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Research Article

# Anthocyanins and their metabolites are weak inhibitors of cytochrome P450 3A4

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The cytochrome P450 enzyme cytochrome P450 3A4 (CYP3A4) controls the metabolism of about 60% of all drugs, and its inhibition may dramatically affect drug safety. Modulation of cytochrome P450 activity has been observed by constituents of fruit extracts including several flavonoids. The present investigation addresses CYP3A4 inhibition by anthocyanins, their aglycons, proanthocyanidins, and phenolic metabolites using a chemiluminescent assay. Test compounds inhibited CYP3A4 activity in a concentration-dependent manner featuring $IC_{50}$ values from 12.2 up to 7,842 μM. In the order of decreasing effect size, anthocyanidins were followed by anthocyanins, proanthocyanidins, and phenolic acids. When compared to earlier data on furanocoumarins from grapefruit extract, the inhibitory activity of tested anthocyanins, and anthocyanidins was shown to be about 10 000-fold weaker, and was negligible for phenolic acids (>100 000-fold weaker). Future studies are invited to address effects of the above flavonoids on other CYP isoforms for more detailed toxicity profiles.

**Keywords:** Anthocyanidin / Anthocyanin / CYP3A4 / Cytochrome P450 / Flavonoid

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## 1 Introduction

Cytochrome P450 enzymes represent a large family of microsomal heme-containing monooxygenase isoenzymes that are primarily expressed on smooth ER membranes by liver hepatocytes, and by cells along the intestinal tract mucosal surface [1]. They are involved in the detoxification of a wide variety of xenobiotics such as drugs, biogenic amines from food sources, environmental toxins, and chemical carcinogens, the oxidation of steroids, fatty acids, prostaglandins, leukotrienes, and fat-soluble vitamins [2–4]. The CYP3A subfamily comprises 30% of the total liver cytochrome P450 enzyme pool in humans [5], and the isoenzyme 3A4 accounts for approximately 60% of drugs metabolized [6]. In addition, an estimated 70% of CYP protein in the small intestinal epithelium is formed by this isoenzyme [7].

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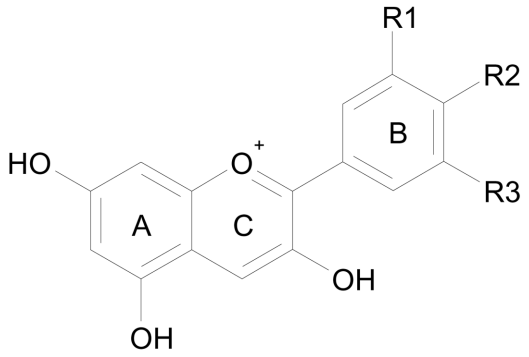
**Abbreviations: CYP3A4,** cytochrome P450 3A4; **glc,** glucoside; **gal,** galactoside; **luciferin-PPXE,** luciferin-6′ phenylpiperazinylyl ether

Coadministration of multiple cytochrome P450 3A4 (CYP3A4) substrates, inducers, or inhibitors, including compounds from food sources, may alter pharmacokinetic and pharmacodynamic parameters of many commonly prescribed drugs [8, 9]. A prominent example of food–drug interactions mediated by CYP3A4 is provided by the inhibitory effects of grapefruit juice on presystemic metabolism, particularly in the intestine [10]. Constituents of grapefruit juice with cytochrome P450 inhibitory activities include furanocoumarins and the flavonoids naringin, quercetin, and kaempferol [11].

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 structures and are most abundant in berries, grapes, and red cabbage, among other foodstuffs [12]. The most common forms found in higher plants are glucosides (glc) and galactosides (gal) of the anthocyanidins cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin, and occur almost exclusively as anthocyanins [13] (Fig. 1). When expressed *per* 100 g fresh weight, approximately 1400 mg of anthocyanins may be obtained from elderberries, 700 mg from black raspberries [14], 600 mg from bilberries [15], and 300 mg from red cabbage [14]. The average dietary per capita and day consumption of anthocyanins was originally estimated at 180–




| anthocyanidin | R1 | R2 | R3 |
|---|---|---|---|
| cyanidin | OH | OH | H |
| delphinidin | OH | OH | OH |
| malvidin | O-$CH_3$ | OH | O-$CH_3$ |
| pelargonidin | H | OH | H |
| peonidin | O-$CH_3$ | OH | H |
| petunidin | O-$CH_3$ | OH | OH |

**Figure 1.** Chemical structures of tested anthocyanidins.

215 mg in Western societies [16], however, more recent calculations from USA surveys have alerted to marked variability due to sociodemographic and lifestyle factors [17].

