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Anthocyanin-rich red grape extract impedes adenoma development in the Apc^{Min} mouse: Pharmacodynamic changes and anthocyanin levels in the murine biophase

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ABSTRACT

Purpose: Red grape pomace extract (oenocyanin) is a cheap and rich source of anthocyanins, the agents suggested to possess cancer chemopreventive properties. Here the hypothesis was tested that oenocyanin added to the diet can interfere with intestinal adenoma development in the Apc^{Min} mouse, a model of intestinal carcinogenesis linked to an *Apc* mutation.

Methods: Mice received oenocyanin (0.3%) in their diet until week 16, when adenoma number and burden were recorded. Expression of Akt and ERK proteins was studied by Western blot in adenomas to discover effects of anthocyanins on cellular signalling *via* the PI3 and MAP kinase pathways. Levels of anthocyanins were measured by HPLC with visible spectroscopic or mass spectrometric detection.

Results: In mice which had consumed oenocyanin, overall adenoma burden was halved and adenoma number was marginally reduced when compared with mice on control diet. The proliferation index in colonic adenomatous crypts, as reflected by Ki-67 staining, was significantly decreased from 88.14% in control mice to 75.6 ± 4% in mice on oenocyanin ($P = 0.014$). Expression of Akt in small intestinal adenomas from Apc^{Min} mice on oenocyanin was reduced by 54% ($P = 0.003$), when compared to controls. Oenocyanin anthocyanins and glucuronide metabolites were found in the urine and intestine but not in plasma.

Conclusions: The results suggest that oenocyanin may be a viable and economical alternative to anthocyanin-rich berry extracts for chemopreventive intervention. Akt and pErk might be suitable biomarkers of anthocyanin target organ efficacy.

1. Introduction

Recent years have seen a considerable resurgence of interest in botanical remedies, exemplified by extracts of plants, fruits and vegetables, rooted in the realisation that such multicomponent and multifunctional 'drugs' may be better suited to prevent or control complex pleiotropic diseases than single active ingredient pharmaceuticals.[1] This realisation has led to a search for plant constituents which may contribute to the pharmacological activity of the source. The health-pro-

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moting properties of fruits, berries and certain vegetables such as purple corn and purple sweet potatoes have been associated with their constituent anthocyanins, water-soluble flavonoid pigments. Anthocyanins possess cancer chemopreventive properties in rodent models of gastrointestinal carcinogenesis.[2–7] Dietary consumption of mirtocyan, a standardised anthocyanin-rich extract of bilberry, or its major constituent cyanidin-3-glucoside, reduced intestinal adenoma development in the *Apc*^{Min} mouse.[7] This mouse, which is characterised by an *Apc* gene mutation, is a model of the human heritable condition familial adenomatous polyposis coli,[8] and it is frequently used in the discovery of novel cancer chemopreventive interventions. In a recent human pilot study, daily consumption of mirtocyan for 7 d by colorectal cancer patients reduced proliferation and induced apoptosis in tumour cells and generated pharmacologically active anthocyanin levels in colorectal tissue.[9] These results encourage further development of anthocyanins as putative colon cancer chemopreventive agents. Red grape pomace extract (oenocyanin) contains high concentrations of the 3-glucosides of the anthocyanidins delphinidin, cyanidin, petunidin, peonidin and malvidin. As an inexpensive byproduct of wine making oenocyanin is an attractive anthocyanin source, but its cancer chemopreventive properties have yet to be studied.

Several mechanisms have been suggested to explain how anthocyanins may prevent malignancies. Amongst them are antiproliferation, induction of apoptosis, antioxidation and inhibition of pathways involving signalling via epidermal growth factor receptors (EGFR), PI3 kinase and MAP kinase.[10] We wished to explore how oenocyanin affects adenoma development in the *Apc*^{Min} mouse and whether it modulates cellular signalling *via* the PI3 and MAP kinases in *Apc*^{Min} mouse adenomas, as reflected by expression levels of Akt and ERK proteins, respectively. Furthermore anthocyanin concentrations were measured in murine biofluids and intestinal mucosa, the chemoprevention target organ, to help interpret the observed chemopreventive efficacy.

