

RESEARCH PAPER

Berry anthocyanins and anthocyanidins exhibit distinct affinities for the efflux transporters BCRP and MDR1

A Dreiseitel¹, B Oosterhuis², KV Vukman^{2,3}, P Schreier⁴, A Oehme⁴, S Locher⁴, G Hajak¹ and PG Sand¹¹Department of Psychiatry, University of Regensburg, Regensburg, Germany, ²SOLVO Biotechnology, Central Hungarian Innovations Center, Budaörs, Hungary, ³Dublin City University, Dublin, Ireland, and ⁴Chair of Food Chemistry, University of Wuerzburg, Wuerzburg, Germany**Background and purpose:** Dietary anthocyanins hold great promise in the prevention of chronic disease but factors affecting their bioavailability remain poorly defined. Specifically, the role played by transport mechanisms at the intestinal and blood–brain barriers (BBB) is currently unknown.**Experimental approach:** In the present study, 16 anthocyanins and anthocyanidins were exposed to the human efflux transporters multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP), using dye efflux, ATPase and, for BCRP, vesicular transport assays.**Key results:** All test compounds interacted with the BCRP transporter *in vitro*. Of these, seven emerged as potential BCRP substrates (malvidin, petunidin, malvidin-3-galactoside, malvidin-3,5-diglucoside, cyanidin-3-galactoside, peonidin-3-glucoside, cyanidin-3-glucoside) and 12 as potential inhibitors of BCRP (cyanidin, peonidin, cyanidin-3,5-diglucoside, malvidin, pelargonidin, delphinidin, petunidin, delphinidin-3-glucoside, cyanidin-3-rutinoside, malvidin-3-glucoside, pelargonidin-3,5-diglucoside, malvidin-3-galactoside). Malvidin, malvidin-3-galactoside and petunidin exhibited bimodal activities serving as BCRP substrates at low concentrations and, at higher concentrations, as BCRP inhibitors. Effects on MDR1, in contrast, were weak. Only aglycones exerted mild inhibitory activity.**Conclusions and implications:** Although the anthocyanidins under study may alter pharmacokinetics of drugs that are BCRP substrates, they are less likely to interfere with activities of MDR1 substrates. The present data suggest that several anthocyanins and anthocyanidins may be actively transported out of intestinal tissues and endothelia, limiting their bioavailability in plasma and brain.*British Journal of Pharmacology* (2009) **158**, 1942–1950; doi:10.1111/j.1476-5381.2009.00495.x; published online 17 November 2009**Keywords:** anthocyanin; anthocyanidin; flavonoid; ABC transporter; MDR1; P-glycoprotein; BCRP**Abbreviations:** ABC, ATP-binding cassette; BBB, blood–brain barrier; BCRP, breast cancer resistance protein; calcein-AM, calcein O,O'-diacetate tetrakis (acetoxymethyl) ester; diglc, diglucosides; ECG, epicatechin gallate; ECGC, epigallocatechin gallate; gal, galactoside; glc, glucoside; HBSS, Hank's Balanced Salt Solution; MDR, multidrug resistance; MDR1, multidrug resistance protein 1; rut, rutinoside; Sf9, *Spodoptera frugiperda*

Introduction

Anthocyanins are flavonoids widely distributed in fruits and vegetables that exhibit antioxidative (Garcia-Alonso *et al.*, 2005; Tarozzi *et al.*, 2007; Salminen and Heinonen, 2008) and anti-inflammatory (Wang and Mazza, 2002; Zafra-Stone *et al.*,

2007; Kim *et al.*, 2008) bioactivities, among others (Shukitt-Hale *et al.*, 2008). A growing body of evidence from animal, epidemiological and clinical investigations suggests that anthocyanins confer neuroprotection (Joseph *et al.*, 1999; Youdim *et al.*, 2004; Matsunaga *et al.*, 2009), but it is not clear how these compounds distribute to tissues and cross the blood–brain barrier (BBB). Daily dietary intake of anthocyanins has been estimated at ~100 mg in Western countries (Thomasset *et al.*, 2009) and can rise several fold depending on seasonal and lifestyle factors (Wu *et al.*, 2006; Chun *et al.*, 2007).

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Based on the current understanding, anthocyanins are either directly absorbed from the gastrointestinal tract (McGhie and Walton, 2007), or are first metabolized by the gut microflora, leading to anthocyanidins and phenolic acid metabolites (Tsuda *et al.*, 1999; Felgines *et al.*, 2002). Native anthocyanins, plus one methylated form, have been identified in rat brain following dietary supplementation with a blackberry extract. Of these, the brain concentration of cyanidin-3-glucoside even exceeded its plasma concentrations (Talavera *et al.*, 2005), suggesting good central nervous system (CNS) bioavailability of selected compounds. A recent study has confirmed the presence of anthocyanins in brains from pigs fed a regular diet, further strengthening this view (Kalt *et al.*, 2008). Uptake in rat brain occurs only 30 min after oral administration (Talavera *et al.*, 2005) and involves regions important for learning and memory (Andres-Lacueva *et al.*, 2005). In pig cerebellum, anthocyanin concentration does not rise uniformly, which implies that anthocyanin absorption may become saturated at high dosage levels (Kalt *et al.*, 2008).

