) for each sample by two independent observers. Analyses were done blinded. Differences in counts between these observers were <10%, and both observed the same differences between cohorts. Acqusition software was Adobe Photoshop version 7. Numbers of epithelial cells counted in each preintervention and

Table 1. Characteristics of 15 patients with colorectal cancer (A) and 10 patients with colorectal liver metastases (B) who received mirtocyan

A.

Characteristics	Value
Age (y)	
Median	64
Range	47-90
Gender	
Female/Male	5/10
Body mass index, kg/m^2	
Median	29
Range	16.7-38.6
ASA grade	
1	3
2	9
3	3
4/5	0
Drug history	
Aspirin	2
Antihypertensives	7
Antianginals	0
Analgesics (non-NSAID)	2
Neoadjuvant therapy	0
Tumor location*	
Caecum/ascending colon	7
Transverse colon	1
Descending colon	1
Sigmoid colon	6
Rectum	1
Tumor differentiation*	
Well	0
Moderate	15
Poor	1
Dukes stage of tumor*	
A	0
B	9
C1	7
C2/D	0

B.

Characteristics	Value
Age, y	
Median	66
Range	50-76
Gender	
Female/Male	2/8
Body mass index, kg/m^2	
Median	29
Range	24-34
ASA grade	
1	3
2	2
3	5
4/5	0

Table 1. Characteristics of 15 patients with colorectal cancer (A) and 10 patients with colorectal liver metastases (B) who received mirtocyan (Cont'd)

B.

Characteristics	Value
Drug history	
Aspirin	1
Antihypertensives	3
Antianginals	0
Analgesics (non-NSAID)	1
Neoadjuvant therapy	
Radiotherapy	0
Chemotherapy	8
Tumor location	
Right lobe	2
Left lobe	2
Bilobar	6
No. of metastases	
1	2
2	3
3/4	0
5 or more	5
Original bowel resection	
Right hemicolectomy	5
Sigmoid colectomy	1
Anterior resection	4
Time from bowel resection to liver resection (mo)	
Median	9.5
Range	6-70

Abbreviation: NSAID, non-steroidal anti-inflammatory drug.
*A total of 16 adenocarcinomas were identified in colorectal resection specimens.

postintervention sample stained for Ki-67 were 1,161 ± 312 and 1,330 ± 465, respectively, and for caspase-3 analysis, they were 1,161 ± 403 and 1,516 ± 703, respectively. Preintervention samples obtained from two patients contained insufficient tissue and were excluded. IGF-I and IGFBP-3 concentrations in plasma were determined using ELISA kits DG 100 and BAF675, respectively (R&D Systems). Assays were validated and done according to the manufacturer's instructions.

Statistical analyses

Statistical comparisons were made by paired Student's *t* test or, in the case of the analysis of plasma IGF-I concentrations, Wilcoxon signed-rank test for nonparametric data, using SPSS version 13 (Windows XP).

Results

Safety of mirtocyan

Consumption of 1.4, 2.8, or 5.6 grams mirtocyan (containing 0.5, 1.0, or 2.0 grams anthocyanins) daily for 7 days by 10, 8, or 7 patients, respectively, was safe and well-tolerated. Patients who consumed the high dose reported the development of dark stool while on treatment, an expected consequence of high anthocyanin consumption. Two adverse events occurred in trial participants. Just before being anaesthetized one colorectal patient, a known arteriopath, developed a supraventricular

tachycardia, and his operation was postponed. Another trial participant suffered a myocardial infarct during liver resection, but he made a full recovery. Both events, which occurred in patients who consumed the low and middle doses, respectively, were considered unrelated to the consumption of mirtocyan.

