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Abstract

The cytochrome P450 CYP2D6 isoform is involved in the metabolism of about 50% of all psychoactive drugs, including neuroleptic agents, selective serotonin reuptake inhibitors, selective norepinephrine reuptake inhibitors and tricyclic antidepressants. Therefore, inhibition of cytochrome P450 activity by foodstuffs has implications for drug safety. The present study addresses inhibitory effects of polyphenolic anthocyanins and their aglycons that are found in many dietary fruits and vegetables. Using a chemiluminescent assay, we obtained IC(50) values ranging from 55 microM to > 800 microM for 17 individual compounds. According to earlier data on furanocoumarins from grapefruit extract, CYP2D6 inhibition is achieved in the range of 190-900 nM. As the tested anthocyanins and anthocyanidins were shown to be about 1,000-fold less potent, they are unlikely to interfere with drug metabolism by CYP2D6. Further studies are warranted to examine the effects of the above flavonoids on other CYP isoforms for more detailed toxicity profiles.
Anthocyanins and anthocyanidins are poor inhibitors of CYP2D6

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Abstract

Background
The cytochrome P450 CYP2D6 isoform is involved in the metabolism of about 50% of all psychoactive drugs, including neuroleptic agents, selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs) and tricyclic antidepressants (TCAs). Therefore, inhibition of cytochrome P450 by foodstuffs has implications for drug safety. The present study addresses inhibitory effects of polyphenolic anthocyanins and their aglycons that are found in many dietary fruits and vegetables.

Methods
Following incubation of membrane preparations containing recombinant human CYP2D6 enzyme with anthocyanins and their aglycons, inhibition of enzyme activity was determined using a chemiluminescent assay.

Results
Both anthocyanins and their aglycons, the anthocyanidins, inhibited CYP2D6 activity in a concentration-dependent manner with IC50 values ranging from 55 µM, for the anthocyanidin pelargonidin, to over 800 µM for diglycoside anthocyanins pelargonin and cyanin.

Conclusions
According to earlier data on furanocoumarins from grapefruit extract, CYP2D6 inhibition is achieved in the range of 190 to 900 nM. In comparison, the tested anthocyanins and anthocyanidins were shown to be about 1000-fold less potent, and unlikely to interfere with drug metabolism by CYP2D6. However, further studies are invited to examine effects of the above flavonoids on other CYP isoforms for more detailed toxicity profiles.
Cytochrome P450 enzymes represent a large family of microsomal heme-containing monooxygenase isoenzymes that are expressed on smooth endoplasmic reticulum membranes by liver hepatocytes, and by cells along the intestinal tract mucosal surface [1]. They catalyze the detoxification of a wide variety of xenobiotics such as drugs, biogenic amines derived from food sources, environmental toxins and chemical carcinogens, plus the oxidation of endogenous substances like steroids, fatty acids, prostaglandins and leukotrienes [2,3]. In addition, some isoforms have been implicated in the activation of procarcinogens [4,5].

Within the P450 super-family, the cytochrome P450 2D6 (CYP2D6) plays a key role in the metabolism of centrally acting drugs, including many neuroleptics [6], selective serotonin reuptake inhibitors (SSRIs) [7], selective norepinephrine reuptake inhibitors (SNRIs) [8] and tricyclic antidepressants (TCAs) [9], by hydroxylation [10], demethylation [11] or dealkylation reactions [3]. The human brain’s CYP2D6 expression pattern maps to the dopaminergic system [12] where CYP2D6 converts the endogenous substrate tyramine, which is formed from tyrosine or phenylalanine, to dopamine. This implies that inhibitors may alter tyrosine and dopamine levels via brain CYP2D6.

During treatment with psychoactive drugs, CYP2D6 inhibitors from food sources may provoke adverse effects and limit the use of medication. Interactions with enzyme inhibitors have been shown to affect both pharmacokinetic and pharmacodynamic parameters of drugs [13,14], and to augment drug toxicity [15]. In an estimated 10% of the general population, CYP2D6 substrate metabolism may be seriously compromised owing to an innate deficiency in enzymatic activity [16,17].