2. Materials and methods

2.1. Materials

Oenocyanin E163, an extract of red grape pomace and a waste product of red wine and grape juice, was produced by Bagnarese SpA (Bagnara di Romagna, Italy), was kindly provided by Indena SpA (Milan, Italy). The anthocyanin content of oenocyanin E163 is standardised at 22% (w/w). It contains the following five major anthocyanins, their approximate relative abundance in percentage of total anthocyanins (=100%) is indicated in brackets: delphinidin-3-glucoside (7%), cyanidin-3-glucoside (16%), petunidin-3-glucoside (12%), peonidin-3-glucoside (20%) and malvidin-3-glucoside (40%) (Bagnarese SpA certificate of analysis). Minor anthocyanin constituents, which altogether amount to 5% of total anthocyanins, are delphinidin-3-glucoside acetate, cyanidin-3-glucoside acetate, petunidin-3-glucoside acetate, peonidin-3-glucoside acetate, malvidin-3-glucoside acetate, delphinidin-glucoside p-coumarate, cyanidin-3-glucoside p-coumarate, petunidin-3-glucoside p-coumarate, peonidin-3-glucoside p-coumarate and malvidin-3-glucoside p-coumarate. Other constituents of oenocyanin E163 are polyphenols other than anthocyanins (flavan-3-ols, i.e. catechins and procyanidins ~12%), carbohydrates (~25%), organic acids (~14%), aliphatic alcohols (~9%), nitrogen compounds (~1%), ash (~1%) with the remaining ~16% undefined. The identity of the major anthocyanins in oenocyanin E163 was confirmed by HPLC-mass spectrometric analysis (data not shown). All antibodies and Western blot reagents were from Cell Signalling Technologies (Beverly MA), Santa Cruz (Heidelberg, Germany) or Geneflow (Staffordshire, United Kingdom).

2.2. Animals and interventions

Breeding colonies were established in the Leicester University Biomedical Services facility using C57BL/6J Min/+ (*Apc*^{Min}) mice originally obtained from the Jackson Laboratory (Bar Harbour, ME). Ear tissue from newborn mice was genotyped for the presence of the mutation using PCR as described previously.[11] Experiments were carried out under animal project licence PPL 80/2167, granted to Leicester University by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the UKCCCR guidelines.[12] *Apc*^{Min} mice (13–14 per group) received standard American Institute of Nutrition (AIN)-93G diet or AIN-93G diet supplemented with oenocyanin at a dietary concentration of 0.3% (w/w) from week 4 to the end of the their life. Animals were culled in the fed state in week 16 by cardiac exsanguination under halothane anaesthesia. The intestinal tract was removed and flushed with phosphate-buffered saline (PBS). Intestinal tissue was cut open longitudinally, and multiplicity, location and size of adenomas were recorded as described previously[11] using a magnifying glass (5×). Polyp volume was derived from polyp diameter; consistent with their histological appearance, a hemispherical shape was assumed for the small bowel polyps and a spherical shape for colon polyps. Tumour burden is the sum of polyp volume per animal. Adenoma size is defined as follows: small <1 mm diameter, medium 1–3 mm diameter and large > 3 mm diameter. The small intestines and colons of some animals were made into Swiss rolls for histochemical analysis.

2.3. Immunohistochemistry

To evaluate the effect of oenocyanin on cell proliferation, formalin-fixed small intestine and colon tissues from *Apc*^{Min} mice were examined. Cell proliferation was assessed using a rabbit polyclonal Ki-67 antibody (NCL-Ki-67p, Novacastra, Leica Biosystems Newcastle Ltd., Newcastle, UK). Briefly, paraffin-embedded sections (4 μm) mounted on Vectabond-coated slides were de-waxed (65 °C, 20 min) and hydrated by a graded series of alcohol rinses. The antigen was unmasked by microwaving sections (20 min) in Tris–EDTA buffer (pH 10). Endogenous peroxidase activity was inactivated by incubation of slides in hydrogen peroxide (3% w/w) for 10 min and non-specific binding was blocked with protein block solution provided with the NovoLink detection kit (Novocatra). Sections were incubated with primary antibody (dilution 1:2000) overnight at 4 °C. After washing (PBS) the sections, staining was detected using the Novolink kit. All slides were scored by two

independent observers blinded to the treatment group. The proliferation index (PI) was quantitated as percentage of Ki-67 positive epithelial cells from 30 crypts and 10 villi of small bowel and 30 crypts of colon or adenomas per section, randomly selected, as described previously.[13]

