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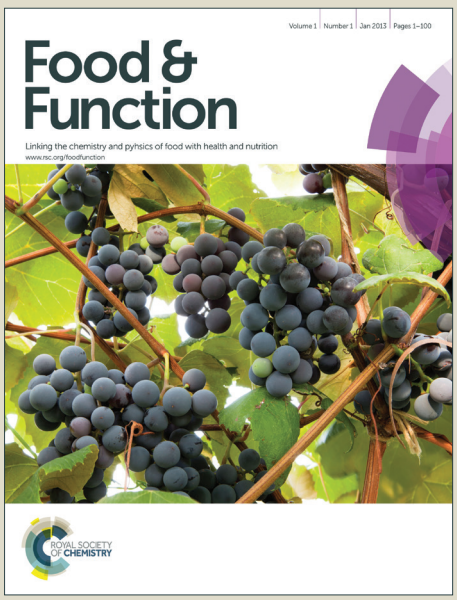

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Effects of anthocyans on the expression of organic anion transporting polypeptides (*SLCOs*/OATPs) in primary human hepatocytes

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Running Title:

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Abstract

Anthocyans (anthocyanins and their aglycones anthocyanidins) are colorful pigments, naturally occurring in fruits. They exhibit many biological effects and have potent health benefits. Anthocyans are widely used as dietary supplements and the safety of products containing them is of great importance. To investigate whether anthocyans influence the expression of hepatic uptake transporters from the organic anion transporting polypeptide (*SLCO* gene/OATP protein) family, we carried out studies on primary cultures of human hepatocytes. The hepato-cellular accumulation of widely used drugs such as statins and some anticancer drugs is mediated by the liver-specific OATP1B1 and OATP1B3, thus any interference with expression of these particular transporters might influence therapeutic outcomes. We evaluated the effects of 21 anthocyanins and their corresponding 6 anthocyanidins on the expression levels of *SLCO1B1/SLCO1B3* by RT-qPCR. Changes in OATP protein levels were confirmed by western blotting. Our data show that OATP1B1 responds differently to anthocyans compared with OATP1B3. We observed the induction of *SLCO1B1* gene and OATP1B1 protein in four hepatocyte samples by the anthocyanins malvin chloride, malvidin-3-*O*-galactoside chloride and cyanidin-3-*O*-sophoroside chloride. For *SLCO1B3*, a reduction in the expression levels was seen with delphin chloride and the anthocyanidin pelargonidin. Although the values varied considerably between primary hepatocyte isolates from different individuals, a mean induction of *SLCO1B1* (up to 60%) and reduction of *SLCO1B3* (by less than 25%) were detected. We propose that the effects of anthocyans derived from high dose dietary supplements may have to be taken into account in patients undergoing a therapy with drugs transported by OATP1B1 and OATP1B3.

Key Words:

Anthocyans, anthocyanins, anthocyanidins, phytochemicals, *SCLO*/OATP1B1, *SCLO*/OATP1B3, *SCLO* expression, OATP levels, human hepatocytes

Introduction

Anthocyans are a sub-group of flavonoids that exist in all tissues of higher plants as water-soluble pigments, responsible for the red, blue or purple colors of berries, grapes, apples, corn and many vegetables.[1] The broad term "anthocyan" encompasses both glycosides (termed anthocyanins) and aglycones (termed anthocyanidins). In higher plants, only anthocyanins are found, and in these, the pigment is linked to one or more sugars, often glucose, galactose, arabinose and xylose, but also to rather rare sugars including rhamnose, sophoroside or sambubioside.[2] The most common anthocyanidins are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin.[1] Many studies have demonstrated that anthocyans exhibit anti-proliferative, anti-apoptotic, anti-tumor, anti-mutagenic, anti-oxidant, anti-inflammatory and nitric oxide inhibitory effects *in vitro* that may be linked to their ability to confer important health benefits. Furthermore they are reported to reduce the incidence of diabetes, cardiovascular disease, arthritis and cancer.[1,3-6]

For the last years, public interest in the cancer chemopreventive properties of dietary constituents has increased as people have sought effective and safe diet-derived alternatives to pharmaceuticals.[7] In a study, the daily intake of anthocyanins in individuals in the U.S. has been estimated to be approximately 180-215 mg/day.[8]

Anthocyan-containing dietary supplements are available as juices, dried juice, dried fruits or water extracts, and are claimed to offer such an alternative.[9] Importantly, the dose of anthocyans in certain dietary supplements is extraordinarily high, e.g. in over-the counter drugs taken for the treatment against diarrhea (blueberry extracts, daily doses of 100-200 mg anthocyans) or against urinary tract infections (cranberry extracts, 60-225 mg per dose). However, there are no clinical studies investigating their effectiveness or potential side effects due to interactions with drug transport. For a critical review of the literature on anthocyans in dietary supplements see Espin et al., 2007.[10]

Anthocyans are effectively absorbed in the intestine and are transported into the liver, where they are metabolized and excreted into bile.[3,11,12] Therefore, it is of special interest to us to discover how these compounds may interact with the transport proteins that mediate the uptake of endogenous and exogenous compounds into hepatocytes. Two proteins, OATP1B1 and OATP1B3, members of the SLC superfamily of uptake transporters (SLC family 21) play an important role in the hepatic uptake of bilirubin, bile acids, conjugated steroids, eicosanoids and thyroid hormones, as well as xenobiotics, phytochemicals and drugs. There is considerable substrate overlap between these two OATPs, and if their substrates are co-administered, they may interfere with their transport capacity. OATP1B1 and OATP1B3 are expressed almost exclusively in hepatocytes and pharmacokinetic consequences are expected for the hepatobiliary elimination of drugs that are preferentially taken up

by either OATP1B1 and/or OATP1B3, e.g. chemotherapeutic drugs such as methotrexate, paclitaxel and statins like atorvastatin.[13-15]