Reports on neuroprotective effects of berry constituents have renewed interest in the mechanisms of BBB penetration and entry into the CNS, by means of a bilitranslocase-like carrier (Talavera *et al.*, 2005) or by cellular transporters for glycosylated anthocyanins (Passamonti *et al.*, 2005). Specifically, the permeability of the BBB may be affected by efflux transporters expressed at the endothelial surface (Youdim *et al.*, 2004).

Efflux transporters of the ATP-binding cassette (ABC) gene family are major determinants of drug distribution to, and elimination from the CNS, of intestinal absorption, and of hepatobiliary excretion (Kusuhara and Sugiyama, 2002; Sun *et al.*, 2003). Localized in the apical membranes of BBB endothelial cells, enterocytes and hepatocytes (Eisenblätter *et al.*, 2003; Dupuy *et al.*, 2006), they mediate the active extrusion of nutrients, toxins, drugs and many metabolites back into the capillary lumen and intestine (del Moral *et al.*, 1998; Begley, 2004; Xu *et al.*, 2005; Breedveld *et al.*, 2006; Fricker, 2008). Moreover, transporters have been implicated in drug interactions that increase the likelihood of adverse effects (Fromm, 2003; Shitara *et al.*, 2005). Among the ABC efflux transporters are the multidrug resistance protein 1 (MDR1, P-glycoprotein) and the breast cancer resistance protein (BCRP). Both are prominently expressed in organs important for absorption (small intestine), distribution (placenta and BBB) and elimination (liver, kidney, small intestine) of drugs (Gottesman *et al.*, 1996; Mao and Unadkat, 2005), and are also found in several tumours (Schinkel and Jonker, 2003; Robey *et al.*, 2007).

We sought to determine whether anthocyanins are substrates for ABC transporters, and whether anthocyanins may interfere with the transport of other substrates. To this end, 16 anthocyanidins and anthocyanins were tested for *in vitro* effects on the human efflux transporters BCRP and MDR1. Compounds were selected so as to include structurally diverse and yet common representatives abundant in dietary sources, such as malvidin-glucoside (glc) from red grapes and cyanidin-glc from elderberries and blackberries (Bub *et al.*, 2001; Wu *et al.*, 2004; Felgines *et al.*, 2005). We also chose substances found in grapes, strawberries and bilberries, as

these have shown positive effects in rodent studies of neuroprotection (Joseph *et al.*, 1999; Ramirez *et al.*, 2005; Shukitt-Hale *et al.*, 2006; Duffy *et al.*, 2008; Rahman *et al.*, 2008; Williams *et al.*, 2008; Matsunaga *et al.*, 2009). Interactions were studied in dye extrusion and ATPase assays, and in vesicular transport assays for BCRP. In combination, these assays give insight into different types of transporter interaction and specify substances' affinities for the ABC transporters under study. Observed inhibitory and substrate-type effects on the BCRP transporter imply a potential of anthocyanidins to alter pharmacokinetics of drugs, and may explain limitations in their plasma and brain bioavailability.

Methods

Assays

Dye extrusion assay. For assessing effects on BCRP, Hoechst fluorescent dye 33342 was added to BCRP transporter-overexpressing MCF7-MX cells (Solvo Biotechnology, Budaörs, Hungary). Modulators of the BCRP transporter activity reduce the rate of Hoechst 33342 extrusion and cause its accumulation inside the cells. Following DNA intercalation, a fluorescent signal may be detected that is proportional to BCRP transporter inhibition. Briefly, cells (1×10^5 per well of standard 96-well tissue culture plates) were incubated with Hoechst 33342 (50 μM) plus the test compound or, alternatively, with DMSO for non-inhibited controls, or Ko143 (1 μM) as a reference inhibitor, at 37°C for 15 min in Hank's balanced salt solution (HBSS). Fluorescence of accumulated Hoechst 33342 inside the cells was measured in real time at excitation and emission wavelengths of 355 and 460 nm, respectively, using a BMG Labtech FluoStar Optima fluorescence photometer (BMG Labtech, Offenburg, Germany).

