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The effect of anthocyans on the expression of selected phase II xenobiotic-metabolizing enzymes in primary cultures of human hepatocytes

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Anthocyans are biologically active constituents of various berry fruits and they are also contained in nutritional supplements derived from extracts or dry matter from berry fruits. In this study we evaluated the effects of anthocyans on the expression of selected drug-metabolizing phase II genes in primary cultures of human hepatocytes by qRT-PCR. Most of the tested anthocyanidins (6) and anthocyanins (21) did not induce the expression of mRNA of UGT1A/2B members in human hepatocytes. The same can be stated for expression of selected GST genes on the mRNA level. However, some of them *e.g.* cyanidin-3-*O*-rutinoside consistently decreased the level of GSTP1 mRNA in all tested cultures. In conclusion, most of the anthocyans did not affect the expression of selected phase II metabolizing enzymes *in vitro*.

Introduction

Anthocyans are biologically active compounds that occur in all tissues of higher plants as water-soluble vacuolar pigments. Structurally, they are flavonoids that differ in the number of hydroxyl groups, degree of methylation of –OH groups, number, nature and position of sugar attachment and the number and nature of aliphatic or aromatic acids fixed to sugars in the molecule.[1] Anthocyans comprise anthocyanins and anthocyanidins, which are aglycon (sugar-free) backbones of anthocyanins. Anthocyans are well known for their various health benefits,[2–4] and for a plethora of biological effects, including anti-proliferative,[5] anti-apoptotic,[6] anti-tumor,[7] anti-mutagenic,[8] anti-oxidant,[9] anti-radical[10] and nitric-oxide inhibitory effects.[11]

Despite numerous studies of anthocyans' biological activities, the systematic study focused on the interactions between anthocyans and drug-metabolizing phase II conjugation enzymes has not yet been carried out. However, there are two recent papers dealing with the effect of anthocyans on the catalytic activity of phase II enzymes.[12,13] Since plant foods, beverages and dietary supplements contain various natural or synthetic xenobiotics, including anthocyans, a phenomenon of food–drug interactions emerged. Dietary xenobiotics can induce both phase I and phase II drug-metabolizing enzymes. We have recently described the induction of drug-metabolizing enzyme CYP1A1 in human cancer cell lines and human hepatocytes by some anthocyanidins[14] and anthocyanins.[15] In the current study, we examined the effects of 27 anthocyans on the expression of selected phase II conjugation enzymes involved in drug metabolism and endogenous processes; *i.e.* 6 isoforms of uridine 5′-diphospho-glucuronosyltransferase (UGT1A1, UGT1A4, UGT1A6, UGT1A9, UGT2B7, UGT2B10) and 5 isoforms of glutathion-*S*-transferase (GSTA1, GSTT1, GSTO1, GSTP1, GSTZ1). We measured the expression of phase II enzymes in primary cultures of human hepatocytes. We tested 6 anthocyanidines (cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin) and 21 anthocyanins (Table 1) in 4 cultures of human hepatocytes and compared the effect of rifampicin or dioxin, the activators of pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR), respectively. The activation of these receptors was reported to increase the level of some phase II enzymes.[16–23]

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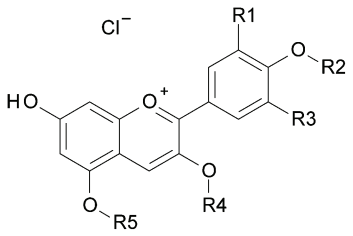
Materials and methods

Compounds and reagents

Dimethylsulfoxide (DMSO) and rifampicin (RIF) were from Sigma-Aldrich (Prague, Czech Republic). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). The following anthocyanins and anthocyanidins were from Extrasynthese (Lyon, France): peonidin-3-*O*-glucoside chloride (PEO-1), peonidin-3-*O*-rutinoside chloride (PEO-2), pelargonidin-3,5-di-*O*-glucoside chloride (PEL-1), pelargonidin-3-*O*-rutinoside chloride (PEL-2), delphinidin-3-*O*-glucoside chloride (DEL-1), delphinidin-3-*O*-rutinoside chloride (DEL-2), delphinidin-3,5-di-*O*-glucoside chloride (DEL-3), delphinidin-3-*O*-sambubioside chloride (DEL-4), delphinidin-3-*O*-rhamnoside chloride (DEL-5), malvidin-3-*O*-glucoside chloride (MAL-1), malvidin-3,5-di-*O*-glucoside chloride (MAL-2), malvidin-3-*O*-galactoside chloride (MAL-3), cyanidin-3-*O*-glucoside chloride (CYA-1), cyanidin-3-*O*-rutinoside chloride (CYA-2),