2.4. Western blot analysis

Intestinal and colonic adenomas were excised, washed (ice-cold PBS), pooled for each mouse and weighed. An aliquot (50 mg) of the tissue was placed in ice-cold RIPA buffer (0.2 ml, 50 mM Tris/HCl pH7.4, 250 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% v/v Igepal) with 1 mM PMSF, 1 mM sodium orthovanadate and 2% (v/v) protease inhibitor cocktail (Sigma–Aldrich, Poole, UK). The resulting lysate was homogenised (hand homogeniser) and centrifuged for 15 min (13,000*g*, 4 °C). Proteins were separated by SDS–PAGE (12% polyacrylamide gel) and transferred onto a nitrocellulose membrane. Western blot analyses were performed using rabbit polyclonal antibodies against Akt, phospho-Erk and Erk. Actin antibody was employed to assess protein loading. Anti-mouse, anti-goat or anti-rabbit IgG peroxidase conjugates were used as secondary antibodies. A chemiluminescent signal was obtained with enhanced chemiluminescence (ECL) detection kit (Amersham Plc, Amersham UK). Detection of the Western signals by chemoluminescence used X-ray films, and band intensity was analysed by densitometry. Densitometry readings in the legend of Fig. 4 are expressed as the ratio of arbitrary band intensity units in each individual mouse relative to the mean band densities in untreated mice (in%), values in adenomas from untreated mice being 100%.

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

Biomatrices were thawed to room temperature, and the samples of plasma, urine and small intestinal mucosa homogenate of 1:1 PBS (300 μl) were centrifuged for 15 min at 13,000 *g*. An aliquot of the supernatants (200 μl) was subjected to solid-phase extraction and was analysed for anthocyanins by HPLC using either visible spectroscopic (VIS) or tandem mass spectrometric (MS/MS) detection as described before.[7] Selected reaction monitoring (SRM) using suitable transitions for parent anthocyanins and their predicted hydroxyl, glucuronide, methyl and sulphate metabolites was performed for the *m/z* values shown in Table 1. Anthocyanins exist in a pH-dependent dynamic equilibrium of at least four tautomers, only one of which is coloured, the flavylium cation (Fig. 1) which predominates at pH < 2.[14] Acidification of biofluid extracts ensured transformation of the colourless tautomers into flavylium ions detectable by HPLC-VIS at 510 nm. Anthocyanin concentrations were semi-quantitated using a standard curve constructed with authentic cyanidin-3-glucoside spiked in control mouse plasma, mucosal homogenate and urine, 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, therefore the steady-state total anthocyanin levels in mucosa and urine are approximate values. The ranges of recovery of the five major oenocyanin anthocyanins from murine plasma, urine and mucosa were 72–87, 86–97 and 54–57%, respectively.

3. Results

3.1. Effect of oenocyanin on adenoma development in Apc^{Min} mice

Apc^{Min} mouse adenomas are primarily located in the small intestine, with very few, mostly large, lesions observed in the colon. Apc^{Min} mice consumed oenocyanin with their diet (0.3%), and the intestinal tumour burden and number were recorded. The consumption of oenocyanin halved overall adenoma burden, with the effect on colonic tumour burden being more prominent than that on small intestinal adenoma burden (Fig. 2A). Oenocyanin reduced the number of adenomas weakly, but not significantly (Fig. 2B), and it failed to decrease tumour number in specific bowel segments (Fig. 2C). Oenocyanin consumption did not affect murine body weight (Fig. 2D).

3.2. Pharmacodynamic effects of oenocyanin

To study the effect of oenocyanin consumption on adenoma cell proliferation, rolled-up intestine from Apc^{Min} mice which had received control diet or diet with oenocyanin was subjected to immunohistochemical inspection following staining for Ki-67. This protein is a granular component of the nucleolus expressed exclusively in proliferating cells and is used as a prognostic marker in human neoplasias. The proliferation index in colonic adenomatous crypts of mice was significantly decreased from 88.1 ± 4% in mice on control diet to 75.6 ± 4% in mice which received oenocyanin (Fig. 3). Proliferation of cells in normal intestinal crypts and villi or small intestinal adenomas was not affected by oenocyanin. Levels of apoptosis in adenomas or normal intestinal tissue of Apc^{Min} mice as reflected by staining for cleaved caspase 3 were

Table 1 – Anthocyanins identified by LC/MS/MS in urine of mice which received oenocyainin with their diet (0.3%).