For quantification of effects on MDR1, calcein-AM was added to MDR1-overexpressing K562-MDR cells (Solvo Biotechnology, Budaörs, Hungary). Modulators of MDR1 activity cause accumulation of calcein-AM inside the cell, whereupon it is cleaved by non-specific esterases to form the fluorescent low permeability dye, calcein. The increase of fluorescent signal inside the cell is proportional to MDR1 inhibition. Briefly, K562-MDR cells (8×10^4 cells per well) and calcein-AM (0.25 μM) were incubated with the test compound or, alternatively, with DMSO for non-inhibited controls, or verapamil (60 μM) as a reference inhibitor, at 37°C for 8 min in HBSS. Fluorescence of accumulated calcein inside the cells was measured at excitation and emission wavelengths of 485 and 538 nm respectively. After the incubation period, propidium iodide was added to all wells (0.01 $\text{mg} \cdot \text{mL}^{-1}$) and fluorescence was measured at 530 and 630 nm excitation and emission wavelengths, respectively, to assess potential cytotoxic effects of the substances tested.

ATPase assay. ATPase activity of wild-type human BCRP and MDR1 was measured according to a protocol modified from Sarkadi *et al.* (1992), by colorimetric detection of inorganic phosphate, the byproduct of ABC transporter function. Membrane preparations from human BCRP transporter- and human MDR1 transporter-expressing *Spodoptera frugiperda*

(Sf9) ovarian cells were supplied by SOLVO Biotechnology (Budaörs, Hungary).

For assessing BCRP and MDR1 transporter-related ATPase activity, incubations were carried out in the presence and absence of sodium orthovanadate (Na_3VO_4) (1.2 mM), a known ABC transporter inhibitor, to distinguish between background ATPase activity and transporter-related ATPase activity. The ATPase assay was performed according to the manufacturer's protocol. Briefly, membranes (20 μg per well) were preincubated at 37°C for 10 min with the test substance or the solvent DMSO in the absence and presence of vanadate, and reactions were started by adding MgATP. Sulphasalazine (10 μM) and verapamil (40 μM) served for activation of BCRP and MDR1 membranes respectively. Final concentrations of assay compounds amounted to 10 mM MgCl_2 , 40 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS)-Tris (pH 7.0), 50 mM KCl, 5 mM dithiothreitol, 0.1 mM EGTA, 4 mM sodium azide, 1 mM ouabain and 5 mM ATP. Reactions were stopped after 10 min at 37°C by the addition of the colour-developing agent followed by a blocking reagent. Another 30 min later, absorbance at 620 nm was detected using a BMG Labtech FluoStar Optima fluorescence photometer (BMG Labtech, Offenburg, Germany). Phosphate standards (0.4 and 0.8 pmol per well) for OD calibration were included on each 96-well plate. For calculation of relative ATPase activity, baseline ATPase activity (ctrl 1), Na_3VO_4 -insensitive ATPase activity (ctrl 2), ATPase activity of fully activated membranes (ctrl 3) and Na_3VO_4 -insensitive ATPase activity of fully activated membranes (ctrl 4) were also determined. The negative control for MDR1 was provided by membrane preparations expressing β -galactosidase (Beta-gal) and for BCRP we used Sf9 cell membranes from cells infected with a baculovirus containing a defective BCRP gene (defBCRP).

Vesicular transport assay. Vesicular transport assays were performed as previously described (Bodo *et al.*, 2003). Membrane vesicles in the inside-out orientation prepared from baculovirus-infected Sf9 cells overexpressing human BCRP transporters were supplied by SOLVO Biotechnology. ^3H -oestrone-3-sulphate was used as a radiolabelled, low permeability, reporter substrate which is transported into the vesicles. Incubations were carried out in the absence and presence of 4 mM ATP to distinguish between transporter-related uptake and passive diffusion into the vesicles. The reference inhibitor Ko143 (300 nM) served as a positive control and defBCRP as negative control. Briefly, membrane vesicle preparations were preincubated with reporter substrate and the test substance or, alternatively, the solvent DMSO at 37°C for 10 min in assay buffer, containing 10 mM MgCl_2 , 10 mM Tris-Cl (pH 7.0) and 250 mM sucrose. Addition of MgATP, or assay buffer for background controls, started the reaction. After 1 min, reactions were stopped by the addition of ice-cold assay buffer. Vesicles were separated by immediate filtration on a 96-well filter plate (0.65 μM , Millipore, Billerica, MA, USA), filter plates were washed, dried and reporter substrate inside the filtered vesicles was quantified by liquid scintillation, using a Perkin Elmer MicroBeta Trilux liquid scintillation counter (Perkin Elmer, Waltham, MA, USA).

Final assay concentrations were 0.07, 0.21, 0.62, 1.85, 5.55, 16.67, 50 and 150 μM for the dye extrusion assays and 0.41,

1.2, 3.7, 11, 33, 100 and 300 μM for ATPase and vesicular transport assays. For each concentration tested, assays were performed in triplicate.