Characterization of anthocyanins in blood, tissue, and urine

Plasma, urine, and normal and malignant colorectal and hepatic tissues obtained from patients were analyzed for presence of anthocyanins by HPLC-VIS and HPLC-MS/MS. Blood and urine were taken 1 hour, and urine 11.5 ± 1.0 hours, after the penultimate mirtocyan dose. Anthocyanins were detected in extracts of peripheral plasma and urine from all patients and of pooled extracts of colorectal tissue from patients on 1.4 or 5.6 grams mirtocyan (Fig. 1*A-D*), but not in liver tissue. Anthocyanin species in portal blood were found by spectroscopic detection in patients for whom the delay between dosing and sampling was 5.7 ± 1 hours ($n = 3$), but they were below the detection limit (~0.5 ng/mL, 1 nmol/L; ref. 20) in patients in whom this delay was 8.1 ± 2.1 hours ($n = 9$). Mass spectrometric analysis and comparison of chromatograms with information contained in the Indena datasheet allows allocation of at least 14 and 17 discrete molecular species to individual peaks in colorectal tissue and urine, respectively (Table 2). The identified species include unaltered constituents of the original mirtocyan mixture such as delphinidin-3-glycosides and cyanidin-3-glycosides, three glucuronide metabolites and glycosides of malvidin, petunidin, and peonidin, which were either unaltered mirtocyan ingredients or products of metabolism of original mirtocyan components.

The most abundant plasma anthocyanin peak (Fig. 1B, *) coeluted with peonidin-3-glucoside. Five other anthocyanin peaks in plasma from patients on the 5.6-gram dose coeluted with delphinidin-3-galactoside, delphinidin-glucoside, delphinidin-arabinoside, and cyanidin-3-galactoside and cyanidin-glucoside (Fig. 1B, *peaks 1-5*). Plasma anthocyanins were not amenable to structural confirmation by HPLC-MS/MS. The most prominent anthocyanin species recovered from colorectal tissue and urine (Figs. 1D and C, *arrows*) gave SRM mass transitions of mass-to charge ratios (m/z) 463>331 (Fig. 1*E*) and 477>301 (Fig. 1*F*), respectively. Both molecules are likely to be anthocyanin metabolites. The former mass transition is consistent with methylpetunidin-3-arabinoside or its positional isomer dimethyldelphinidin-3-arabinoside (Fig. 1*E*), the latter with peonidin glucuronide or isomeric methylcyanidin glucuronide (Fig. 1*F*). Analysis of normal colorectal tissue yielded chromatograms (data not shown) very similar to those obtained for tumor (Fig. 1*D*).

Concentrations of anthocyanins in blood, tissue, and urine

Total anthocyanin levels measured semiquantitatively in peripheral plasma and urine were approximately related to mirtocyan dose (Fig. 2). In the plasma of patients on the low dose of mirtocyan, the species tentatively characterized as peonidin-3-glucoside (see above) was the only anthocyanin amenable to semiquantitation; in patients on 2.8 and 5.6 grams mirtocyan, it constituted ~50 and ~30%, respectively, of total anthocyanins (data not shown). At the highest dose, total anthocyanin plasma concentration was ~117 ± 57 ng/mL (266 pmol/mL; Fig. 2).

Pooled tumor and normal colorectal tissue taken either proximal or distal to the tumor from five patients on the highest dose afforded ~179, 96, and 123 ng anthocyanins per gram tissue (0.40, 0.22, and 0.28 nmol/g), respectively. In colorectal tissue from these patients, the amount of the most prominent anthocyanin, methylpeonidin-3-arabinoside, or dimethylcyanidin-3-arabinoside (see above) was ~94 ng/g (0.21 nmol/g) in tumor and ~39 ng/g (0.09 nmol/g) in normal tissue. In tissue samples from patients on 1.4 or 2.8 grams mirtocyan, anthocyanin species were too low for semiquantitation.

Effect of mirtocyan on tumor proliferation and circulating IGF-I

Immunohistochemical observations were made in biopsy samples preintervention versus resection tissue postintervention. In colorectal tumors from all patients who had received mirtocyan, the proliferation index reflected by Ki-67 staining was significantly decreased by 7% ($P = 0.003$) compared with the preintervention value (Fig. 3A). The decrease in tumor tissue proliferation in patients on 1.4 grams mirtocyan was 9% ($P = 0.021$), but the decrease was not significant in the 2.8 and 5.6-gram dose cohorts. The apoptotic index in colorectal cancer samples from all patients increased from 3.6% to 5.3% of epithelial cells ($P = 0.04$, $n = 12$; data not shown). However, this increase may, at least to some extent, be the consequence of inherent procedural differences in measurements, as tissue pharmacodynamics were compared between biopsy samples and resection material. In a recent study in rectal cancer patients, caspase-3 activity in biopsies was found to be lower than that in the corresponding resected tumor specimens (21), implying a stimulating effect of tumor resection on tissue apoptosis. In the absence of a zero dose control group in our study, we cannot exclude the possibility that the increase in apoptosis observed here in postintervention compared with preintervention tissue samples was due to the surgical procedure. Nevertheless, it is unlikely that resection affected tissue proliferation.