Table 1 Chemical structures of anthocyanins and anthocyanidins



	Anthocyanins	R1	R2	R3	R4	R5
PEO-1	Peonidin-3-*O*-glucoside chloride	OCH_3	H	H	Glucoside	H
PEO-2	Peonidin-3-*O*-rutinoside chloride	OCH_3	H	H	Rutinoside	H
PEL-1	Pelargonidin-3,5-di-*O*-glucoside chloride	H	H	H	Glucoside	Glucose
PEL-2	Pelargonidin-3-*O*-rutinoside chloride	H	H	H	Rutinoside	H
DEL-1	Delphinidin-3-*O*-glucoside chloride	OH	H	OH	Glucoside	H
DEL-2	Delphinidin-3-*O*-rutinoside chloride	OH	H	OH	Rutinoside	H
DEL-3	Delphinidin-3,5-di-*O*-glucoside chloride	OH	H	OH	Glucoside	Glucose
DEL-4	Delphinidin-3-*O*-sambubioside chloride	OH	H	OH	Sambubioside	H
DEL-5	Delphinidin-3-*O*-rhamnoside chloride	OH	H	OH	Rhamnoside	H
MAL-1	Malvidin-3-*O*-glucoside chloride	OCH_3	H	OCH_3	Glucoside	H
MAL-2	Malvidin-3,5-di-*O*-glucoside chloride	OCH_3	H	OCH_3	Glucoside	Glucose
MAL-3	Malvidin-3-*O*-galactoside chloride	OCH_3	H	OCH_3	Galactoside	H
CYA-1	Cyanidin-3-*O*-glucoside chloride	OH	H	H	Glucoside	H
CYA-2	Cyanidin-3-*O*-rutinoside chloride	OH	H	H	Rutinoside	H
CYA-3	Cyanidin-3,5-di-*O*-glucoside chloride	OH	H	H	Glucoside	Glucose
CYA-4	Cyanidin-3-*O*-sophoroside chloride	OH	H	H	Sophoroside	H
CYA-5	Cyanidin-3-*O*-arabinoside chloride	OH	H	H	Arabinoside	H
CYA-6	Cyanidin-3-*O*-rhamnoside chloride	OH	H	H	Rhamnoside	H
CYA-7	Cyanidin-3-*O*-galactoside chloride	OH	H	H	Galactoside	H
CYA-8	Cyanidin-3-*O*-sambubioside chloride	OH	H	H	Sambubioside	H
CYA-9	Cyanidin-3-*O*-lathyroside chloride	OH	H	H	Lathyroside	H
	Anthocyanidins	R1	R2	R3	R4	R5
	Pelargonidin chloride	H	H	H	H	H
	Cyanidin chloride	OH	H	H	H	H
	Delphinidin chloride	OH	H	OH	H	H
	Petunidin chloride	OCH_3	H	OH	H	H
	Malvidin chloride	OCH_3	H	OCH_3	H	H
	Peonidin chloride	OCH_3	H	H	H	H

cyanidin-3,5-di-*O*-glucoside chloride (CYA-3), cyanidin-3-*O*-sophoroside chloride (CYA-4), cyanidin-3-*O*-arabinoside chloride (CYA-5), cyanidin-3-*O*-rhamnoside chloride (CYA-6), cyanidin-3-*O*-galactoside chloride (CYA-7), cyanidin-3-*O*-sambubioside chloride (CYA-8), cyanidin-3-*O*-lathyroside chloride (CYA-9), cyanidin chloride (CYA), delphinidin chloride (DEL), malvidin chloride (MAL), peonidin chloride (PEO), petunidin chloride (PET), and pelargonidin chloride (PEL). Oligonucleotide primers used in RT-PCR reactions were synthesized by Generi Biotech (Hradec Kralove, Czech Republic). LightCycler 480 Probes Master was from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). All other chemicals were of the highest quality commercially available.