Data analysis

Dye extrusion assay. The rate of dye accumulation was defined as the slope of the fluorescence versus time plot. For calculation of relative transporter inhibition (%), values from DMSO and control inhibitors (Ko143 and verapamil) were defined as 0% and 100% inhibition, respectively, by the following equation:

$$\frac{\text{flavonoid} - \text{DMSO}}{\text{control inhibitor} - \text{DMSO}} \cdot 100$$

IC_{50} was defined as the test substance concentration required for inhibiting the transport of the reporter substrate by 50%. Efficacy describes the maximal inhibition achieved by a test compound in per cent of the maximal inhibition observed in the presence of the reference inhibitors Ko143 and verapamil.

ATPase assay. For calculation of relative activation and inhibition, the vanadate-sensitive baseline ATPase activity and the maximal vanadate-sensitive ATPase activity after stimulation with 10 μM sulphasalazine, for BCRP, or 40 μM verapamil, for MDR1, were defined as 0% and 100% transporter ATPase activity respectively. Relative activation (%) was calculated as follows:

$$\frac{\{[\text{flavonoid} - (\text{flavonoid} + \text{vanadate})] - (\text{ctrl1} - \text{ctrl2})\}}{(\text{ctrl3} - \text{ctrl4}) - (\text{ctrl1} - \text{ctrl2})} \cdot 100$$

EC_{50} was defined as the test substance concentration needed to reach 50% of its own maximal activation and efficacy was defined as the compound's maximal activation, as compared with the activation by the respective reference.

Relative inhibition values (%) were obtained as follows:

$$\frac{\{[(\text{flavonoid} + \text{reference activator}) - (\text{flavonoid} + \text{reference activator} + \text{vanadate})] - (\text{ctrl1} - \text{ctrl2})\}}{(\text{ctrl3} - \text{ctrl4}) - (\text{ctrl1} - \text{ctrl2})} \cdot 100$$

IC_{50} was defined as the test substance concentration at which half-maximal inhibition occurred and efficacy was defined as the maximal inhibitory effect achieved by the test substance relative to the baseline activity.

Vesicular transport assay. Substrate transport relative to the non-inhibited control (%) was calculated according to the following equation:

$$\frac{(\text{flavonoid} + \text{ATP}) - \text{flavonoid}}{(\text{DMSO} + \text{ATP}) - \text{DMSO}} \cdot 100$$

IC_{50} was defined as the test substance concentration required for inhibiting transport of the reporter substrate by

50%. Efficacy specifies the maximal inhibition achieved by the test compound in per cent of the maximal activity.

ISIS/Draw V2.1.4 (MDL Information Systems, CA, USA) served to illustrate chemical structures of anthocyanidins (Figure 1).

Statistics. GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA) was used for curve fitting, determination of reaction parameters and statistical analysis.

To address a putative structural effect of tested anthocyanins' sugar component on BCRP ATPase activation and vesicular transport, substances were grouped by the presence or absence of sugar moieties, that is, anthocyanins versus anthocyanidins. EC₅₀ and IC₅₀ values, respectively, were compared with an unpaired *t*-test following confirmation of Gaussian distributions. To correct for unequal variances (*F*-test), Welch's correction was performed. In order to minimize the

risk of type I error, a Bonferroni correction was applied. Statistical significance was set at *P* = 0.05.

Materials

Cyanidin, cyanidin-3,5-diglucoside (cyanidin-3,5-diglc), cyanidin-3-galactoside (cyanidin-3-gal), cyanidin-3-glucoside (cyanidin-3-glc), cyanidin-3-rutinoside (cyanidin-3-rut), delphinidin, delphinidin-3-glucoside, malvidin, malvidin-3,5-diglucoside, malvidin-3-galactoside, malvidin-3-glucoside, peonidin, peonidin-3-glucoside, pelargonidin, pelargonidin-3,5-diglucoside and petunidin were purchased from Extrasynthese (Genay, France). Stock solutions of the flavonoids (15 mM) and serial dilutions were prepared in DMSO.

Radiochemicals were purchased from Perkin Elmer (Waltham, MA, USA). Other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) or were of analytical grade.

Results

None of the test compounds showed any affinity for either the BCRP or the MDR1 transporter in the dye efflux assay. For the remaining BCRP and MDR1 assays, calculated reaction parameters are shown in Table 1.