This is especially important for drugs with a narrow therapeutic index, such as cytostatics and statins, for which high concentrations in blood plasma can result in severe toxic side effects and in case of statins in severe myopathy.[16,17] In addition to the consequences of altered substrate competition, altered levels of expression of these OATPs will also change the intracellular concentration and the efficacy of these therapeutics. In this study, we focused on the effects of the anthocyans on these two "liver-specific" OATPs with a unique substrate specificity, although other OATPs, e.g. OATP2B1 are also highly expressed in liver.[18]

It has been suggested that the expression of *SLCO1B1* and *SLCO1B3* genes and the corresponding proteins can be modified by drugs, xenobiotics and natural compounds.[19] Studies in rodents showed that activation of nuclear receptors, such as the pregnane X receptors (PXR) by natural compounds, for example the polyphenol hyperforin, and synthetic drugs such as the antibiotic rifampicin modulate the expression of these transporters.[20] However, a recent study using human hepatocytes revealed that rifampicin, although an effective stimulator of PXR, has little effect on *SLCO1B1* and *SLCO1B3* expression.[21]

To the best of our knowledge, there are no reports describing the effects of anthocyans on the expression of any OATPs in human liver cells, and so we investigated whether the levels of expression of OATP1B1 and OATP1B3 mRNA and protein are modified by common anthocyans (21 anthocyanins and their corresponding six anthocyanidins) in human hepatocytes isolated from four healthy donors.

Materials and methods

Chemicals

Dimethyl sulfoxide (DMSO) and rifampicin (RIF) were purchased from Sigma-Aldrich (Prague, Czech Republic). The following anthocyanins and anthocyanidins were purchased from Extrasynthese (Lyon, France): peonidin-3-*O*-glucoside chloride, peonidin-3-*O*-rutinoside chloride, pelargonidin-3,5-di-*O*-glucoside chloride, pelargonidin-3-*O*-rutinoside chloride, delphinidin-3-*O*-glucoside chloride, delphinidin-3-*O*-rutinoside chloride, delphinidin-3,5-di-*O*-glucoside chloride, delphinidin-3-*O*-sambubioside chloride, delphinidin-3-*O*-rhamnoside chloride, malvidin-3-*O*-glucoside chloride, malvidin-3,5-di-*O*-glucoside chloride, malvidin-3-*O*-galactoside chloride, cyanidin-3-*O*-glucoside chloride, cyanidin-3-*O*-rutinoside chloride, cyanidin-3,5-di-*O*-glucoside

chloride, cyanidin-3-*O*-sophoroside chloride, cyanidin-3-*O*-arabinoside chloride, cyanidin-3-*O*-rhamnoside chloride, cyanidin-3-*O*-galactoside chloride, cyanidin-3-*O*-sambubioside chloride, cyanidin-3-*O*-lathyroside chloride, cyanidin chloride, delphinidin chloride, malvidin chloride, peonidin chloride, petunidin chloride, and pelargonidin chloride. All other chemicals and solvents were commercially available, of analytical grade, and used without further purification.

Human hepatocytes

Human hepatocytes were isolated from human liver obtained from multiorgan donors LH45 (M, 46 years), LH46 (M, 37 years), LH47 (M, 47 years) and LH49 (M, 38 years). The tissue acquisition protocol was in accordance with the requirements stated by the local ethical commission in the Czech Republic. Long-term preserved human hepatocytes were obtained from Biopredic International (Rennes, France) as monolayer batch HEP220670 (F, 64 years).

Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator. All hepatocytes were treated in a serum-free medium for 24 h with the tested compounds and/or vehicle (DMSO; 0.1% v/v).[3,22]

No toxicity was found using these conditions in human hepatocytes even at the high concentration of 50 μM using trypan blue exclusion and the MTT-test for toxicity measurements.[3]

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent® and cDNA was synthesized according to the common protocol, using M-MLV Reverse Transcriptase F-572 (Finnzymes, Thermo Scientific, Portsmouth, NH, USA) and random hexamers 3801 (Takara, Saint-Germain-en-Laye, France).[23] TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) were purchased for *SLCO1B1* and *SLCO1B3* (containing intron-spanning primers: *SLCO1B1*: Hs00272374_m1, *SLCO1B3*: Hs00251986_m1). To select appropriate reference genes, the expression levels of 12 different human housekeeping mRNAs were analyzed using a geNorm reference gene selection kit with PerfectProbeTM (PrimerDesign Ltd., Southampton, UK). *GAPDH* (glyceraldehyde-3 phosphate dehydrogenase), *YWHAZ* (14-3-3 protein zeta/delta) and *TOP1* (DNA topoisomerase 1) were selected as acceptable reference genes for TaqMan® q-PCR analysis of the samples as previously described.[23] Data were analyzed by the delta–delta Ct method. Results are expressed as fold-induction over DMSO-treated cells. Data were visualized as a heat map using Java TreeView.[24]

Western blotting

Total protein extracts were prepared as described elsewhere.[25] After SDS-PAGE separation and Western blot transfer, filters were probed with rabbit polyclonal antisera against OATP1B1 (rabbit polyclonal; LS-C161285; LifeSpan BioSciences, Seattle, WA, USA) or OATP1B3 (rabbit polyclonal; LS-C159033; LifeSpan BioSciences) giving immunoreactive bands at approx.. 70 and 90 kDa, respectively. Chemiluminescent detection was performed using a horseradish peroxidase-conjugated secondary antibody and an ECL detection kit both from Thermo Scientific (Portsmouth, NH, USA). A VersaDoc 4000MP Imaging System (Bio-Rad Laboratories Inc., California, US) was used to capture images. As a loading control, the blots were probed to detect β-actin (band at 42kDa) using an anti-beta actin antibody from Abcam (Oxford, G.B). Densitometric analysis of immunoblots was done by VersaDoc 4000MP Imaging System.

Statistics

Data are given either as means ± SD or as means (min-max values) as indicated in the legends to Figures and Tables. Significance ($p < 0.05$) was calculated by applying the Mann-Whitney U test (GraphPad Prism 5 software, La Jolla, CA).