The opportunity for a food-drug interaction is an everyday occurrence, of which grapefruit juice provides a prominent example. Coadministration of grapefruit juice with many
Therapeutic agents is known to increase the oral bioavailability of common drugs by altering presystemic metabolism, particularly in the intestine [18]. Compounds from grapefruit juice that exhibit cytochrome P450 inhibitory activity include furanocoumarins and the flavonoids naringin, quercetin and kaempferol [15]. Inhibitory compounds are equally found in black raspberry juice, wild grape juice, black mulberry juice [19], red wine [20] and, presumably, in cranberry juice [21]. Recognition of such drug-food interactions has raised concerns over risks that may be posed by functional foods, and specifically, by food enriched with secondary plant metabolites.

Among the phenolic compounds for which P450 inhibitory activity is still unknown count anthocyanins. These water-soluble glycosidic derivates of flavylium salts occur in different pH-dependent conformations and are most abundant in berries, grapes and red cabbage, among other foodstuffs [22]. When expressed per 100 g fresh weight, approximately 1480 mg of anthocyanins may be obtained from chokeberries [23], 588 mg from bilberries [24], 476 mg from black currant, and 322 mg from red cabbage [23]. The average dietary per capita and day consumption of anthocyanins was originally estimated at 180 – 215 mg in Western societies [25]. More recent calculations from U.S. American surveys have concluded to a daily intake of only 12.5 mg [23] but the actual amount has been shown to vary considerably with sociodemographic and life-style factors [26], plus the seasonal availability of anthocyanin-rich fruits and vegetables [23].

In recent years, numerous studies have testified to anthocyanins’ health benefits, greatly promoting their popularitity. Favourable effects have been described on free radical scavenging and antioxidant systems [27,28,29], on immune defense [30], on inflammation [31,32], on sensory functions [33,34], and on parameters of neuroprotection [35,36,37]. It has been shown that anthocyanins themselves are not metabolized by cytochrome P450 enzymes.
but their ability to interfere with the metabolism of centrally acting drugs via inhibition of CYP2D6 has not been examined.
Methods

Chemicals

P450-Glo\textsuperscript{TM} screening system was purchased from Promega (Mannheim, Germany).

Quinidine was obtained from Sigma-Aldrich (Schnelldorf, Germany).

Cyanidin, cyanidin-3,5-diglucoside (cyanin), cyanidin-3-galactoside (ideain), cyanidin-3-glucoside (kuromanin), cyanidin-3-rutinoside (keracyanin), delphinidin, delphinidin-3-glucoside (myrtillin), malvidin, malvidin-3,5-diglucoside (malvin), malvidin-3-galactoside, malvidin-3-glucoside (oenin), peonidin, peonidin-3-glucoside, pelargonidin, pelargonidin-3,5-diglucoside (pelargonin), petunidin, and procyanidin B2 were purchased from Extrasynthese (Genay, France). All test substances were dissolved and diluted in DMSO.

CYP2D6 Assay

Effects of test substances on CYP2D6 activity were determined using a validated and isoenzyme-specific screening system (P450-Glo\textsuperscript{TM}) \cite{39} according to the manufacturer’s protocol. At room temperature, a membrane preparation, containing recombinant human CYP2D6 and cytochrome P450 reductase, was preincubated for 10 minutes with the compound under study and the ethylene glycol ester of luciferin-6’-methylether (luciferin-ME EGE), a luminogenic substrate. The assay was performed in 96-well microtiter plates in KPO\textsubscript{4} buffer. NADPH, glucose-6-phosphate, MgCl\textsubscript{2}, and glucose-6-phosphate dehydrogenase served as a NADPH regeneration system, and were added to start the enzymatic reaction. Final concentrations in the assay were 0.25 pmol CYP2D6, 100 mM KPO\textsubscript{4}, 30 µM luciferin-ME EGE, 1.3 mM NADPH, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl\textsubscript{2}, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 50 µM sodium citrate. Subsequent to incubation at room temperature for 45 min, the reaction was stopped and a luminescent signal was initiated by adding a detection reagent, containing an esterase and a firefly luciferase. After another 20
minutes, chemiluminescence values, displayed as relative light units (RLU), were recorded on a
Anthos Lucy 1 microplate luminometer (Anthos Labtech, Salzburg, Austria) with a measuring time of 12 s for each well.

Anthocyanins, anthocyanidins and procyanidin B2 were diluted with DMSO to yield final concentrations ranging from 20 to 800 µM in the assay. Quinidine was diluted to final concentrations of 1, 10, 100 and 1000 nM.