In BCRP ATPase assays, baseline ATPase activity was stimulated by several anthocyanidins and their glycosides (Table 1). The presence or absence of a sugar moiety failed to predict half-maximal ATPase activation values in this assay (*P* = 0.1504, *t* = 1.78, *df* = 4, *p*_{corr} = 0.3008). With the exception of cyanidin-3,5-diglc, however, only anthocyanidins elicited BCRP ATPase inhibition at IC₅₀ < 50 µM. Potent inhibitors comprised cyanidin and peonidin, intermediate inhibition was exhibited by pelargonidin and delphinidin, with minimal inhibition observed for cyanidin-3-rut, delphinidin-3-glc, malvidin-3-glc, pelargonidin-3,5-diglc, malvidin-3-gal (eff. <44%). Interestingly, two compounds, malvidin and

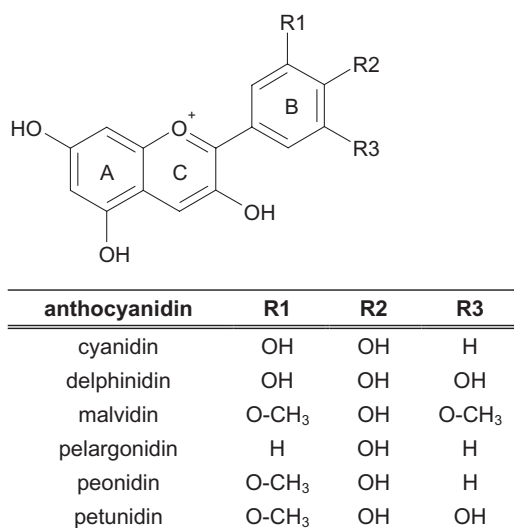


Figure 1 Chemical structures of tested anthocyanidins.

Table 1 Reaction parameters derived from MDR1 or BCRP ATPase and BCRP vesicular transport (VT) assays

Test compound	MDR1 ATPase	BCRP ATPase		BCRP VT
	Inhibition [IC ₅₀] (eff.)	Inhibition [IC ₅₀] (eff.)	Activation [EC ₅₀] (eff.)	Inhibition [IC ₅₀] (eff.)
Cyanidin	58 (100)	7.6 (100)	– (–)	5 (100)
Delphinidin	104 (100)	87 (100)	– (–)	13 (98)
Malvidin	220 (62)	49 (100)	1.1 (59)	3 (98)
Pelargonidin	83 (100)	76 (79)	– (–)	17 (97)
Peonidin	100 (100)	30 (100)	– (–)	4 (96)
Petunidin	230 (65)	193 (87)	3.3 (125)	5 (100)
Cyanidin-3-glc	– (–)	– (–)	119 (142)	49 (87)
Cyanidin-3-gal	– (–)	– (–)	15 (237)	25 (89)
Cyanidin-3-rut	– (–)	– (35)	– (–)	95 (73)
Cyanidin-3,5-diglc	– (–)	43 (100)	– (–)	83 (76)
Delphinidin-3-glc	– (–)	– (44)	– (–)	47 (93)
Malvidin-3-glc	– (42)	– (35)	– (–)	43 (94)
Malvidin-3-gal	– (24)	– (14)	6.3 (73)	31 (88)
Malvidin-3,5-diglc	– (–)	– (–)	12 (87)	23 (81)
Pelargonidin-3,5-diglc	– (14)	– (24)	– (–)	130 (66)
Peonidin-3-glc	– (20)	– (–)	45 (133)	24 (93)

Half-maximal inhibition and activation values are displayed in µM. Efficacies are given in brackets at the highest anthocyanidin/anthocyanin concentration tested. –, no effect observed; BCRP, breast cancer resistance protein; MDR1, multidrug resistance protein 1.

petunidin, which stimulated the BCRP ATPase at low concentrations (1–3 μM), displayed bimodal activities, inhibiting ATPase activity at higher concentrations (IC_{50} = 49 μM and 193 μM). Of the identified inhibitors, delphinidin, cyanidin, malvidin and peonidin inhibited both the maximal ATPase activity after sulphasalazine stimulation, and the baseline ATPase activity at higher concentrations.

In MDR1 ATPase assays, cyanidin-3-glc, cyanidin-3-gal and delphinidin-3-glc showed weak stimulation of both baseline and verapamil-induced activity (eff. = 35%, 23% and 24% respectively). Only cyanidin, pelargonidin, peonidin and delphinidin fully inhibited verapamil-stimulated ATPase activity within the tested concentration range (<300 μM). Partial and very weak MDR1 inhibition was observed for malvidin, petunidin, malvidin-3-glc, malvidin-3-gal, peonidin-3-glc and pelargonidin-3,5-diglc. Finally, cyanidin-3-rut, cyanidin-3,5-diglc and malvidin-3,5-diglc did not exhibit any measurable inhibitory effects on MDR1 ATPase.