So far, an *in vitro* CYP3A inhibitory potential has been described for black raspberry juice, wild grape juice, black mulberry juice [18], and red wine [19], and has been suggested for cranberry juice [20], but a contributory role of anthocyanins remains to be elucidated.

Proanthocyanidins, natural anthocyanin precursors, are also common constituents of many foods, such as cocoa, tea, apples, and grape seeds [21]. Studies of their impact on CYP3A4 activity were therefore included in the present investigation, along with phenolic metabolites of anthocyanins and procyanidins.

## 2 Materials and methods

### 2.1 Chemicals

Ketoconazole, 3-(4-hydroxyphenyl)propionic acid, 3-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid, syringic acid, vanillic acid, protocatechuic acid, and 4-hydroxybenzoic acid were obtained from Sigma – Aldrich (Steinheim, Germany).

Cyanidin, cyanidin-3,5-diglucoside (cyanin), cyanidin-3-gal (ideain), cyanidin-3-glc (kuromanin), cyanidin-3-rutinoside (keracyanin), delphinidin, delphinidin-3-glc (myrtillin), malvidin, malvidin-3,5-diglucoside (malvin), malvidin-3-gal, malvidin-3-glc (oenin), peonidin, peonidin-3-glc, pelargonidin, pelargonidin-3,5-diglucoside (pelargonin), petunidin, procyanidin B1, and procyanidin B2 were purchased from Extrasynthese (Genay, France). Ketoconazole and phenolic acids were dissolved in DMSO/$H_2O$ (1:1), and the remaining compounds in DMSO.

### 2.2 Enzyme and substrates

Membrane preparations containing recombinant human CYP3A4 obtained from a baculovirus expression system, and the specific luminogenic cytochrome enzyme substrate luciferin-6′ phenylpiperazinylyl ether (luciferin-PPXE) were purchased from Promega (Mannheim, Germany).

### 2.3 CYP3A4 Assay

Effects of test substances on CYP3A4 activity were determined using the P450-Glo™ Screening System. Briefly, a membrane preparation containing recombinant human CYP3A4, cytochrome P450 reductase, and cytochrome b5 as an enhancer, was preincubated for 10 min at room temperature with the compound under study and luciferin-PPXE, a luminogenic substrate. Assays were performed in 96-well microtiter plates in $K_3PO_4$ buffer. $NADP^+$, glucose-6-phosphate, $MgCl_2$, and glucose-6-phosphate dehydrogenase served as an NADPH regeneration system and were added to start the enzymatic reaction. Final substance concentrations in the assay were 200 mM $K_3PO_4$, 24 mM Tris-HCl, 3.3 mM $MgCl_2$, 10 pmol/mL CYP3A4, 25 μM luciferin-PPXE, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.4 U/mL glucose-6-phosphate dehydrogenase. Subsequent to further incubation at room temperature for 20 min, the reaction was stopped and a luminescent signal was initiated by adding a detection reagent, containing a firefly luciferase. After another 20 min, chemiluminescence values, displayed as relative light units (RLU), were recorded on an Anthos Lucy 1 microplate luminometer (Anthos Labtech, Salzburg, Austria) with a measuring time of 12 s for each well.

Anthocyanins, anthocyanidins, phenolic acids, procyanidin B1 and B2 were diluted with DMSO/$H_2O$ (1:1) to yield final concentrations ranging from 2 to 800 μM in the assay for tested polyphenols, and from 100 to 10 000 μM for phenolic acids. Ketoconazole was diluted to final concentrations from 2 up to 200 nM.

### 2.4 Data analysis

For quantification of CYP3A4 inhibition, enzyme activity was calculated using the light signal generated by oxidation of luciferin which, in turn, is produced by CYP3A4 dealkylation of the substrate luciferin-PPXE. As the amount of light is directly proportional to the amount of luciferin released in the reaction with CYP3A4, the following equation applies:

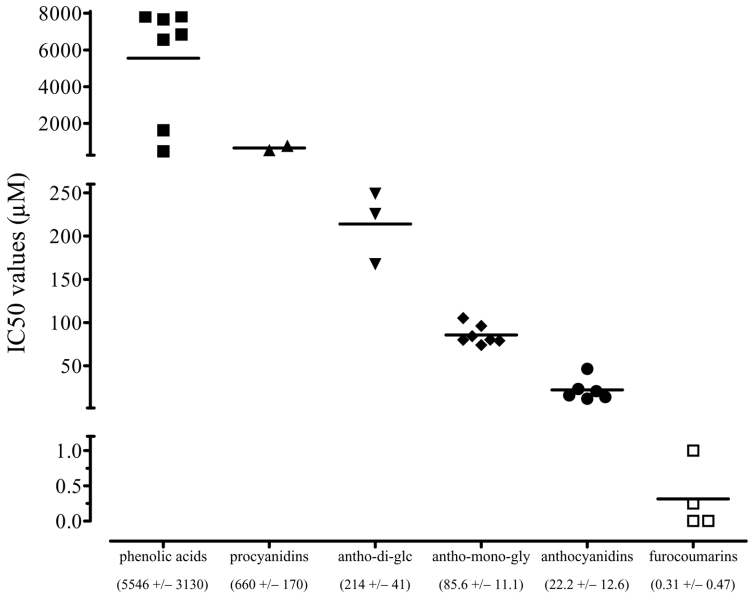



**Figure 2.** $IC_{50}$ values of phenolic acids, procyanidins, anthocyanidin-3,5-diglucosides (antho-di-glc), anthocyanidin-3-glycosides (antho-mono-gly), and anthocyanidins compared to $IC_{50}$ values of furocoumarins [11]. Mean $IC_{50}$ values ± SD (µM) are given in brackets.

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

where $\%A$ is the percentage of the CYP3A4 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.

For each substance tested, mean values were analyzed from three separate experiments performed in triplicate at up to seven concentrations steps, using a nonlinear regression model to determine the concentration inhibiting 50% of maximum CYP3A4 activity (Prism V 4.00, GraphPad Software, CA, USA).

To address a putative structural effect of anthocyanins' sugar component, substances were grouped by the number of sugar moieties, *i.e.*, anthocyanins, anthocyanidin monoglycosides and anthocyanidin-diglucosides, and analysis of variance (ANOVA) was performed (Stata 8, Stata, 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 chemical structures of anthocyanidins.

## 3 Results

In total, ten anthocyanins', six anthocyanidins', and seven phenolic acids' modulatory effects on cytochrome P450 3A4 activity were investigated in the present study, plus the effects of the natural anthocyanin precursors, procyanidins B1 and B2, two diastereomeric epicatechin-epicatechin dimers.

Test compounds inhibited CYP3A4 activity in a concentration-dependent manner and gave distinct profiles for the substance groups under study. For anthocyanidins, $IC_{50}$ values ranged from 12.2 to 46.5 µM (pelargonidin 12.2 µM, malvidin 14.0 µM, peonidin 16.1 µM, cyanidin 20.8 µM, petunidin 23.4 µM, delphinidin 46.5 µM) (mean 22.2 ± 12.6 µM), for anthocyanidin monoglycosides from 74.1 to 105.3 µM (delphinidin-3-glc 74.1 µM, peonidin-3-glc 79.2 µM, malvidin-3-glc 80.0 µM, cyanidin-3-gal 80.2 µM, malvidin-3-gal 84.3 µM, cyanidin-3-rutins 96.1 µM, cyanidin-3-glc 105.3 µM) (mean 85.6 ± 11.1 µM), for anthocyanidin diglucosides from 167.5 to 249.0 µM (cyanidin-3,5-diglc 167.5 µM, malvidin-3,5-diglc 225.6 µM, pelargonidin-3,5-diglc 249.0 µM) (mean 214 ± 41 µM), for phenolic acids from 472.3 up to 7842 µM (mean 5546 ± 3130 µM) and for procyanidins B2 and B1 539.8 and 779.9 µM (mean 660 ± 170 µM). Thus phenolic acids acted as the weakest inhibitors, followed by procyanidins, anthocyanins, and anthocyanidins (Fig. 2). Ketoconazole, a widely used inhibitor that had been chosen for a reference, reached an $IC_{50}$ value of 18.4 nM.

When the number of sugar moieties per compound was used to predict $IC_{50}$ values of anthocyanins, a significant impact was noted on CYP3A4 inhibitory functionality ($p < 0.0001$, $F = 94.69$, $R^2 = 0.94$).

## 4 Discussion

Growing evidence suggests that diets rich in berry fruits protect against cancer, cardiovascular disease, and neurodegenerative disease [22]. Research on the functionality of fruit extracts containing high amounts of anthocyanins has so far addressed radical scavenging and antioxidant [23 – 27], anti-inflammatory [28 – 31], anticarcinogenic [31, 32], and neuroprotective activities [33 – 38] among others, but not CYP450 inhibition. It has been shown that anthocyanins

themselves are not metabolized by cytochrome P450 enzymes [39], but the rapidly growing interest in dietary anthocyanins calls for a more thorough understanding of their cellular targets and possible hazards posed by interference with the metabolism of common drugs.