**Data analysis**

For quantification of CYP2D6 inhibition, enzyme activity was calculated using the light signal generated by oxidation of luciferin which, in turn, is produced by CYP2D6 demethylation of the substrate luciferin-ME EGE. As the amount of light is directly proportional to the amount of luciferin released in the reaction with CYP2D6, the following equation applies:

\[
\%A = 100 \times \left(1 - \frac{A_I}{A_{DMSO}}\right)
\]

where \(\%A\) is the percentage of CYP2D6 activity remaining after the exposure to test substances, \(A_I\) is the activity in the presence of an inhibitor, and \(A_{DMSO}\) is the enzyme activity in the absence of inhibitors (control).

For each substance tested, mean values were analyzed from three separate experiments performed in triplicate at up to five concentrations steps, using a non-linear regression model to determine the concentration inhibiting 50% of maximum CYP2D6 activity (Prism v. 2.01, GraphPad Software, CA, USA). For assessing possible effects of structural features (anthocyanin sugar moiety and substitution pattern of the flavonoids’ B-ring) on CYP2D6 inhibition, we performed analysis of variance (ANOVA) (Stata 8, Stata Corp., College Station, TX, USA). Statistical significance was set at \(p = 0.05\). ISIS/Draw v. 2.1.4 (MDL Information Systems, CA, USA) served to illustrate structures of anthocyanidins.
In total, modulatory effects of sixteen anthocyanins and anthocyanidins on cytochrome P450 2D6 activity were investigated, plus effects of the natural anthocyanin precursor procyanidin B2, an epicatechin-epicatechin dimer (figure 1). IC$_{50}$ values of all flavonoids under study are summarized in table 1. Test compounds inhibited CYP2D6 activity in a concentration-dependent manner with IC$_{50}$ values starting at 55 µM (pelargonidin), and exceeding 800 µM (diglucosides of cyanidin and pelargonidin, procyanidin B2).

The four most potent inhibitors were the anthocyanidins pelargonidin, peonidin, delphinidin and cyanidin, with IC$_{50}$ values of 55, 59, 66, and 69 µM, respectively. For the remaining anthocyanidins, IC$_{50}$ values of 77 (malvidin) and 150 µM (petunidin) were determined. Regarding the tested anthocyanins, IC$_{50}$ values from 70 to 266 µM were obtained in the order of decreasing potency for malvidin-3-glucoside, malvidin-3-galactoside, delphinidin-3-glucoside, malvidin-3,5-diglucoside, cyanidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-galactoside, and cyanidin-3-rutinoside. Finally, the least potent inhibitors were identified as the diglucosidic anthocyanins pelargonidin-3,5-diglucoside, plus cyanidin-3,5-diglucoside and procyanidin B2, all of which featured IC$_{50}$ values greater than 800 µM. Quinidine, a widely used inhibitor that had been chosen for a reference, reached an IC$_{50}$ value of 6 nM.

To investigate the modulatory effects of substances’ sugar moiety or B-ring substitution pattern, analysis of variance (ANOVA) was performed, excluding cyanidin-3,5-diglucoside and pelargonidin-3,5-diglucoside, for which IC$_{50}$ values could not be calculated (> 800 µM). When only presence vs. absence of the sugar moiety was used to predict IC$_{50}$ values, the model reached significance (p = 0.03, F = 5.7, R$^2$ = 0.32). Fitting was improved when the number of B-ring substituents, plus an interaction term (sugar moiety*substituents) were included in the model (p = 0.006, F = 7.73, R$^2$ = 0.78). Specifically, anthocyanins were shown to be weaker CYP2D6 inhibitors than anthocyanidins (table 1), and CYP2D6 inhibition
decreased with more substituents on anthocyanidins’ B-ring, whereas it increased with more substituents on anthocyanins’ B-ring.
Dietary supplementations with anthocyanin-rich fruit extracts have been advocated in the light of multiple health-promoting effects including antioxidant, anti-inflammatory, and neuroprotective activities [40,41,42,43,44]. However, limited data exist on possible adverse effects as a consequence of food-drug interactions. Foods that contain complex mixtures of phytochemicals, such as fruits, vegetables, herbs, spices and teas have great potential to induce or inhibit the activity of drug-metabolizing enzymes, including cytochrome P450 enzymes [45,46]. Such interactions are exemplified by effects of grapefruit juice, red wine, black mulberry juice and black raspberry juice [13,15,18,19,20,47,48,49]. Amidst growing safety concerns, the present study addresses in vitro interactions with CYP2D6, an isoform highly relevant to the metabolism of many psychoactive drugs [17,50,51,52,53]. In continuation of earlier studies with grapefruit extract [15] and the polyphenolic apple constituent quercetin [54,55], CYP2D6 activity was determined in the presence of anthocyanins, the corresponding aglycons, and the procyanidin dimer B2. Our data show that anthocyanins and anthocyanidins are weak inhibitors of CYP2D6, with IC$_{50}$ values in the micromolar range, starting at 55 µM and exceeding 800 µM for several compounds. When anthocyanins and anthocyanidins were compared, mean inhibitory potency of anthocyanins was lower ($p = 0.03$, $F = 5.7$, $R^2 = 0.32$). Of all molecules under study, diglucosides of cyanidin and pelargonidin, plus procyanidin B2 exhibited the weakest effects. Analysis of variance confirmed a modulatory role of the B-ring substitution pattern (number of substituents), plus the interaction term (sugar moiety*substituents). Anthocyanidins with three hydroxyl- or methoxyl-substituents on their B-ring acted as weaker inhibitors than did agents with fewer substituents. In contrast, anthocyanidin-3-glycosides and anthocyanidin-
3,5-diglycosides achieved lower IC$_{50}$ values when the B-ring was substituted to a greater extent.