In view of the overall poor affinities of these compounds for MDR1, further characterizations by transport assays to rule out non-specific stimulation of ATPase were conducted only for BCRP. All test compounds inhibited BCRP-mediated transport of ^3H -oestrone-3-sulphate into vesicles, dose-dependently (far right hand column, Table 1). Effects were most pronounced for malvidin, peonidin, petunidin and cyanidin with essentially identical IC_{50} values and full efficacy. The remainder of the compounds tested were less potent with IC_{50} values ranging from 13 M to 130 M and efficacies from 98% to 66%. With regard to structural features in the vesicular transport assay, anthocyanidins and their glycosides exhibited a significant difference in mean IC_{50} values (P = 0.0029, t = 4.05, df = 9, p_{corr} = 0.0058).

Discussion and conclusions

We used a combination of assays to examine the interactions of 16 flavonoids with two key proteins involved in the transport of xenobiotics.

Passive permeability is a prerequisite for eliciting a response in dye extrusion assays as has previously been shown for other compounds (Polli *et al.*, 2001; Mahar Doan *et al.*, 2002). The absence of effects in the calcein-AM and Hoechst 33342 assays, suggests that anthocyanins and their aglycones cannot cross the cell membrane passively and, therefore, will not reach the substrate binding site of the transporter.

We identified seven potent stimulants of the BCRP ATPase activity, with half-maximal activation in the low micromolar range for two anthocyanidins. For four flavonoids, stimulation of baseline ATPase activity exceeded 100% as defined by the reference activator substrate. While this was in part a non-specific effect that was also observed in the defBCRP negative control for cyanidin-3-gal and cyanidin-3-glc (data not shown), actual effects may be lower for these compounds. However, petunidin and peonidin-3-glc emerged as genuine stimulants of BCRP ATPase activity that led to a higher level of activation than is achieved with 10 μM sulphasalazine. Stimulation of ATPase activity by substrates is considered a direct correlate of the actual transport process (Sarkadi *et al.*, 1992; Mao and Unadkat, 2005) and therefore an indicator of

substrate functionality. However, stimulation of BCRP- and MDR1-associated ATPase activity may, on occasion, occur independently of substrate transport (Adachi *et al.*, 2001; Ozvegy *et al.*, 2002). In other cases, induction of ABC transporter activity has been observed (Zhou *et al.*, 2004). This appears unlikely for anthocyanins and anthocyanidins, as reporter substrate transport was not increased in the vesicular transport assay. In addition to some stimulants of BCRP ATPase, potent inhibitors were also identified, with IC_{50} values of cyanidin, peonidin, cyanidin-3,5-diglc and malvidin ranging from 7.6 to 49 μM . Moreover, malvidin and petunidin, both of which stimulated baseline ATPase activity at low concentrations (EC_{50} of 1.1 and 3.3 μM respectively), inhibited sulphasalazine-stimulated ATPase activity at higher concentrations (IC_{50} values of 49 and 193 μM respectively). It cannot be entirely ruled out, however, that these two anthocyanidins are not true inhibitors, but rather slowly transported substrates which compete with the assay substrate for transport. With only one exception, cyanidin-3,5-diglc, the absence of sugar moieties was associated with higher levels of BCRP ATPase inhibition, as compared with those achieved by other compounds.

Results of the vesicular transport assays confirmed significantly higher affinities of anthocyanidins for BCRP (mean IC_{50} = 7.8 μM) when compared with affinities of glycosylated test compounds (mean IC_{50} = 55.0 μM , P = 0.0029, t = 4.05, df = 9). Vesicular transport assays also served to verify affinities of all compounds for BCRP. Moreover, none of the tested anthocyanins or aglycones enhanced transport of the reporter substrate. This further supports the hypothesis that the seven compounds activating BCRP ATPase are true BCRP substrates, rather than mere stimulants of BCRP activity.

In contrast to our results for BCRP ATPase, the effects of the test compounds on MDR1 ATPase activity were weak. Three compounds enhanced both baseline and verapamil-stimulated MDR1 ATPase activity, but the latter effect is rarely observed in practice. Our findings are best explained by non-specific interactions with the MDR1 transporter, such as stimulation of an endogenous ATPase. For these flavonoids, a slight stimulation of ATPase activity in the beta-gal control membranes was also observed. All tested aglycones reached IC_{50} values from 58 to 230 μM for MDR1 ATPase inhibition. Thus, anthocyanidins are either moderate MDR1 inhibitors or slowly transported MDR1 substrates, while even lower levels of inhibition were observed for glycosylated compounds.