Anthocyanin species	Retention time (min)	MRM transition (*m/z*)
Cyanidin-3-glucoside	4.7	449 > 287
Petunidin-3-glucoside	6.4	479 > 317
Peonidin-3-glucoside	9.4	463 > 301
Malvidin-3-glucoside	11.6	493 > 331
Peonidin-3-glucoside glucuronide	4.9	639 > 301
Methyl cyanidin-3-glucoside glucuronide	3.1	639 > 301

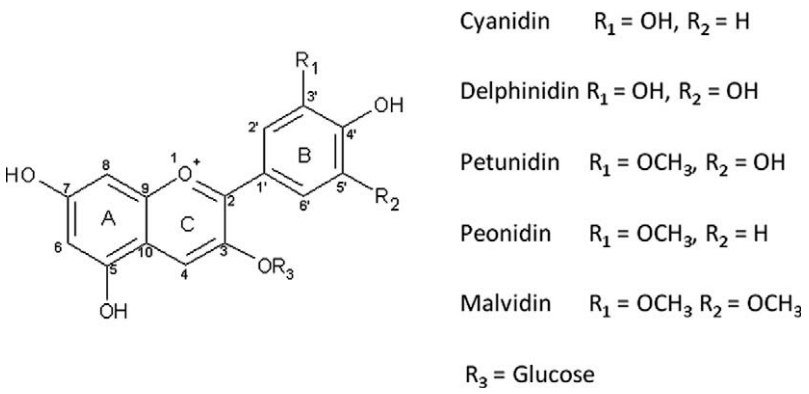

Fig. 1 – Structures of major anthocyanin constituents of oenocyanin.

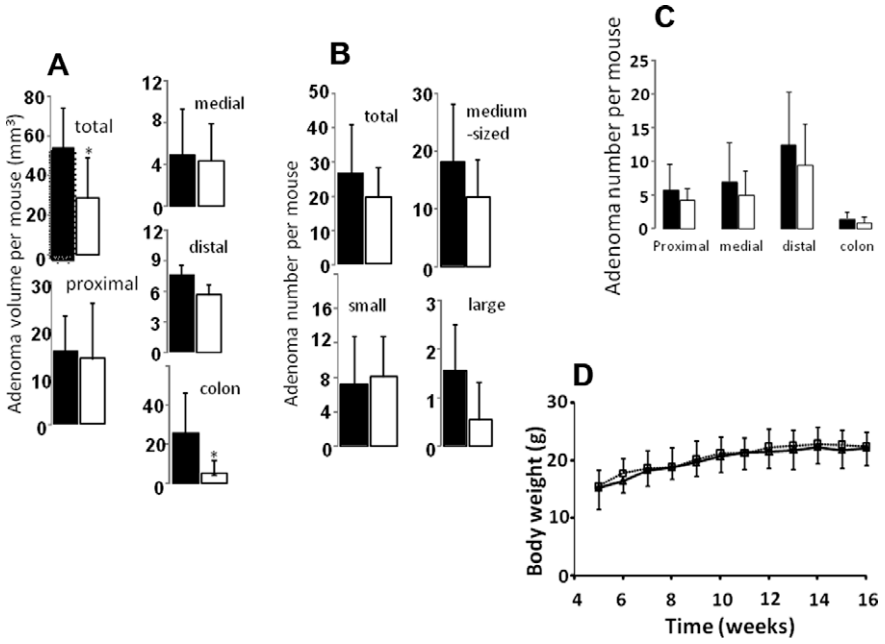

Fig. 2 – Effect of dietary administration of oenocyanin (0.3%) on adenoma burden in the total intestinal tract or intestinal sections (A), on total adenoma number or number of small (>1 mm diameter), medium (1–3 mm diameter) or large (>3 mm diameter) adenomas in the whole intestine (B) and in intestinal sections (C) and on body weight (D) of Apc^{Min} mice. Mice received control diet (black bars in A–C, solid line in D) or diet with oenocyanin (open bars in A–C, dotted line in D) from week 4 to 16. Results are the mean ± SD, number of mice were 13 and 14 in the control and oenocyanin groups, respectively. Asterisk indicates that value is significantly different from control ($P < 0.001$). For details of experimental design and assessment of adenoma burden and number see Section 2.

extremely low, confounding the detection of meaningful differences between mice on control diet and those which had ingested oenocyanin.