Mirtocyan did not change serum IGF-I (Fig. 3B) or IGFBP-3 concentrations or the ratio of IGF-I to IGFBP-3 significantly (data not shown). However, there was a hint that circulating levels of IGF-I might be lower than the preintervention values, with P value of 0.06 for all patients combined. Mirtocyan failed to affect levels of oxidative DNA damage as reflected by leukocytic malondialdehyde-DNA adducts or urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (data not shown).

Discussion

Because of their excellent safety record, mixtures of dietary constituents, exemplified by mirtocyan, are attractive as potential cancer chemopreventive alternatives to drugs such as aspirin and celecoxib. It seems likely that an increasing number of such mixtures will be subjected to clinical cancer intervention studies, although their rational development provides more challenges than that of a single agent. Often discrete pharmacologically active constituents responsible for the putative efficacy of a mixture are not clearly defined, confounding the pharmacokinetic and pharmacodynamic objectives of such studies. The clinical pilot study of a mixture

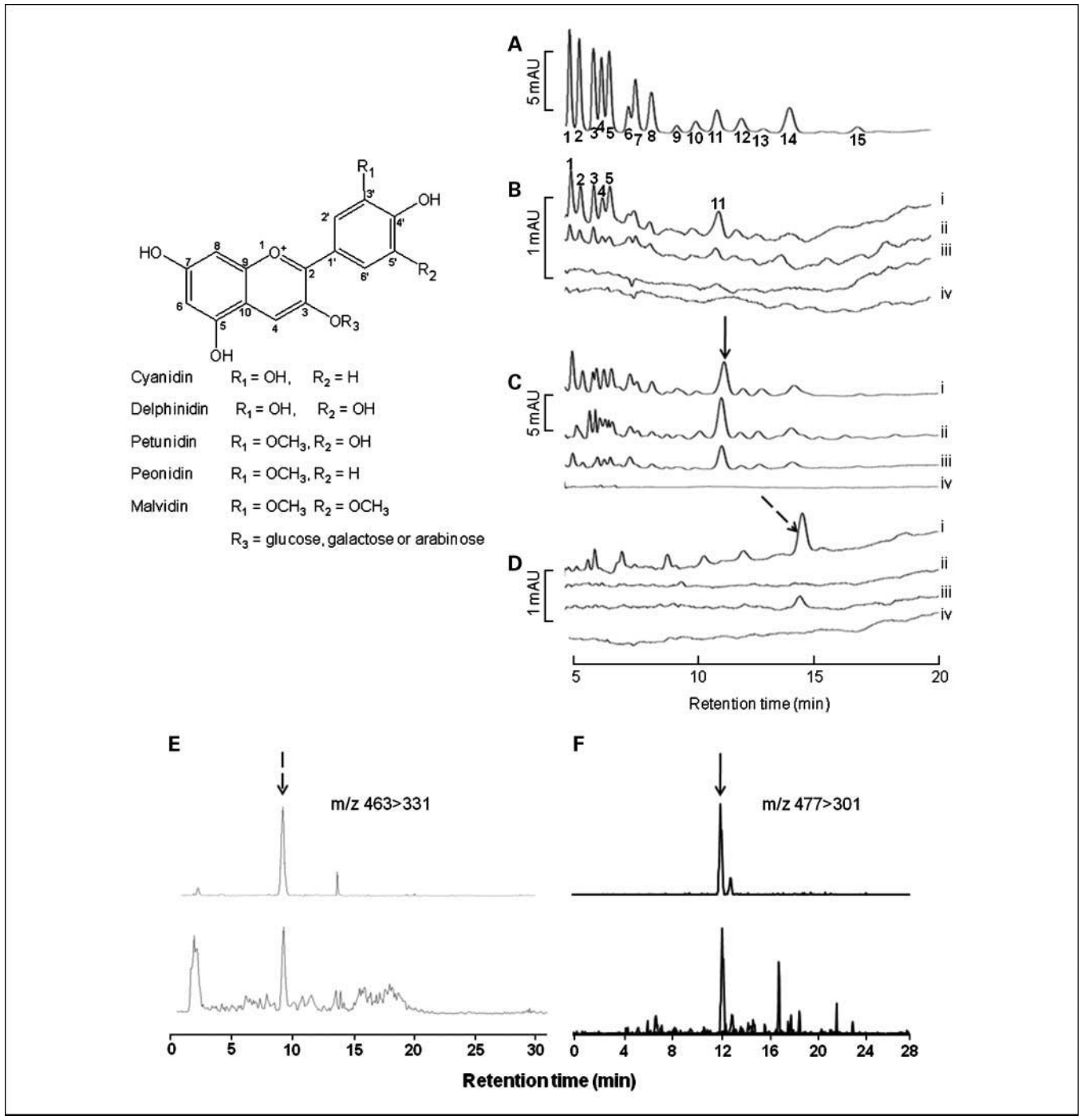

Fig. 1. HPLC-VIS chromatograms of extracts of authentic mirtocyan (*A*) and of plasma (*B*), urine (*C*), or colorectal tumor tissue (*D*) from patients who received 1.4 (*i*), 2.8 (*ii*), or 5.6 grams (*iii*) of mirtocyan daily for 7 d; and identification by HPLC-MS/MS of major anthocyanin species in extracts of tumor tissue (*E*) and urine (*F*) from patients on 5.6 grams of mirtocyan. *Inset,* structures of anthocyanins contained in mirtocyan; glycosidic sugars are attached via C3 in the anthocyanidin. Traces represent representative samples (*B* and *C*) of 10 (low dose, *i*), 8 (medium dose, *ii*), or 7 patients (high dose, *iii*), or pooled tumor tissue samples (*D*) of 4 (*i*) or 5 patients (*ii* and *iii*). *iv,* samples of predose "control" biofluids (*A* and *B*) and for tumor tissue. *D,* pooled presurgery biopsy samples. Numbers below/above peaks in *A* and *B* identify mirtocyan anthocyanin species (by HPLC-MS/MS and comparison with chromatographic data in the Indena datasheet) as follows: *1,* delphinidin-3-galactoside; *2,* delphinidin-3-glucoside; *3,* cyanidin-3-galactoside; *4,* delphinidin-3-arabinoside; *5,* cyanidin-3-glucoside; *6,* petunidin-3-galactoside; *7,* cyanidin-3-arabinoside; *8,* petunidin-3-glucoside; *9,* peonidin-3-galactoside; *10,* petunidin-3-arabinoside; *11,* peonidon-3-glucoside; *12,* malvidin-3-galactoside; *13,* peonidon-3-arabinoside; *14,* malvidin-3-glucoside; *15,* malvidin-3-arabinoside. Peonidin-3-glucoside (compound 11) is the major anthocyanin in plasma (*B*). *Arrows,* major anthocyanin species in urine (*C*) and tumor (*D*), which were identified by HPLC-MS/MS with SRM (*E* and *F*). Extracts of pooled tissue from 5 patients in *E*, and of one urine sample representative of 7 patients in *F* were analyzed for 28 mass transitions spanning conceivable anthocyanins and metabolites generated by methylation, glucuronidation, and sulfation of mirtocyan anthocyanins. Top traces in *E* and *F* show peaks at specific mass transitions, and bottom traces total ion chromatogram (*E*) or composite chromatogram of all 28 channel transitions (*F*). Transition m/z 463>331 characterizes the major peak in tumor tissue (*E*) as methylpetunidin arabinoside or isomeric dimethyldelphinidin arabinoside (Rt, 9.0 min), with a less abundant peak for malvidin-3-arabinoside (Rt, 13.8). Transition m/z 477>301 identifies the most abundant species in urine (*F*) as peonidin glucuronide/methylcyanidin glucuronide (Rt, 12-13 min; two species). Note that chromatographic conditions (column length, detector) in *E* and *F* differed from those used to yield the results shown in *A* to *D*; therefore, there are slight discrepancies in retention time.