A variety of dietary polyphenols are known to interact with BCRP or MDR1 *in vitro*. Of these, the stilbene resveratrol, the flavanons hesperetin (Cooray *et al.*, 2004) and naringenin (Zhang *et al.*, 2004a), the flavons luteolin and chrysin (Imai *et al.*, 2004), and the flavonols kaempferol and quercetin (Zhang *et al.*, 2004b) have emerged as inhibitors of BCRP, among others. The isoflavone genistein is considered as a natural substrate that competitively inhibits drug efflux by BCRP (Imai *et al.*, 2004). With respect to MDR1, resveratrol (Nabekura *et al.*, 2005), hesperetin, naringenin, chrysin (Mitsunaga *et al.*, 2000), the tea catechins epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), plus the anthocyanidin cyanidin (Kitagawa, 2006) have previously displayed inhibitory activity. Regarding bimodal functions, as observed for malvidin and petunidin with respect to BCRP ATPase

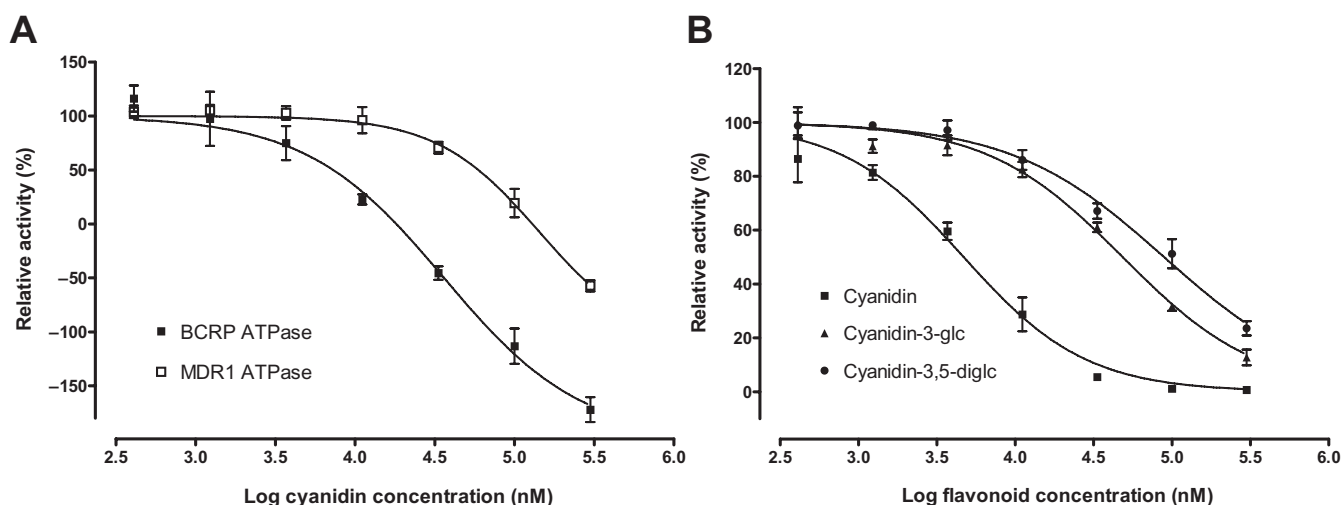


Figure 2 Dose-response curves for cyanidin with respect to BCRP and MDR1 ATPase inhibition (A), and for cyanidin, cyanidin-3-glc and cyanidin-3,5-diglc with respect to inhibition of BCRP vesicular transport (B). BCRP, breast cancer resistance protein; MDR1, multidrug resistance protein 1.

activity, similar effects have been noted for quercetin and kaempferol on MDR1 activity (Mitsunaga *et al.*, 2000; Yoo *et al.*, 2007). Polyphenolic MDR1 stimulants include (-)-epicatechin (Wang *et al.*, 2002) and the flavonol galangin (Critchfield *et al.*, 1994).

Owing to the mostly semi-quantitative and heterogenous assays employed in many earlier studies (Conseil *et al.*, 1998), however, a cautious comparison of flavonoid affinities for BCRP and MDR1 is warranted. Among the consistent findings was the modulatory role of sugar moieties on efflux transporter activity. Thus, for most flavonoids that interacted with either BCRP or MDR1, the corresponding glycosides proved either inactive, or less active (Mitsunaga *et al.*, 2000; Imai *et al.*, 2004; Zhang *et al.*, 2004b; Kitagawa, 2006; Yoo *et al.*, 2007). In a further parallel to earlier work on other polyphenols (Imai *et al.*, 2004; Ahmed-Belkacem *et al.*, 2005; Katayama *et al.*, 2007; Brand *et al.*, 2008), we noted prominent effects of anthocyanidins on BCRP (Figure 2). As BCRP ATPase inhibition was up to sevenfold that for MDR1, and substrate-like behaviour was limited to BCRP, we suggest that anthocyanidins and anthocyanins exhibit BCRP-specific functions. This may be relevant to the intestinal barrier and to brain microvessels where BCRP is expressed at higher levels than MDR1 (Albermann *et al.*, 2005; Hilgendorf *et al.*, 2007; Dauchy *et al.*, 2008). Despite the difference in transporter expression, however, the role played by BCRP in substrate efflux at the BBB *in vivo* is less firmly established (Lee *et al.*, 2005; Zhao *et al.*, 2009). Few investigations have addressed the selectivity of xenobiotics for BCRP, relative to MDR1. Distinct affinities are currently being discussed for the substrates flavopiridol, mitoxantrone and topotecan (Litman *et al.*, 2000; Doyle and Ross, 2003; Nakanishi *et al.*, 2003), plus the inhibitory fumitremorgin C analogue Ko143 and tariquidar analogues (Allen *et al.*, 2002; Kuhnle *et al.*, 2009). For these agents, anthocyanidins may alter the pharmacokinetics, depending on their mode of interaction with BCRP.