The hypothesis was tested that oenocyanin consumption affects the expression and/or activation of different ErbB family members, down-stream signalling elements of the PI3 K/Akt signalling pathway and the mitogen-activated protein kinase cascade in intestinal adenoma tissue of Apc^{Min} mice. Intestinal levels of expression of pAkt or ErbB receptors (EGFR, ErbB2, ErbB3) were too low to allow detection, even after protein immunoprecipitation. Consistent with these findings, ErbB proteins were also undetectable in APC10.1 cells (data not shown), an established cell line derived from Apc^{Min} mouse intestinal adenoma.[15] Fig. 4 shows that expression of Akt in small intestinal adenomas from Apc^{Min} mice on oenocyanin was significantly reduced by 50%, when compared to mice on control diet, whilst ERK was not affected. Intervention seemed to elevate the expression of pERK in Apc^{Min} adenomas, but on densitometric evaluation there was no significant difference compared to controls.

3.3. *Anthocyanin concentrations in biofluids and intestinal mucosa*

Plasma, urine and intestinal mucosa of Apc^{Min} mice which had received dietary oenocyanin were subjected to analysis

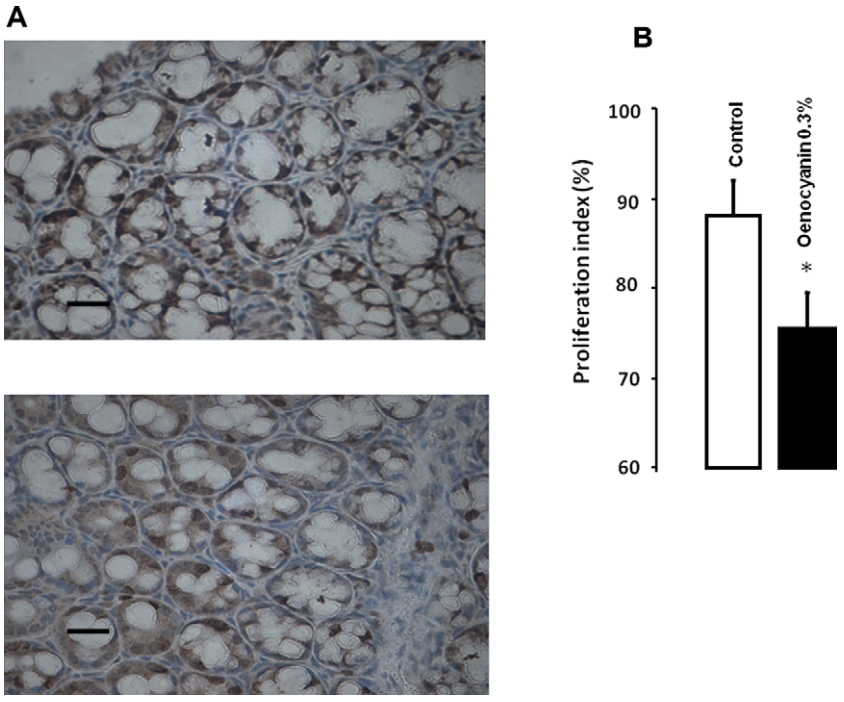

Fig. 3 – Representative photomicrographs of histological sections of colonic adenomatous crypts immuno-stained for Ki-67 (A) and proliferative index (B) in *Apc*^{Min} mice which had received either control diet (top graph in A, open bar in B) or diet containing oenocyanin (0.3%) from week 4 to 16 (bottom graph in A, closed bar in B). Bars in A = 25 μm. Note that the ordinate in B commences at 60%. Results in B are the mean ± SD of *n* = 4 mice; statistical comparison was by Students *t*-test, $^{*}P = 0.014$ with respect to controls.

of anthocyanins by HPLC with VIS light detection. Anthocyanins were recovered from the urine and intestine (Fig. 5), but could not be detected in the plasma (result not shown). Urinary anthocyanins were identified by LC/MS/MS exploiting suitable mass transitions as four of the five major oenocyanin constituents and two isomeric glucuronide metabolites of methyl cyanidin-3-glucoside, one probably being peonidin-3-glucoside (Table 1). The extracts of intestinal mucosa did not furnish peaks on LC/MS/MS analysis because of insufficient amount of biomatrix, but HPLC-VIS co-chromatography