Results

To assess the effects of anthocyans on the expression of liver-specific *SLCO1B1*/OATP1B1 and *SLCO1B3*/OATP1B3 in a system that reflects the situation in patients, we used primary hepatocytes from different multi-organ donors for the investigations.[26,27] Previous studies showed that expression of these OATPs is greatly reduced in liver cancer cell lines and different mechanisms regulating their expression differ between hepatocytes and human liver cell lines.[28]

Effects of anthocyanidins on OATP expression

Initially, we elucidated the effect of the aglycones, namely the anthocyanidins cyanidin, peonidin, petunidin, pelargonidin, delphinidin, and malvidin on SLCO1B1 and SLCO1B3 expression levels in LH45, LH46, and LH47 cells. For comparison, we also used long-term preserved HEP220670 (HEP) cells derived from one donor. Cells were incubated for 24h with anthocyanidins (50 μM), and the

vehicle (DMSO, 0.1% v/v), before mRNA and protein were isolated and *SLCO/*OATP1B1 and *SLCO/*OATP1B3 levels were determined.

As shown in Table 2, treatment with pelargonidin reduced *SLCO1B3* gene levels by approximately 25%. In HEP cells, no significant alterations in the mRNA expression levels were seen with any anthocyanidin.

Effects of anthocyanins on OATP expression

In the next series of experiments, the effects of 21 anthocyanins (50 μM) were tested on four different human hepatocyte cultures (LH45, LH46, LH47, LH49). The induction pattern of *SLCO1B1* and *SLCO1B3* (see heat map in Fig. 2) was highly variable between hepatocytes obtained from different donors and the effect of anthocyanins on *SLCO1B3* gene expression was more variable between the LH-cultures than it was on *SLCO1B1*. The majority of anthocyanins increased the level of *SLCO1B1* in LH49 cells, whereas hardly any induction of *SLCO1B3* was observed in these cells.

By contrast, on using LH45 cells, *SLCO1B3* levels increased in response to many anthocyanins, and strong induction of *SLCO1B1* expression was observed after cyanidin-3-*O*-sophoroside chloride exposure, with more-moderate increases for other anthocyanins. In LH46-hepatocytes, malvin chloride caused a pronounced increase of *SLCO1B1* and *SLCO1B3* levels. In LH47 cells, increased levels of *SLCO1B1*, but not of *SLCO1B3* occurred in response to cyanidin-3-*O*-sambubioside chloride, malvidin-3-*O*-galactoside chloride and pelargonidin-3-*O*-rutinoside chloride.

The mean values and minimum and maximum values for *SLCO1B1* and *SLCO1B3* levels in the LH cells are summarized in Table 3. The data indicate that the effects of anthocyanins on *SLCO1B1* expression are rather moderate, revealing a maximal induction of 1.6-fold (mean values) of *SLCO1B1* with malvidin-3-*O*-galactoside chloride. A small, but significant increase in the mean levels of *SLCO1B1* was also produced by two other anthocyanins: malvin chloride (1.51-fold) and cyanidin-3-*O*-sophoroside chloride (1.38-fold).

Protein levels corresponded with the mRNA response pattern in hepatocyte samples. Higher levels of immunoreactive OATP1B1 after malvidin chloride and malvidin-3-*O*-galactoside chloride treatment reflected the higher mRNA levels. Incubation with pelargonin chloride and pelargonidin-3-*O*-rutinoside chloride, which had no effect on the *SLCO1B1* gene, also did not alter OATP1B1 protein levels (Fig. 3 and Table 4).

In contrast to *SLCO1B1/*OATP1B1, mean *SLCO1B3* expression in LH45, LH46, LH47 and LH49 cells was not significantly increased by anthocyanins (Table 3). Notably, *SLCO1B3* expression was

reduced by nearly 20% (mean value 0.817-fold) after delphin chloride incubation. Again, OATP1B3 protein levels corresponded with the mRNA levels (Fig. 3 and Table 4).

In control experiments in the hepatocytes, we also noticed that rifampicin does not induce the expression of either *SLCO1B3* or *SLCO1B3* (n-fold induction 0.74-0.99). This is not surprising, as in a previous study, rifampicin derivatives, known to be common inducers of some drug metabolising P450 enzymes (CYPs) and transporters [20,21,29], did also not show any specific upregulation of *SLCO1B1/SLCO1B3* genes. [21]

Discussion

Our investigation into the effects of 27 anthocyans on the expression of *SLCO/*OATP1B1 *and SLCO/*OATP1B3 in primary human hepatocytes revealed that these phytochemicals can indeed influence the expression of these liver-specific transporters. Interestingly, we observed induction of *SLCO*/OATP1B1 expression, but reduction of *SLCO*/OATP1B3 expression with some anthocyans.

Because the expression levels of liver-specific OATP1B1 and OATP1B3 is generally reduced in hepatocellular cancer cell lines such as HepG2 [27] we studied the effects of anthocyans on transporters expression in a system that reflects the situation in patients by using primary hepatocytes from different multi-organ donors.

In all hepatocyte cell samples tested, increases of *SLCO/*OATP1B1 levels of at least 10% occurred in response to the anthocyanins malvin chloride, malvidin-3-*O*-galactoside chloride, and cyanidin-3-*O*-sophoroside chloride. For *SLCO*/OATP1B3, a reduction in the expression levels was seen with one anthocyanidin, pelargonidin, and one anthocyanin, delphin chloride (Table 2 and 3). Only the glycosides but not the corresponding aglycones were able to affect increases in expression, indicating that the molecule together with the sugar moiety is necessary for the stimulation.