BCRP has been implicated in multidrug resistance of tumours (Ahmed-Belkacem *et al.*, 2005; Robey *et al.*, 2007),

and co-determines responsiveness to the treatment of CNS disease (Loscher and Potschka, 2005; Lazarowski *et al.*, 2007). Targeting of BCRP may affect barriers in the placenta (Gupta *et al.*, 2004), secretory organs (Sarkadi *et al.*, 2004), the digestive tract and the brain (Maliepaard *et al.*, 2001) with regard to permeability for toxins (Jonker *et al.*, 2005), other dietary compounds (Mao and Unadkat, 2005; Sugimoto *et al.*, 2005; Cervenak *et al.*, 2006) and environmental carcinogens (van Herwaarden *et al.*, 2006).

While substrate-type affinity of anthocyanidins and anthocyanins for BCRP is liable to limit their intestinal absorption and, possibly, brain uptake, the moderate levels of interaction with MDR1 would appear not to be able to reduce bioavailability. Moreover, anthocyanidins may compete with and thereby limit transport of drugs with high affinity for BCRP.

Compounds that cause efflux transporter inhibition may on the one hand dismantle protection against toxins (Kusuhara and Sugiyama, 2007), or may pose a risk of food-drug interactions including those mediated by dietary supplements (Zhang *et al.*, 2004b; Alvarez *et al.*, 2009). On the other hand, transporter inhibitors have restored chemosensitivity to tumour cells (Schinkel and Jonker, 2003; Zhang *et al.*, 2004b) and may enhance brain uptake of drugs. As adjuvants to therapy, inhibitors thus hold promise in the pharmacotherapy of epilepsy (Aronica *et al.*, 2005; Loscher and Potschka, 2005; Lazarowski *et al.*, 2007) and primary brain tumours (Aronica *et al.*, 2005; Breedveld *et al.*, 2006; de Vries *et al.*, 2007).

However, for inhibition of BCRP and MDR1 transporters by anthocyanidins to take effect, we would need to assume that anthocyanidins enter intestinal epithelial and brain endothelial cells at micromolar concentrations. Existing data point to relatively high anthocyanin concentrations in the intestine (Kahle *et al.*, 2006), whereas peak plasma concentrations are in the nanomolar range, and less than 1% is usually excreted in urine (Matsumoto *et al.*, 2001; McGhie and Walton, 2007; Mullen *et al.*, 2008). These estimates may be considered conservative in that analytical challenges are posed (i) by

anthocyanin accumulation in tissues as shown for rats (El Mohsen *et al.*, 2006); (ii) by metabolic transformation to molecular structures that are not routinely detected; and (iii) by protein binding (Satué-Gracia *et al.*, 1997; Murkovic *et al.*, 2000).

For extrapolations to the *in vivo* situation, functional genetic variation in transporter proteins must also be taken into account as a likely confounder of the functionalities addressed (Siddiqui *et al.*, 2003; Pauli-Magnus and Kroetz, 2004; Kusuhara and Sugiyama, 2007). More detailed investigations of anthocyanin and anthocyanidin transporter binding sites and interaction mechanisms are desirable, including studies on reversibility of effects. Possible mechanisms include competitive inhibition or steric blockage of substrate binding to the transporter or ATPase, and allosteric effects on substrate recognition, translocation, dissociation or ATP hydrolysis. Other areas of future research would include the effects of natural exposure to combinations of anthocyanins, as in standardized extracts, and to anthocyanin degradation products such as phenolic acids. Finally, direct transport assays and studies on BCRP knockout mice to rationalize our assumptions and comparative studies involving other members of the ABC transporter family are warranted.

In summary, we have demonstrated moderate to high affinities of anthocyanins and anthocyanidins for the human efflux transporter BCRP, and moderate to low affinities for MDR1. These results add to our understanding of anthocyanin bioavailability and put into perspective the potential of those flavonoids to interfere with the transport and the pharmacokinetics of other MDR1 and BCRP substrates.

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Conflicts of interest

None.

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