Human hepatocytes

Human hepatocytes were isolated from human livers, obtained from two sources: (i) multiorgan donors LH44 (F, 57 years), LH45 (M, 46 years) and LH46 (M, 37 years); the tissue acquisition protocol was in accordance with the requirements issued by local ethical commission in the Czech Republic; (ii) *Long-term human hepatocytes in monolayer* Batch HEP220670 (F, 64 years) (Biopredic International, Rennes, France). Cells were cultured in a serum-free medium. Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator. Hepatocytes were incubated with the tested compounds, inducers and/or vehicle (DMSO; 0.1% v/v) for 24 h and 48 h. TCDD was not used as a positive control in Hep220670 culture only.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent® (Molecular Research Center, USA). cDNA was synthesized from 1000 ng of total RNA using M-MuLV Reverse Transcriptase (F-572, Finnzymes) at 42 °C for 60 min in the presence of random hexamers (3801, Takara). qRT-PCR was carried out on a LightCycler

Table 2 List of primers with corresponding UPL probes used for PCR

Name of the gene	Primer sequences (F/R)	UPL number
UGT1A1	ATATGGTTTTTGTTGGTGGAATC	8
	GCATTAATGTAGGCTTCAAATTCCT	
UGT1A4	CAAGTCTTGCCTCTGAGCTTTT	138
	ACACGGATGCATAGCTGACA	
UGT1A6	GGCAAAATCCCTCAGACAGT	47
	GTTCGCAAGATTCGATGGTC	
UGT1A9	ACTATCCCAAACCCGTGATG	119
	TCTCCAGAAGCATTAATGTAGGC	
UGT2B7	ACCAAATGTTGATTTTGTTGGA	86
	CACCACAACACCATTTTCTCC	
UGT2B10	TCCTCATCCATTCTTACCAAATG	86
	TCTGTACAAACTCCTCCATTTCC	
GSTA1	ACGGTGACAGCGTTTAACAA	53
	CCGTGCATTGAAGTAGTGGA	
GSTP1	CACTCAAAGCCTCCTGCCTAT	24
	TGCTGGTCCTTCCCATAGAG	
GSTT1	ACGGGGACTTCACCTTGAC	15
	GACCTTATATTTGCGCGTCAG	
GSTO1	CTGCAAACCCCAGAGGAG	60
	GGCAGAACCTCATGCTGTAGA	
GSTZ1	TTTCTGACCTCATCGCTGGT	42
	TCTCCCACTTGCTTCAGGAC	
GAPDH	CTCTGCTCCTCCTGTTCGAC	60
	ACGACCAAATCCGTTGACTC	

480 apparatus (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of all mRNAs were determined using primers and Universal Probes Library (UPL; Roche Diagnostic Corporation, Prague, Czech Republic) probes described in Table 2. The following program was used for monitoring the expression of all genes: an activation step at 95 °C for 10 min was followed by 45 cycles of PCR (denaturation at 95 °C for 10 s; annealing with elongation at 60 °C for 30 s). The measurements were performed in duplicate. Gene expression was normalized *per* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by the delta–delta method. Results are expressed as fold induction over DMSO-treated cells.

Results

Effects of anthocyanidins on the expression of phase II biotransformation genes in human hepatocytes