Only few data are available on CYP2D6 inhibitory effects of defined polyphenolic compounds. Of these, quercetin exhibited an IC$_{50}$ value of 24 µM [55], while flavonols and flavonol glycosides from wild ginger exhibited IC$_{50}$ values ranging from 5 to 51 µM [56]. In contrast, furanocoumarins from grapefruit juice achieved 50% inhibition in the nanomolar range. Specifically, bergamottin, 6',7'-dihydroxybergamottin (DHB) and the dimersic GF-I-1 (4-[[6-hydroxy-7-[(1-hydroxy-1-methyl)ethyl]-4-methyl-6-(7-oxo-7H-furo[3,2-
4 g][1]benzopyran-4-y1]-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-furo[3,2-
5 g][1]benzopyran-7-one) and GF-I-4 (4-[[6-hydroxy-7-[[4-methyl-1-(1-methylethenyl)-6-(7-
6 oxo-7H-furo[3,2-g][1]benzopyran-4-y1]-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-
7 furo[3,2-g][1]benzopyran-7-one) reached IC$_{50}$ values of 190 nM, 900 nM, 200 nM and 300 nM, respectively [15]. For comparison, psychoactive drugs have yielded CYP2D6 IC$_{50}$ values of 1.2 (paroxetine), 1.5 (perphenazine), 2.7 (thioridazine) and 6.5 nM (haloperidol) [57,58,59] (figure 2).

Inhibitory effects of anthocyanins on CYP2D6 are weaker by several orders of magnitude, ranging from 70 µM to over 800 µM. According to in silico prediction models, structural features required for effective CYP2D6 inhibition comprise a tertiary amine fragment plus a positive charge on nitrogen and flat hydrophobic region [60]. These ligand descriptors are not met by the compounds investigated here, and relative to the cinchona alkaloid quinidine, the inhibitory potential of anthocyanins and their aglycons was at least 10 000-fold lower. This suggests that anthocyanins pose a limited risk of food-drug interactions mediated by CYP2D6 as compared to above mentioned grapefruit and apple constituents, or other phytochemicals.

For an extrapolation of in vitro data to in vivo effects, additional parameters must be taken into account. Thus it has been proposed that CYP2D6 may be less susceptible to inhibition by food constituents than other isoforms, since substances must cross the intestinal barrier before
they can act on hepatic CYP2D6, whereas high-level expression of other cytochromes in the intestine allows interactions during digestion [61]. Depending on anthocyanins’ *in vivo* portal availability which has yet to be determined, the impact on metabolic processes may be even smaller than anticipated.

Clinical relevance of CYP450 inhibition is subject to individual differences in enzyme activity, i.e. the prevalence of functional genetic variants. The present data obtained with recombinant human CYP2D6 count against CYP2D6 modulatory effects of anthocyanins and anthocyanidins for subjects carrying the CYP2D6*1 allele [62], i.e. for the majority of the Caucasian population. Whether similar effects can be expected in carriers of other CYP2D6 alleles remains to be determined.