Recent papers show that after intake of food supplements, high concentrations of anthocyans are attained in the gastrointestinal tract, and most likely high concentrations of anthocyans occur in the portal vein, liver and, even more, in bile.[3,11] Studies in animals revealed that plasma levels of anthocyans reach approx. 4 μM after intake of bilberry extracts (360 mg per kg body weight). This amount would be equal to the intake of 250 g for a person with a weight of 70 kg. Since anthocyans are metabolized by the liver cells, local concentrations there may greatly exceed the plasma concentrations. Although it is unlikely that drug interactions caused by the interaction with the transporters may occur at doses used under normal conditions, the risk of interactions with OATP substrates in patients cannot be excluded if the liver function is impaired. For example during

cholestasis or hepatic inflammation, the polarity of hepatocytes is no longer maintained and the permeability between blood and bile is changed.[30] These processes lead to an accumulation of compounds in the liver with the risk of further interactions between dietary compounds and drugs on the transporter level. These might be even more important if patients take preparations with a better bioavailability as dietary supplements. Such conclusions were also drawn from data showing the effects of silymarin from milk thistle preparations on hepatic uptake transporters.[31]

The transporters OATP1B1 and OATP1B3 mediate the intracellular accumulation of widely used drugs such as statins and chemotherapeutic agents, so our findings are important because altered expression of OATP1B1 and OATP1B3 by these dietary constituents might change the efficacy of a drug therapy. However, it must be considered that the SLCO/OATP expression pattern varies considerably between hepatocytes from individual donors[27] and those variations may have a more severe impact on the OATP-mediated cellular accumulation of drugs in patients. It is also important that in the liver, OATP1B1 is expressed in all hepatocytes throughout the lobules, whereas OATP1B3 expression is highest in hepatocytes located around the central vein.[32] This implicates that their expression in liver is regulated differently, which could also account for the different reaction of these SLCOs/OATPs to anthocyans.

In general, our data concur with previous studies on the effect of anthocyans on biotransformation pathways that showed only small effects of anthocyans on selected enzymes. Kamenickova et al. recently reported the effects of cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin and their most common anthocyanins on the aryl hydrocarbon receptor (AhR)–cytochrome P450 CYP1A1 pathway.[3,22] They showed, using human hepatocytes and HepG2 cells, that pelargonidin is a weak ligand/agonist of the AhR and of AhR-dependent gene expression. Pelargonidin moderately induced *CYP1A1* mRNA expression in all of their primary hepatocyte cultures. Among the anthocyanins, pelargonidin-3-*O*-rutinoside chloride and cyanin chloride were weak inducers of *CYP1A1* gene expression.[3,22] Studies of the effect of anthocyans on expression of CYP2C9, CYP2A6, CYP2B6, and CYP3A4 in human hepatocytes and liver microsomes did not reveal significant alterations of mRNA or protein levels.[1]

In summary, our data demonstrate that some anthocyans are capable of altering the expression of *SLCO*/OATP1B1 and *SLCO*/OATP1B3 in cultured primary human hepatocytes. However, the effects varied considerably among individual cell isolates. Therefore, we conclude that, within the range of commonly ingested nutritional supplements, possible effects on OATP1B1 and OATP1B3 expression may not present major problems. However, in patients under therapy with OATP

substrates (e.g. taxols, methotrexate, statins) consequences of interactions between these OATP transporters and anthocyans taken at high doses in dietary supplements cannot be excluded.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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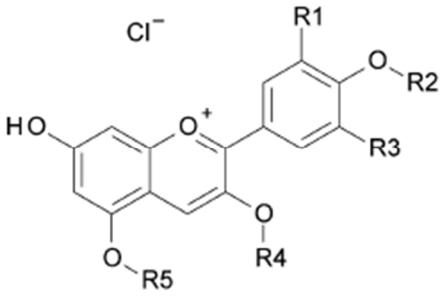
Tables

Table 1

Chemical structures of anthocyans



Anthocyanins	R1	R2	R3	R4	R5
Cyanidin-3-*O*-arabinoside chloride	OH	H	H	Arabinoside	H
Cyanidin-3-*O*-lathyroside chloride	OH	H	H	Lathyroside	H
Cyanidin-3-*O*-rhammnoside chloride	OH	H	H	Rhammnoside	H
Cyanidin-3-*O*-sambubioside chloride	OH	H	H	Sambubioside	H
Cyanidin-3-*O*-sophoroside chloride	OH	H	H	Sophoroside	H
Delphinidin-3-*O*-rhamnoside chloride	OH	H	OH	Rhammnoside	H
Delphinidin-3-*O*-rutinoside chloride	OH	H	OH	Rutinoside	H
Delphinidin-3-*O*-sambubioside chloride	OH	H	OH	Sambubioside	H
Malvidin-3-*O*-galactoside chloride	OCH_3	H	OCH_3	Galactoside	H
Pelargonidin-3-*O*-rutinoside chloride	H	H	OH	Rutinoside	H
Peonidin-3-*O*-glucoside chloride	OCH_3	H	H	Glucoside	H
Peonidin-3-*O*-rutinoside chloride	OCH_3	H	H	Rutinoside	H
Cyanin chloride (Cyanidin-3,5-di-*O*-glucoside chloride)	OH	H	H	Glucoside	Glucose
Delphin chloride (Delphinidin-3,5-di-*O*-glucoside chloride)	OH	H	OH	Glucoside	Glucose
Ideain chloride (Cyanidin-3-*O*-galactoside chloride)	OH	H	H	Galactoside	H
Keracyanin chloride (Cyanidin-3-*O*-rutinoside chloride)	OH	H	H	Rutinoside	H
Kuromanin chloride (Cyanidin 3-*O*-glucoside chloride)	OH	H	H	Glucoside	H
Malvin chloride (Malvidin-3,5-di-*O*-glucoside chloride)	OCH_3	H	OCH_3	Glucoside	Glucose
Myrtillin chloride (Delphinidin-3-*O*-glucoside chloride)	OH	H	OH	Glucoside	H
Oenin chloride (Malvidin-3-*O*-glucoside chloride)	OCH_3	H	OCH_3	Glucoside	H
Pelargonin chloride (Pelargonidin-3,5-di-*O*-glucoside chloride)	H	H	H	Glucoside	Glucose
Anthocyanidins					
Cyanidin chloride	OH	H	H	H	H
Delphinidin chloride	OH	H	OH	H	H
Malvidin chloride	OCH_3	H	OCH_3	H	H

Pelargonidin hloride	H	H	H	H	H
Peonidin chloride	OCH_3	H	H	H	H
Petunidin chloride	OCH_3	H	OH	H	H

Table 2

Effects of anthocyanidins on *SCLO1B1* and *SCLO1B3* levels in primary human hepatocytes treated for 24 h with tested compounds. Results are expressed as n-fold change compared to DMSO-treated cells (control). Data, normalized to the reference genes, are expressed as mean ± SD (n=3) for HEP220670 and for LH45, LH46, LH47 as means (min-max values), calculated from two separate determinations. Values in bold are significantly different ($p<0.05$) as assessed with the Mann-Whitney U test.