Table 2. Anthocyanins identified by HPLC-MS/MS in tumor tissue and urine of patients who received mirtocyan (5.6 grams) daily for 7 d

Anthocyanin species	Retention time (min)	SRM transition (m/z)*	Tumor	Urine
Delphinidin-3-galactoside	4.9	465 > 303	+	+
Delphinidin-3-glucoside	5.5	465 > 303	+	+
Delphinidin-3-arabinoside	6.3	435 > 303		+
Cyanidin-3-galactoside	5.9	449 > 287	+	+
Cyanidin-3-glucoside	6.7	449 > 287	+	+
Cyanidin-3-arabinoside	7.7	419 > 287	+	+
Petunidin-3-galactoside	7.1	479 > 317	+	+
Petunidin-3-glucoside	8.2	479 > 317	+	+
Petunidin-3-arabinoside	10.0	449 > 317	+	+
Peonidin-3-galactoside	8.9	463 > 301	+	
Methylcyanidin-3-galactoside	9.2	463 > 301		+
Peonidin-3-glucoside	10.8	463 > 301	+	
Methylcyanidin-3-glucoside	11.1	463 > 301		+
Peonidin -3-arabinoside	12.1	433 > 301		+
Malvidin-3-galactoside	11.4	493 > 331	+	+
Malvidin-3-glucoside	13.4	493 > 331	+	+
Methylpetunidin-3-arabinosideor dimethyldelphinidin-3-arabinoside	9.0	463 > 331	+†	
Malvidin-3-arabinoside	13.8	463 > 331	+	
Cyanidin glucuronide	6.4	463 > 287		+
Peonidin glucuronide and/or methylcyanidin glucuronide	11.2, 12.6	477 > 301		+†
Malvidin glucuronide	13.8	507 > 331		+

*The SRM transitions monitored correspond to loss of the sugar/glucuronide fragment from the parent $[M+H]^+$ molecular ion.
†Major species in respective biomatrix.

of bilberry constituents described here was designed to address some of the difficulties inherent in the clinical development of such mixtures. Anthocyanins are the putative cancer chemopreventive ingredients of bilberry, as borne out by their efficacy in preclinical models, especially those of gastrointestinal malignancies: anthocyanins and anthocyanin-rich fruit extracts decreased aberrant crypt foci and reduced colonic adenocarcinoma burden in rats that had received the chemical carcinogens azoxymethane, 1,2-dimethylhydrazine or 2-amino-1-methyl-6-phenylimidazo[4, 5-*b*]pyridine (3–5). Anthocyanin-rich berry extracts protected against esophageal cancer in rodents (6) and reduced adenoma development in Apc^{Min} mice (7), a model of human familial adenomatous polyposis coli. Mirtocyan and cyanidin-3-glucoside as a single agent were also efficacious in the Apc^{Min} model (8). Additionally, cyanidin-3-glucoside interfered with experimental skin tumorigenesis and reduced lung tumor growth and metastasis in the A549 nude mouse xenograft model (22). These preclinical data in conjunction with the safety record of berry extracts justify consideration of the clinical development of berry anthocyanins as colorectal cancer chemopreventive agents.

Anthocyanin species were identified in patients' plasma, colorectal tissue, and urine. In addition to molecules contained in the original mirtocyan mixture including glycosides of delphinidin and cyanidin, products of metabolic glucuronidation and *O*-methylation of anthocyanins were found. Little is known about differences in biological potency between individual anthocyanins including their metabolic conjugates, and it is conceivable that methylpetunidin-3-arabinoside or dimethyldelphinidin-3-arabinoside, the major metabolite(s) identified here in colorectal tissue after mirtocyan consumption, may constitute one of several anthocyanin species responsible for pharmacodynamic effects.