Three different primary cultures of human hepatocytes (*i.e.* cultures LH44, LH45, HEP220670) were incubated for 24 h with six tested anthocyanidins in the concentration of 50 μM (*i.e.* cyanidin, peonidin, petunidin, pelargonidin, delphinidin, malvidin), with model inducers of drug-metabolizing genes rifampicin (RIF; 10 μM) and dioxin (TCDD; 5 nM) and DMSO (0.1% v/v) as a vehicle for control. These model inducers worked well with phase I drug metabolizing enzymes (CYP3A4 and CYP1A1, respectively).[14,24] However, the effect on members of the UGT family was quite controversial. While TCDD induced UGT1A1 about 1.6 fold over control in two hepatocyte cultures, the induction of UGT1A4/1A6/1A9 was not either present or was not reproducible (Fig. 1A). However, TCDD consistently down-regulated the UGT2B7/2B10 mRNA level in both cultures. In contrast, rifampicin induced all measured UGTs in one culture only, Hep220670. This was probably due to the longer stabilization of this culture before the start of the treatment. With an exception of culture Hep220670, where all anthocyanidins induced UGT2B7/2B10 quite strongly, their presence either decreased mRNA levels or had no effect (Fig. 1A). Regarding the GST genes, TCDD caused a modest decrease and the effect of RIF on mRNA levels followed a similar pattern like in the case of UGT genes, *i.e.* there was an effect in Hep220670 culture only (Fig. 1B). The level of expression after incubation with anthocyanidins was without the effect with an exception of pelargonidin (PLDIN), which caused massive induction of GSTP1 in two cultures (Hep220670, LH44) (Fig. 1B).

Effects of anthocyanins on the expression of phase II biotransformation genes in human hepatocytes

In the next series of experiments, we tested the effect of 21 anthocyanins in three different human hepatocyte cultures (LH44, LH45, LH46). We incubated human hepatocytes for 24 h with tested compounds (50 μM), rifampicin (RIF; 10 μM), dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% v/v). The effect of anthocyanins on the mRNA level of UGTs or GSTs was quite variable (Fig. 2). Some compounds caused relatively strong induction but this was not reproducible in other cultures. Thus, it is logical to state that the variability likely comes from donor-specific properties of the cultures. Probably the most consistent and strongest effect was observed for cyanidin-3-*O*-rutinoside chloride (CYA-2), which down-regulated substantially UGT2B10 and GSTP1/A1 mRNAs with modest or irreproducible effect on other UGT/GST gene expression in all cultures tested (Fig. 2).

Discussion

In the current paper we investigated the effect of 6 anthocyanidins and 21 anthocyanins on the expression of selected phase II biotransformation enzymes in summary 4 cultures of human hepatocytes. In general, the effect of these compounds (Table 1) was in the majority of cases irreproducible and almost any of the compounds demonstrated a consistent effect in all tested cultures.

The main reason for irreproducibility probably comes from inter-individual variability and different quality and viability of the individual cultures with likely different metabolizing properties. Each culture could have been and probably was unique concerning the basal expression and polymorphism of biotransformation enzymes and transporters. These two factors contributed likely to different and often inconsistent pattern seen in cultures. When taking into account the fact that phase II enzymes are usually abundantly expressed in hepatocytes and are only slightly induced, in contrast to cytochrome P450 members, then this finding is not very surprising. However,