	Changes in mRNA expression			
	LH45, LH46 and LH47		**HEP220670**	
	SCLO1B1	*SCLO1B3*	*SCLO1B1*	*SCLO1B3*
Control	1.151 (0.77-1.62)	1.006 (1.00-1.01)	1.016±0.43	1.015±0.01
Cyanidin	1.027 (0.90-1.29)	0.912 (0.71-1.28)	0.782±0.15	0.929±0.38
Delphinidin	0.916 (0.54-1.39)	1.145 (0.76-1.55)	0.813±0.24	0.794±0.68
Malvidin	0.991 (0.66-1.36)	1.029 (0.72-1.63)	0.942±0.22	0.956±0.32
Pelargonidin	0.962 (0.66-1.26)	**0.756 (0.62-0.89)**	1.025±0.35	0.797±0.52
Peonidin	0.865 (0.70-0.98)	1.037 (0.70-1.45)	1.130±0.43	1.290±0.40
Petunidin	0.947 (0.67-1.10)	1.189 (0.70-1.97)	1.114±0.43	1.148±0.19

Table 3

Effects of anthocyanins on the levels of *SLCO1B1* and *SLCO1B3* in primary human hepatocytes LH45, LH46, LH47 and LH49 treated for 24 h with the indicated compounds. Results are expressed as n-fold changes compared to vehicle-controls. Data are mean ± SD from triplicate measurements.

Values in bold are significantly different ($p<0.05$), determined by Mann-Whitney U test.

	Changes in mRNA expression			
Anthocyanins	*SLCO1B1*		*SLCO1B3*	
Control	1.006	(1.00-1.02)	1.005	(1.00-1.01)
Cyanidin-3-*O*-arabinoside chloride	1.219	(0.66-1.92)	0.882	(0.66-1.02)
Cyanidin-3-*O*-lathyroside chloride	1.041	(0.97-1.12)	0.890	(0.55-1.40)
Cyanidin-3-*O*-rhammnoside chloride	1.078	(0.77-1.28)	0.860	(0.73-1.09)
Cyanidin-3-*O*-sambubioside chloride	1.193	(0.91-1.56)	1.026	(0.97-1.08)
Cyanidin-3-*O*-sophoroside chloride	**1.505**	**(1.14-1.72)**	1.027	(0.66-1.45)
Delphinidin-3-*O*-rhamnoside chloride	1.007	(0.83-1.18)	1.239	(0.70-1.78)
Delphinidin-3-*O*-rutinoside chloride	1.039	(0.91-1.15)	0.933	(0.69-1.24)
Delphinidin-3-*O*-sambubioside chloride	1.005	(0.81-1.38)	1.038	(0.59-1.49)
Malvidin-3-*O*-galactoside chloride	**1.606**	**(1.19-2.01)**	0.781	(0.47-1.17)
Pelargonidin-3-*O*-rutinoside chloride	1.104	(0.83-1.62)	1.107	(0.70-1.87)
Peonidin-3-*O*-glucoside chloride	1.097	(0.91-1.20)	1.051	(0.69-1.32)
Peonidin-3-*O*-rutinoside chloride	1.343	(1.04-1.82)	1.006	(0.64-1.43)
Cyanin chloride	0.993	(0.84-1.15)	0.897	(0.65-1.03)
Delphin chloride	1.066	(0.92-1.20)	**0.817**	**(0.69-0.90)**
Ideain chloride	1.065	(0.75-1.44)	0.834	(0.63-1.55)
Keracyanin chloride	1.175	(0.83-1.78)	0.864	(0.68-1.14)
Kuromanin chloride	0.981	(0.85-1.21)	0.932	(0.75-1.22)
Malvin chloride	**1.380**	**(1.13-1.86)**	1.564	(0.87-2.32)
Myrtillin chloride	1.163	(1.03-1.30)	0.926	(0.73-1.20)
Oein chloride	1.242	(0.98-1.88)	1.165	(0.73-1.97)
Pelargonin chloride	1.149	(0.91-1.30)	0.988	(0.55-1.48)

Table 4

Effects of selected anthocyanins (50 μM) on immunoreactive OATP1B1 and OATP1B3. Data from the densitometric analysis of immunoblots (given as % of control ; * $p<0.05$).

Changes in OATP1B1 and OATP1B3 levels	
Anthocyanins	Means ± S.D.
OATP1B1 (% of control)	
Pelargonidin chloride	98.23 ± 12.94
Pelargonidin-3-*O*-rutinoside chloride	97.75 ± 18.20
Malvin chloride	119.24 ± 12.71*
Malvin-3-*O*-galactoside chloride	128.70 ± 17.97 *
Cyanidin-3-*O*-sophoroside chloride	121.10 ± 17.50 *
OATP1B3 (% of control)	
Delphin chloride	89.25 ± 8.50 *