Finally, characterization of anthocyanin effects on additional CYP450 isoforms is invited to foster our understanding of polyphenolics’ biological activity. Thus inhibitors of procarcinogen-activating isoforms (e.g., CYP19, CYP1A or CYP1B) may limit the generation of harmful metabolites from estradiol [5], aromatic amines, heterocyclic amines [4], and polycyclic aromatic hydrocarbons [5]. More detailed toxicity profiles will help to unveil the related benefits and novel dietary or medical applications of anthocyanins.
Conclusions

Anthocyanin-rich extracts from berry fruits have become popular food supplements warranting investigations of possible interactions with drug metabolism. The present \textit{in vitro} study argues against such interactions with regard to metabolism by the human cytochrome 2D6 isoenzyme. In comparison to established enzyme inhibitors, the flavonoids examined proved weaker by several orders of magnitude. However, further studies are invited to examine effects of anthocyanins on other CYP isoforms for more detailed toxicity profiles.
Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

AD carried out the CYP2D6 inhibition measurements, performed statistical analyses and drafted the manuscript. PS, GR, HP and GH participated in study design, revised the manuscript critically and finalized it for submission. AO and SL assisted with conceiving the study, selecting test substances and writing the manuscript. PGS was responsible for conception and design of the study, supervision of statistical analysis and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgement

This investigation was funded by the German Federal Ministry of Education, Science, Research and Technology, BMBF – grant No. 0313848C.

Table 1. Inhibition of CYP2D6 activity by anthocyanins and anthocyanidins and mean IC$_{50}$ values.

<table>
<thead>
<tr>
<th>Anthocyanin/Anthocyanidin</th>
<th>IC$_{50}$ in µM (Anthocyanidins)</th>
<th>IC$_{50}$ in µM (Anthocyanins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin</td>
<td>55 [51 - 59]</td>
<td></td>
</tr>
<tr>
<td>Peonidin</td>
<td>59 [51 - 67]</td>
<td></td>
</tr>
<tr>
<td>Delphinidin</td>
<td>66 [65 - 68]</td>
<td></td>
</tr>
</tbody>
</table>
cyanidin 69 [64 - 74]
malvidin-3-glucoside (oenin) 70 [68 - 72]
malvidin-3-galactoside 73 [67 - 80]
malvidin 77 [69 - 85]
delphinidin-3-glucoside (myrtillin) 109 [87 - 137]
malvidin-3,5-diglucoside (malvin) 145 [133 - 157]
petunidin 150 [138 - 162]
cyanidin-3-glucoside (kuromanin) 151 [114 - 199]
peonidin-3-glucoside 217 [199 - 237]
cyanidin-3-galactoside (ideain) 245 [217 - 278]
cyanidin-3-rutinoside (keracyanin) 266 [169 - 420]

95% confidence intervals are given in brackets. Procyanidin B2, plus the anthocyanins pelargonidin-3,5-
diglucoside (pelargonin) and cyanidin-3,5-diglucoside (cyanin) lacked CYP2D6 inhibitory activity (IC₅₀ > 800 µM).

<table>
<thead>
<tr>
<th>anthocyanidin</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanidin</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>delphinidin</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
</tbody>
</table>
malvidin O-CH$_3$ OH O-CH$_3$

pelargonidin H OH H

peonidin O-CH$_3$ OH H

petunidin O-CH$_3$ OH OH

IC$_{50}$ values in µM

0.0 2.5 5.0 7.5 10.0 100 200 300

anthocyanins anthocyanidins psychoactive drugs furanocoumarins quinidine

10.0 7.5 5.0 2.5 0.0
References


Figure 1 – Chemical structures
Chemical structures of tested anthocyanidins (A) and procyanidin B2 (B), an epicatechin-epicatechin dimer.

Figure 2 – IC<sub>50</sub> values of anthocyanins, anthocyanidins, psychoactive drugs (haloperidol, thioridazine, perphenazine, paroxetine), furanocoumarins (bergamottin, 6',7'-DHB, GF-I-1, GF-I-4) and quinidine.
Only those compounds are shown for which IC<sub>50</sub> values were available, i.e. excluding cyanidin-3,5-diglucoside and pelargonidin-3,5-diglucoside.

Table
Table 1 - Inhibition of CYP2D6 activity by anthocyanins and anthocyanidins and mean IC<sub>50</sub> values
95% confidence intervals are given in brackets. Procyanidin B2, plus the anthocyanins pelargonidin-3,5-diglucoside (pelargonin) and cyanidin-3,5-diglucoside (cyanin) lacked CYP2D6 inhibitory activity (IC<sub>50</sub> > 800 µM).