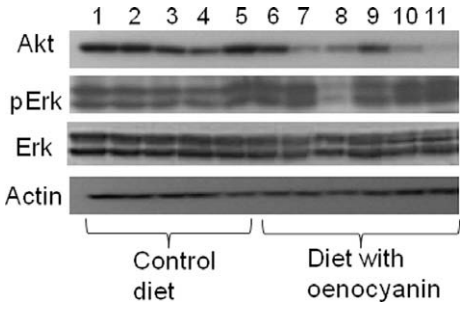

Fig. 4 – Western blot analysis of ERK, pERK and Akt in colonic adenomas of *Apc*^{Min} mice which received either control diet (*n* = 5, lanes 1–5) or diet containing oenocyanin (0.3%) (*n* = 6, lanes 6–11). Quantitation of protein band intensity by densitometry afforded the following percentage values, as compared to diet-only controls: 46 ± 22% for Akt (*P* = 0.003), 85 ± 13% for pERK and 98 ± 12% for ERK.

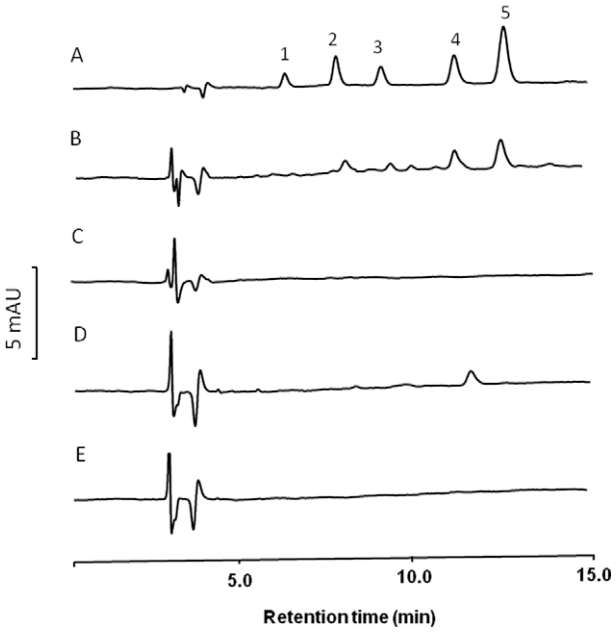

Fig. 5 – Representative HPLC-VIS chromatograms of a solution of authentic oenocyanin (A) and of extracts of urine (B and C) or intestinal mucosa (D and E) from *Apc*^{Min} mice which received control diet (C and E) or diet fortified with oenocyanin (0.3%; B and D). Peaks in A were identified by co-chromatography and LC/MS/MS as delphinidin-3-glucoside (1), cyanidin-3-glucoside (2), petunidin-3-glucoside (3), peonidin-3-glucoside (4) and malvidin-3-glucoside (5).

suggests that the major anthocyanin peak in mucosa was probably peonidin-3-glucoside. When anthocyanin peaks were semi-quantitated on the basis of a standard curve constructed with cyanidin-3-glucoside, steady-state levels in urine and intestinal mucosa were estimated as 3.2 ± 1.2 μg/ml (7.0 ± 2.8 μM, $n = 4$) and 6.2 ± 10.9 μg/g (13.8 ± 24.3 nmol/g, $n = 8$), respectively.