References

1. A. Srovnalova, M. Svecarova, M. Kopecna Zapletalova, P. Anzenbacher, P. Bachleda, E. Anzenbacherova and Z. Dvorak, Effects of Anthocyanidins and Anthocyanins on the Expression and Catalytic Activities of CYP2A6, CYP2B6, CYP2C9, and CYP3A4 in Primary Human Hepatocytes and Human Liver Microsomes, *J Agric Food Chem*, 2014.
2. C. R. Welch, Q. Wu and J. E. Simon, Recent Advances in Anthocyanin Analysis and Characterization, *Curr. Anal. Chem.*, 2008, **4**, 75-101.
3. A. Kamenickova, E. Anzenbacherova, P. Pavek, A. A. Soshilov, M. S. Denison, M. Zapletalova, P. Anzenbacher and Z. Dvorak, Effects of anthocyanins on the AhR-CYP1A1 signaling pathway in human hepatocytes and human cancer cell lines, *Toxicol. Lett.*, 2013, **221**, 1-8.
4. R. L. Prior and X. Wu, Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities, *Free Radic. Res.*, 2006, **40**, 1014-1028.
5. Y. Zhang, S. K. Vareed and M. G. Nair, Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables, *Life Sci.*, 2005, **76**, 1465-1472.
6. L. Kaume, L. R. Howard and L. Devareddy, The blackberry fruit: a review on its composition and chemistry, metabolism and bioavailability, and health benefits, *J. Agric. Food Chem.*, 2012, **60**, 5716-5727.
7. S. Thomasset, N. Teller, H. Cai, D. Marko, D. P. Berry, W. P. Steward and A. J. Gescher, Do anthocyanins and anthocyanidins, cancer chemopreventive pigments in the diet, merit development as potential drugs?, *Cancer Chemother. Pharmacol.*, 2009, **64**, 201-211.
8. S. Zafra-Stone, T. Yasmin, M. Bagchi, A. Chatterjee, J. A. Vinson and D. Bagchi, Berry anthocyanins as novel antioxidants in human health and disease prevention, *Mol. Nutr. Food Res.*, 2007, **51**, 675-683.
9. L. S. Wang and G. D. Stoner, Anthocyanins and their role in cancer prevention, *Cancer Lett*, 2008, **269**, 281-290.
10. J. C. Espin, M. T. Garcia-Conesa and F. A. Tomas-Barberan, Nutraceuticals: facts and fiction, *Phytochemistry*, 2007, **68**, 2986-3008.
11. S. Talavera, C. Felgines, O. Texier, C. Besson, C. Manach, J. L. Lamaison and C. Remesy, Anthocyanins are efficiently absorbed from the small intestine in rats, *J. Nutr.*, 2004, **134**, 2275-2279.
12. T. K. McGhie and M. C. Walton, The bioavailability and absorption of anthocyanins: towards a better understanding, *Mol. Nutr. Food Res.*, 2007, **51**, 702-713.
13. D. Keppler, The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia, *Drug Metab. Dispos.*, 2014, **42**, 561-565.
14. E. van de Steeg, A. van Esch, E. Wagenaar, K. E. Kenworthy and A. H. Schinkel, Influence of human OATP1B1, OATP1B3, and OATP1A2 on the pharmacokinetics of methotrexate and paclitaxel in humanized transgenic mice, *Clin Cancer Res*, 2013, **19**, 821-832.
15. M. Karlgren, A. Vildhede, U. Norinder, J. R. Wisniewski, E. Kimoto, Y. Lai, U. Haglund and P. Artursson, Classification of inhibitors of hepatic organic anion transporting polypeptides (OATPs): influence of protein expression on drug-drug interactions, *J Med Chem*, 2012, **55**, 4740-4763.

16. B. Hagenbuch and C. Gui, Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family, *Xenobiotica*, 2008, **38**, 778-801.
17. C. Gui, A. Obaidat, R. Chaguturu and B. Hagenbuch, Development of a cell-based high-throughput assay to screen for inhibitors of organic anion transporting polypeptides 1B1 and 1B3, *Curr. Chem. Genomics.*, 2010, **4**, 1-8.
18. V. Kumar, B. Prasad, G. Patilea, A. Gupta, L. Salphati, R. Evers, C. E. Hop and J. D. Unadkat, Quantitative Transporter Proteomics by LC-MS/MS: Addressing Methodological Issues of Plasma Membrane Isolation and Expression-activity Relationship, *Drug Metab. Dispos.*, 2014.
19. M. Svoboda, J. Riha, K. Wlcek, W. Jaeger and T. Thalhammer, Organic anion transporting polypeptides (OATPs): regulation of expression and function, *Curr. Drug Metab.*, 2011, **12**, 139-153.
20. L. M. Aleksunes and C. D. Klaassen, Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARalpha-, and Nrf2-null mice, *Drug Metab. Dispos.*, 2012, **40**, 1366-1379.
21. B. Williamson, K. E. Dooley, Y. Zhang, D. J. Back and A. Owen, Induction of influx and efflux transporters and cytochrome P450 3A4 in primary human hepatocytes by rifampin, rifabutin, and rifapentine, *Antimicrob. Agents Chemother.*, 2013, **57**, 6366-6369.
22. A. Kamenickova, E. Anzenbacherova, P. Pavek, A. A. Soshilov, M. S. Denison, P. Anzenbacher and Z. Dvorak, Pelargonidin activates the AhR and induces CYP1A1 in primary human hepatocytes and human cancer cell lines HepG2 and LS174T, *Toxicol. Lett.*, 2013, **218**, 253-259.
23. K. Wlcek, M. Svoboda, T. Thalhammer, F. Sellner, G. Krupitza and W. Jaeger, Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma, *Cancer Biol Ther*, 2008, **7**, 1450-1455.
24. A. J. Saldanha, Java Treeview--extensible visualization of microarray data, *Bioinformatics*, 2004, **20**, 3246-3248.
25. P. Pavek, L. Cerveny, L. Svecova, M. Brysch, A. Libra, R. Vrzal, P. Nachtigal, F. Staud, J. Ulrichova, Z. Fendrich and Z. Dvorak, Examination of Glucocorticoid receptor alpha-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines, *Placenta*, 2007, **28**, 1004-1011.
26. S. Imai, R. Kikuchi, Y. Tsuruya, S. Naoi, S. Nishida, H. Kusuhara and Y. Sugiyama, Epigenetic regulation of organic anion transporting polypeptide 1B3 in cancer cell lines, *Pharm Res*, 2013, **30**, 2880-2890.
27. K. Wlcek, M. Svoboda, J. Riha, S. Zakaria, U. Olszewski, Z. Dvorak, F. Sellner, I. Ellinger, W. Jager and T. Thalhammer, The analysis of organic anion transporting polypeptide (OATP) mRNA and protein patterns in primary and metastatic liver cancer, *Cancer Biol. Ther.*, 2011, **11**, 801-811.
28. M. Le Vee, E. Jouan, B. Stieger and O. Fardel, Differential regulation of drug transporter expression by all-trans retinoic acid in hepatoma HepaRG cells and human hepatocytes, *Eur. J. Pharm. Sci.*, 2013, **48**, 767-774.
29. W. J. Burman, K. Gallicano and C. Peloquin, Comparative pharmacokinetics and pharmacodynamics of the rifamycin antibacterials, *Clin. Pharmacokinet.*, 2001, **40**, 327-341.
30. L. Wang and J. L. Boyer, The maintenance and generation of membrane polarity in hepatocytes, *Hepatology*, 2004, **39**, 892-899.
31. K. Kock, Y. Xie, R. L. Hawke, N. H. Oberlies and K. L. Brouwer, Interaction of silymarin flavonolignans with organic anion-transporting polypeptides, *Drug Metab. Dispos.*, 2013, **41**, 958-965.