This is illustrated by adverse effects of drugs that are precipitated by the consumption of grapefruit juice, meanwhile a well-known modulator of CYP3A4 metabolic activity [6, 8, 10, 11, 19, 40 – 42]. To extend earlier studies of grapefruit and polyphenolic green tea, wine, and apple constituents, the present study investigated CYP3A4 activity of anthocyanins, their aglycons, the procyanidin dimers B1 and B2, and several phenolic acids [43]. Natural anthocyanins and procyanidins are unstable in the intestinal environment and under physiological conditions. Therefore, the role of phenolic acid metabolites after intestinal microflora and metabolic degradation is of particular interest [43 – 46].

Our data show that anthocyanins and anthocyanidins are weak inhibitors of CYP3A4, featuring $IC_{50}$ values from 12.2 up to 249.0 μM. Procyanidins' effects were even weaker (539.8 and 779.9 μM), and the phenolic acids under study exhibited only negligible inhibitory activity with $IC_{50}$ values ranging from 472.3 to 7,842 μM. To judge by these results, anthocyanins' and their metabolites' contribution to CYP3A4 inhibitory activity in wine and berry juices is only minor [18, 19, 47].

To the best of our knowledge, these are the first in vitro studies of anthocyanins' and phenolic acids' potential to interfere with drug metabolism. Of the polyphenolic compounds previously investigated, naringenin, (–)-epigallocatechin-gallate (EGCG), quercetin, epicatechin-gallate, curcumin, and resveratrol have exhibited $IC_{50}$ values of approximately 87 [48], 40 [49], 38 [50], 20 [49], 16 [51], and 4 μM [52], respectively. Furanocoumarins from grapefruit juice, however, set an activity benchmark in the nanomolar range. Specifically, bergamottin, 6′,7′-dihydroxybergamottin (DHB) and the dimerics GF-I-1 (4-[[6-hydroxy-7-[[1-[(1-hydroxy-1-methyl)ethyl]-4-methyl-6-(7-oxo-7H-furo[3,2-g][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-furo[3,2-g][1]benzopyran-7-one) and GF-I-4 (4-[[6-hydroxy-7-[[4-methyl-1-(1-methylethenyl)-6-(7-oxo-7H-furo[3,2-g][1]benzopyran-4-yl)-4-hexenyl]oxy]-3,7-dimethyl-2-octenyl]oxy]-7H-furo[3,2-g][1]benzopyran-7-one) have exhibited $IC_{50}$ values as low as 1000, 250, 3, and 3 nM for CYP3A4, respectively [11].In comparison, CYP3A4 inhibitory effects of anthocyanins, their aglycons, dimeric procyanidins and phenolic acids are weaker by several orders of magnitude. Relative to ketoconazole, an antifungal and known inhibitor, the flavonoids investigated here featured a 1000 – 10 000-fold lower potential for interactions (> 100 000-fold lower for phenolic acids).

Thus our results suggest that anthocyanins, procyanidins, and phenolic acids pose only a limited risk of food – drug interactions mediated by CYP3A4 as compared to other grapefruit, wine, green tea, and apple juice constituents.

Before an extrapolation of in vitro data to *in vivo* effects can be attempted, additional parameters must be taken into account. For instance, CYP3A4 may be more susceptible to inhibition by food constituents than other mainly hepatic cytochrome P450 isoforms. As high levels of CYP3A4 expression in the intestine allow interactions during the digestive process [53], substances need not cross the intestinal barrier in order to interfere with metabolism. Even when oral bioavailability is low, compounds may act as potent inhibitors, provided that they are able to penetrate epithelial cells in the intestine. Therefore, anthocyanins' and anthocyanidins' impact on metabolic drug processing by CYP3A4 is also dependent on transport into intestinal epithelium cells.

Anthocyanidins' glycosylation may play a dual role with regard to *in vivo* effects. In addition to impacting on the absorption of compounds [54, 55], sugar moieties have been shown to enhance cytochrome inhibitory activity of some flavonoids [56]. In contrast, in the present investigations, the number of sugar moieties predicted a decline in anthocyanidins' effects on CYP3A4, which underscores the need for more detailed data on structure – activity relationships.

Finally, further characterization of anthocyanins' and their metabolites' effects on CYP450 isoforms other than CYP3A4 is invited to add to our understanding of polyphenolics' multiple functionalities and to promote the safe use of these compounds in food supplementation.

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