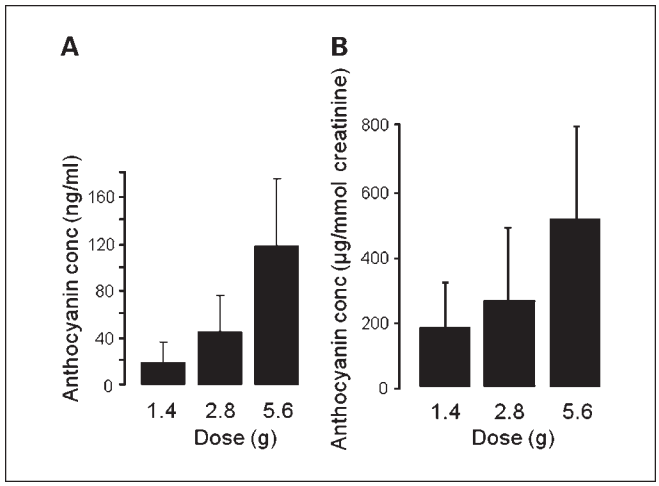

Fig. 2. Approximate concentrations of anthocyanins in the peripheral plasma (*A*) and urine (*B*) of patients who received mirtocyan 1.4 (n = 10), 2.8 (n = 8), or 5.6 grams (n = 7) daily for 7 d. Plasma samples were obtained 1 h, and urine 11.5 ± 1.0 h, after the penultimate dose of mirtocyan. Anthocyanins were semiquantitated by HPLC analysis using a calibration curve for cyanidin-3-glucoside, assuming that the spectroscopic absorption of all anthocyanins detected here are similar. In the case of the plasma, only one species was quantified for the 1.4-gram dose, characterized tentatively as peonidin-3-glucoside by cochromatography. Urinary concentrations are standardized to creatinine.