32. T. Abe, M. Unno, T. Onogawa, T. Tokui, T. N. Kondo, R. Nakagomi, H. Adachi, K. Fujiwara, M. Okabe, T. Suzuki, K. Nunoki, E. Sato, M. Kakyo, T. Nishio, J. Sugita, N. Asano, M. Tanemoto, M. Seki, F. Date, K. Ono, Y. Kondo, K. Shiiba, M. Suzuki, H. Ohtani, T. Shimosegawa, K. Iinuma, H. Nagura, S. Ito and S. Matsuno, LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers, *Gastroenterology*, 2001, **120**, 1689-1699.

Legends to Figures

Figure 1:

Chemical structures of the anthocyanins malvin chloride, malvidin-3-O-galactoside chloride and cyanidin-3-O-sophoroside chloride.

Figure 2:

Heat map showing mRNA expression levels for *SLCO1B1* and *SLCO1B3* in LH45, LH46, LH47 and LH49 human hepatocyte cultures.

The image map (Java TreeView) shows the pattern of *SLCO1B1* and *SLCO1B3* gene expression as determined by TaqMan RT-PCR. Data shows upregulation (red) or downregulation (blue) of the *SLCO* gene after incubation with anthocyanins, compared to the median expression level (black) of DMSO-treated hepatocyte cultures.

Figure 3:

Effects of selected anthocyanins on the expression of OATP1B1 and OATP1B3 proteins in human hepatocytes.

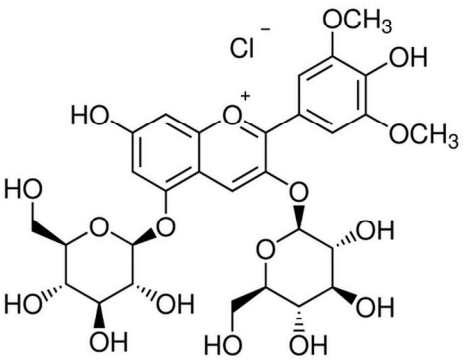
Primary human hepatocyte cultures (LH45, LH46, LH47, LH49) were incubated for 24 h with 50 μM of pelargonin chloride, pelargonidin-3-O-rutinoside chloride, malvidin chloride malvidin-3-O-galactoside chloride (blot A and B), delphin chloride (blot C), and DMSO (0.1% v/v) as a vehicle control.

A: Western blots show the analysis of OATP1B1 and OATP1B3 proteins (giving single bands at approx. 70 kDa and 90 kDa, respectively) in LH45, LH46, LH47, LH49.

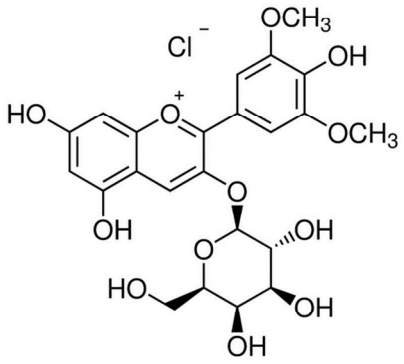
B: ß-actin in LH45, LH46, LH47, LH49 at 42 kDa was used as a loading control.

C: Immunoreactive OATP1B3 in delphin chloride-treated LH samples

Malvin chloride



Malvidin-3-*O*-galactoside chloride



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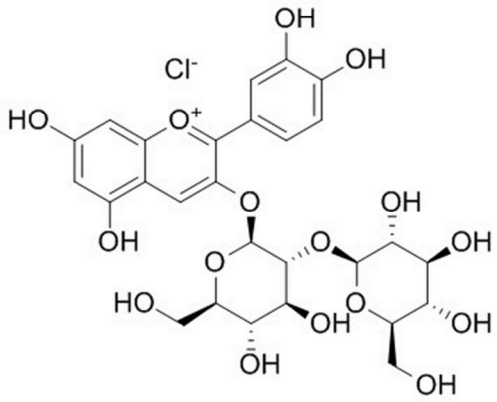

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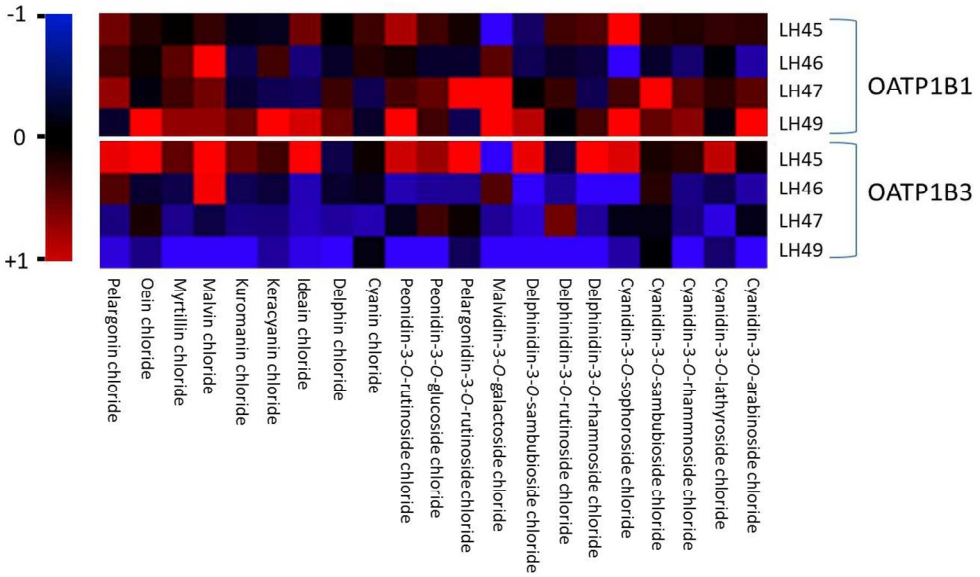