4. Discussion

Cancer chemopreventive agents need to be administered over protracted periods of time, and in principle, for a plant-derived preparation to constitute a viable cancer preventive intervention in humans, it needs to have a low cost. *De novo* extracts of fruits such as bilberry are expensive, as their production is labour intensive, a fact which may somewhat militate against their wide-spread use in cancer chemoprevention. As a rich source of anthocyanins, oenocyanin has a considerable advantage over berry extracts in that it is inexpensive, being a byproduct of wine and grape juice production. Oenocyanin has been commercially available in Italy for over 130 years and is used as a nutritional colorant or supplement.[16] We report here for the first time that consumption of oenocyanin interferes with adenoma development in the Apc^{Min} mouse. It affected Apc^{Min} tumour burden more prominently than tumour number, suggesting that it may compromise adenoma development mainly at a relatively late stage. The efficacy of oenocyanin reported here is comparable to that published previously for the bilberry extract mirtocyan.[7] Oenocyanin efficacy was accompanied by decreased adenoma cell proliferation and down-regulation of expression of the PI3 pathway component Akt, which supports cell proliferation. Therefore the decrease in adenoma development and adenoma proliferation caused by oenocyanin anthocyanins may be the consequence of decreased signalling through this pathway. Our results do not allow conclusions to be drawn as to how anthocyanins interfere with this pathway. Previously oenocyanin anthocyanins have been shown to act as broad spectrum receptor tyrosine kinase inhibitors, exemplified by their effects on members of the ErbB (EGFR, ErbB2, ErbB3) and VEGF (VEGFR 2 and 3) receptor families.[17] Oenocyanin substantially decreased the phosphorylation of these receptors in human-derived A431 cells *in vitro*, thus potentially compromising signalling *via* the MAPK and PI3 K pathways. It is conceivable that anthocyanins blocked ErbB receptor phosphorylation also in Apc^{Min} adenomas *in vivo*, but protein levels in these mice were below the limits of detection achievable with the murine ErbB receptor antibodies used here, confounding the study of oenocyanin effects. Consistent with the effects of oenocyanin on Akt protein described here, delphinidin, an anthocyanidin closely related to the oenocyanin constituents, potently reduced ErbB receptor phosphorylation in A431 and HT29 cells.[18,19] Suppression of receptor phosphorylation was accompanied by a decrease in phosphorylated Erk1/2[18] and an even more potent reduction of Akt expression (Teller et al., unpublished results). Delphinidin has also been reported to interfere with the activity of the MAPK cascade in murine epidermal cells.[20] All these results suggest the possibility that Akt constitutes a key molecule in the mediation of the antiproliferative signalling effects of oenocyanin anthocyanins. The sensitivity of Akt levels on exposure to anthocyanins renders it a potential biomarker of their antiproliferative properties.

The dose of oenocyanin employed here, 0.3% in the diet, which translates approximately into 360 mg/kg containing 79 mg/kg anthocyanins per day based on a 25 g mouse eating about 3 g diet, engendered measurable levels of anthocyanins in the urine and intestinal mucosa. Plasma levels were below the limits of quantitation (20 ng/ml by HPLC-VIS following SPE). Anthocyanin levels in the urine and intestinal mucosa of mice which received oenocyanin were similar to those measured in Apc^{Min} mice after dietary administration of mirtocyan at 0.3% containing 130 mg/kg anthocyanins.[7] The anthocyanin concentration (6.2 μg/g) measured in the intestinal mucosa of Apc^{Min} mice after exposure to oenocyanin is comfortably above the IC_{50} values for inhibition by oenocyanin anthocyanins of activities associated with ErB2 and EGFR kinases in a cell-free system *in vitro*, which were in the ranges of approximately 0.22–1.1 μg/ml and 0.02–0.2 μg/ml, respectively.[17] So the anthocyanin concentration measured in the intestinal tract of the Apc^{Min} mice *in vivo* can explain the inhibitory effect of oenocyanin on Akt in Min adenomas. In contrast, the IC_{50} concentrations for oenocyanin anthocyanins which characterised inhibition of phosphorylation of ErB2, ErB3 or EGFR in intact A431 cells *in vitro* were 22–44 μg/ml, and 108 μg/ml was required to marginally compromise cell growth, values which considerably exceed mucosal levels of oenocyanin anthocyanins seen *in vivo*. Comparisons of results obtained in cells *in vitro* with observations made in intestinal tissues *in vivo* are tenuous and need to be interpreted with utmost caution. In the *in vivo* paradigm, intestinal tissue is exposed to oenocyanin for several months rather than a few days, as is the case in cell culture experiments, and this difference may explain the greater sensitivity of tissues *in vivo* than that of cells *in vitro* to the effects of anthocyanins.

Overall the results presented here suggest that oenocyanin may be a viable and economical alternative to anthocyanin-rich berry extracts for chemopreventive intervention. Akt and pErk might be suitable biomarkers of anthocyanin target organ efficacy. Whether changes in levels of these molecules can be exploited in surrogate tissue remains to be studied.

Conflict of interest statement

None declared.

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