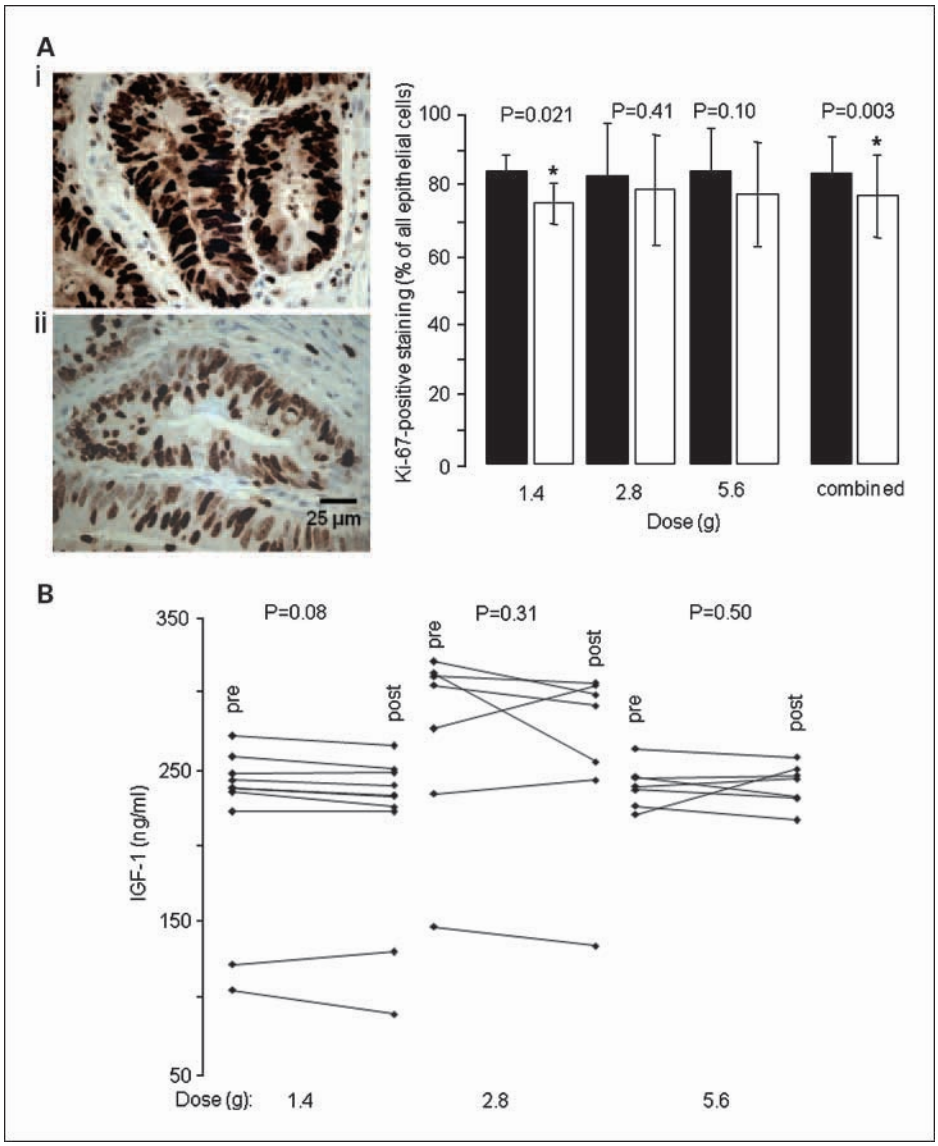

Fig. 3. Effect of consumption of mirtocyan (1.4, 2.8, or 5.6 grams) daily for 7 d on colorectal tumor tissue proliferation as reflected by Ki-67 staining (*A*) and circulating levels of IGF-I (*B*). *A,* immunohistographs of colorectal tumor tissue from a representative patient after staining for Ki-67 before (*i*) and after (*ii*) consumption of 1.4 grams mirtocyan; magnification, ×400. *Open* and *closed bars,* preintervention and postintervention values, respectively; *B,* concentrations of IGF-I preintervention and postintervention for individual patients. Number of patients in *A* was 4 per dose level and 12 for the combined doses; *B,* 10 patients in the 1.4-gram dose and 7 in each of the 2.8 and 5.6-gram dose groups. Note that in the case of the low dose in *B*, the fifth line from the top (or *bottom*) describes identical values in two patients. Statistical significance of differences was evaluated using the Student's *t* test in *A* and Wilcoxon signed-rank test in *B*. *P* values are indicated above the graphs. In *B*, the *P* value for all patients combined was 0.06. A significant difference compared with the preintervention value is denoted by an asterisk.

Comparison of the doses of mirtocyan used here with consumption of anthocyanins in the diet suggests that the 1.4-gram dose containing ~0.5 gram anthocyanins equates to ~370 grams fresh bilberries (23). Average daily consumption of anthocyanins with the Western diet has been estimated at around a fifth of that amount (~100 mg; refs. 24, 25). In Table 3, anthocyanin concentrations measured after consumption of mirtocyan in a recent study in Apc^{Min} mice (8) are juxtaposed with those described here in humans. Approximate concentrations of anthocyanins in tumor and normal tissue proximal or distal to the tumor from patients on mirtocyan at the highest dose were only 2.2%, 1.2%, and 1.5%, respectively, of the total anthocyanin concentration observed in the intestinal mucosa of Apc^{Min} mice that had received mirtocyan 0.3% (equating to a dose of ~450 mg/kg) in the diet for their lifetime (8). This dietary dose of mirtocyan, which is almost identical to the medium dose administered here (2.8 g = 485 mg/kg, by body surface extrapolation; see ref. 18), caused significant reduction in Apc^{Min} adenoma number (8). According to this comparison, a dose of mirtocyan that was twice as high as an efficacious dietary dose in Apc^{Min} mice generated levels in the human target organ of only ~1/50th of those observed in mice. This discrepancy indicates that the handling of anthocyanins in humans may be fundamentally different from that in mice, tentatively militating against a potential role of these agents in human cancer chemoprevention. The difference may, at least in part, be the consequence of the fact that mirtocyan was given in mice with the diet, whereas in the trial, the final dose was administered in patients who were fasting, and diet may conceivably impede anthocyanin absorption. When the adenoma development retarding efficacy of cyanidin-3-glucoside as single agent was determined in the Apc^{Min} mouse (8), intestinal mucosal levels associated with the adenoma number-retarding dietary dose of 0.3% were 43 ng/gram tissue. This value is of the same order of magnitude as the colorectal tissue total anthocyanin concentration determined here in humans on the highest dose of mirtocyan. The plasma concentration of anthocyanins measurable in Apc^{Min} mice on dietary mirtocyan (0.3%; ref. 8) was almost identical to the value observed here in the plasma of patients who received the