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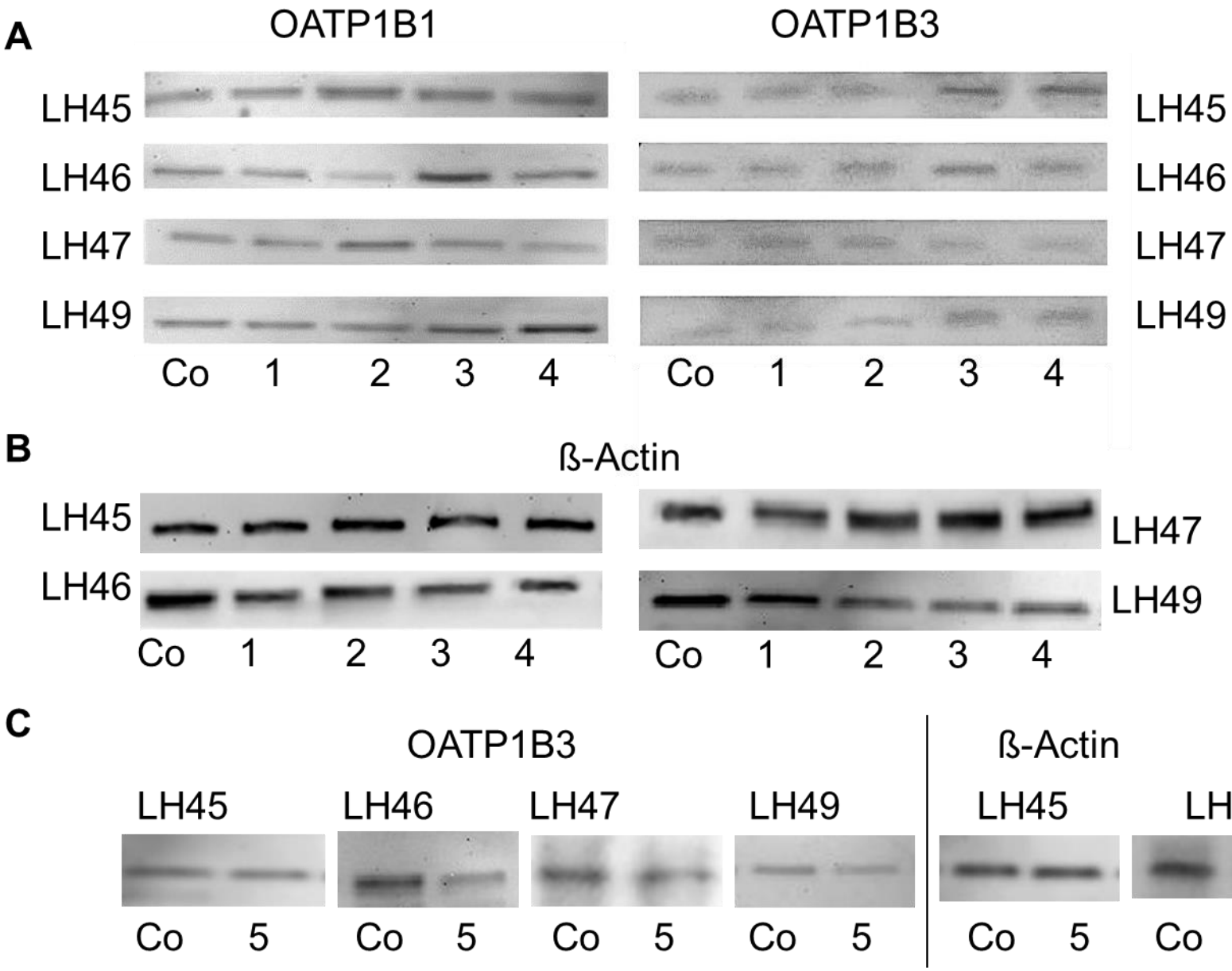

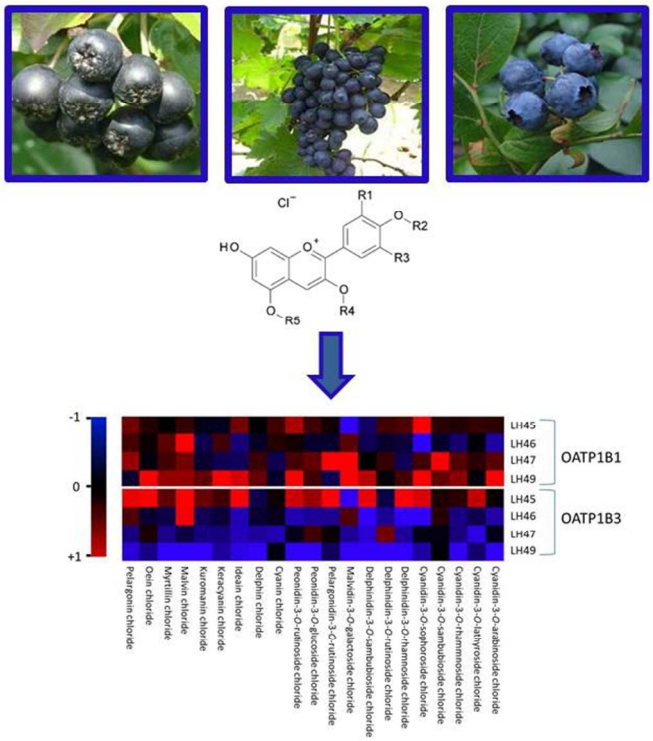

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