Table 3. Comparison of anthocyanin concentrations in plasma and intestinal tissue between *Apc*^{Min} mice and humans after consumption of mirtocyan

	Mean anthocyanin concentration (ng/mL or ng/g)	
	Mouse	**Human**
Plasma*	46	48
Intestine†	8,100	96-179

*Mirtocyan dose: ~450 mg/kg in mice, 485 mg/kg in humans.
†Mirtocyan dose: ~450 mg/kg in mice, 970 mg/kg in humans.

medium dose of mirtocyan (Table 3; Fig. 3A). It is important to emphasize the tenuous nature of such quantitative comparisons, not least because the amounts of individual mirtocyan anthocyanins and their metabolites in the biomatrices are bound to differ considerably between mice and humans. Furthermore, in the above comparisons, the implicit assumption is made that different mirtocyan anthocyanin glycosides possess comparable adenoma-reducing potency, a notion that is probably simplistic and needs experimental verification.

The recovery of quantifiable amounts of anthocyanins from urine at all three dose levels suggests that it should be feasible in future intervention trials to use urinary anthocyanin concentrations as an indicator of adherence. In the light of the intense color of the anthocyanin molecule as the flavylium tautomer, development of a simple and inexpensive colorimetric method for anthocyanin determination in urine extracts seems possible.

The results of the pharmacodynamic part of the investigation presented above hint at the possibility that in humans bilberry anthocyanins, in addition to being well-tolerated, may exert effects consistent with colorectal cancer chemoprevention. Consumption of mirtocyan led to a small decrease in proliferation in target tissue. Although the biological importance of such a slight decrease is debatable, its observation supports the notion that, in principle, bilberry anthocyanins can exert a pharmacologic effect in humans. Antiproliferative effects of mirtocyan anthocyanins are consistent with reports of the ability of anthocyanins to arrest growth and induce apoptosis *in vitro* in cells derived from a variety of malignancies (9), notably those of the gastrointestinal tract (26, 27). The increase in apoptosis observed here in postintervention compared with preintervention tumor samples are difficult to interpret as it may have been the consequence of the surgical procedure rather than agent intervention. Consumption of mirtocyan also reduced, albeit not significantly, IGF-I serum concentrations. In view of the putative positive association between IGF-I serum levels with colorectal cancer incidence (28), the indication that anthocyanins may reduce IGF-I in the biophase warrants experimental confirmation.

In conclusion, administration of an anthocyanin-rich bilberry extract may cause pharmacodynamic changes in humans commensurate with colorectal cancer chemoprevention. The dose range used here generated concentrations of anthocyanins in human plasma that resembled those seen previously in *Apc*^{Min} mice. The pharmacodynamic changes observed seemed to be more prominent in patients at the dose of 0.5 gram anthocyanins, which elicited target tissue levels below the detection limit, than at the 2.0 gram dose, which furnished detectable anthocyanin levels in colorectal tissue. These levels were ~1/50th of the intestinal levels associated with reduction of tumor incidence in *Apc* ^{Min} mice. A potential explanation of this conundrum is that in the case of the low dose, a constituent of the anthocyanin mixture, which can counteract the pharmacodyamic changes elicited by anthocyanins, would be more diluted in the biophase, thus less inhibitory, than after the high dose. Alternatively, the pharmacology of anthocyanins may be characterized by an unusual dose-response relationship, which has been described before for other dietary agents. For example, in a nested case-control study of colorectal patients with matched references, the relationship between plasma folate levels and colorectal cancer risk observed a bell-shaped curve, with decreased risk in the lowest and highest quintiles (29). Further clinical studies of pharmacodynamic effects of bilberry anthocyanins administered at doses lower than those used here should help decide whether they warrant evaluation as potential colorectal cancer chemopreventive intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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