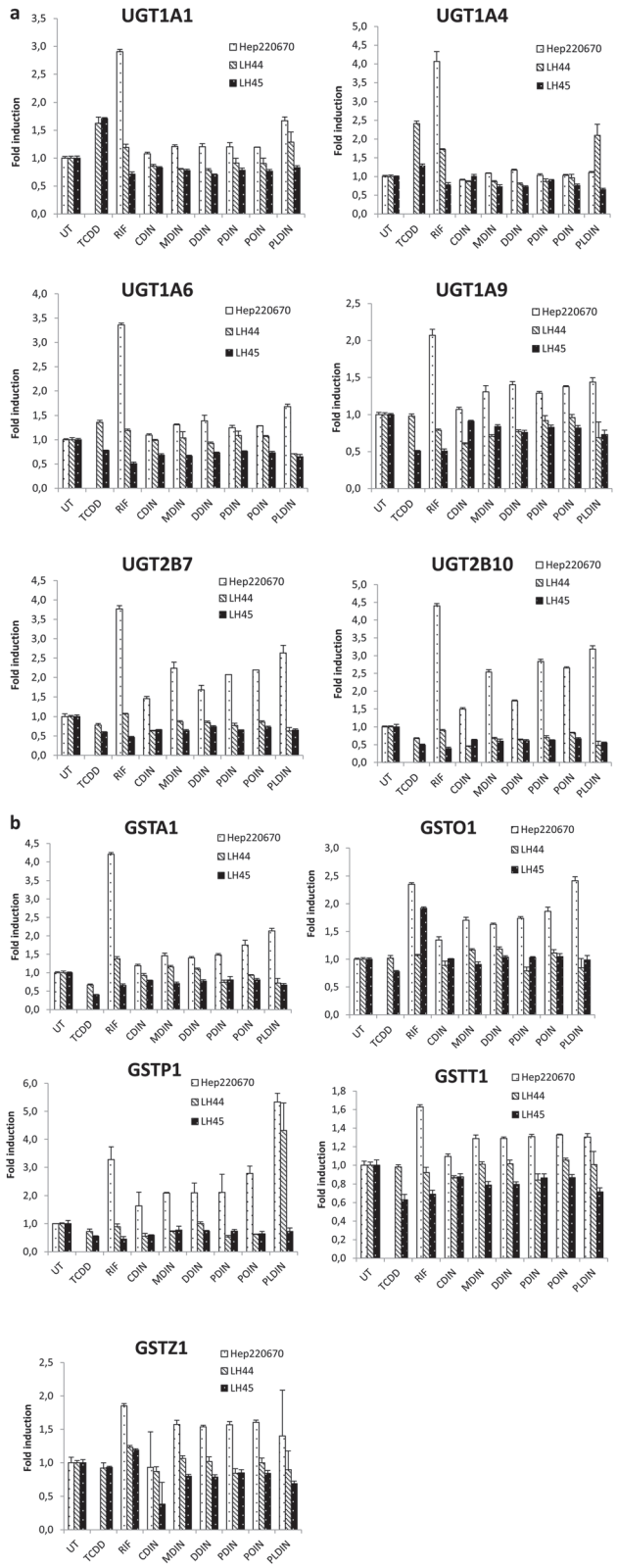


Fig. 1 Effect of anthocyanidins on the mRNA expression of phase II genes in primary cultures of human hepatocytes. Primary human hepatocyte cultures (HEP220670, LH44, LH45) were incubated for 24 h with cyanidin (CDIN, 50 μM), peonidin (POIN, 50 μM), petunidin (PDIN, 50 μM), pelargonidin (PLDIN, 50 μM), delphinidin (DDIN, 50 μM), malvidin (MDIN, 50 μM), rifampicin (10 μM), TCDD (5 nM) and DMSO (0.1% v/v; UT) as a vehicle for control. Bar graphs show RT-PCR analyses of: Panel A: UGT1A1, UGT1A4, UGT1A6, UGT1A9, UGT2B7, and UGT2B10 mRNAs; Panel B: GSTA1, GSTT1, GSTP1, GSTO1, and GSTZ1 mRNAs. The data are the mean from triplicate measurements and are expressed as a fold-induction as compared to DMSO-treated cells. The data were normalized *per* GAPDH mRNA level. Non-represented bars = not determined.

there are two points of this screening study worth of mentioning deeply.

The first point worth of discussion would be the induction of GSTP1 mRNA by pelargonidin (PLDIN) (Fig. 1B). As it was demonstrated recently, PLDIN activates AhR, which leads to induction of CYP1A1.[14] This is not probably very surprising since it was demonstrated that β-naftoflavone (β-NF), an activator of AhR, induces GSTP1 in the rat liver.[25] The controversial thing is why there is no induction by TCDD, the most potent AhR activator known so far. The possible explanation may lie in different regulation of rat *vs.* human GSTP1 (described in ref. 26). Moreover, it was reported that the reporter construct with the promoter region of rat GSTA2 (containing XRE – an element for AhR) was responsive for TCDD and β-NF but when XRE was deleted from the construct, it was not responsive for TCDD but still for β-NF.[27] Nevertheless, the presence of AhR and CYP1A1 enzymatic activity (presumably in order to oxidize the flavonoid) was still needed for the induction of GSTP1. In addition, the induction of GSTP1 in the rat liver is stronger with phenolic antioxidants than with β-NF[25] and thus it is likely that the induction of GSTP1 in human hepatocytes of our study by PLDIN is not even mediated by AhR but instead by NF-E2-related factor 2 (Nrf2), which reacts to the presence of antioxidants.

The second point, the authors would like to highlight, is the effect of cyanidin-3-*O*-rutinoside chloride (CYA-2) on UGT2B10 and GSTP1 mRNAs (Fig. 2). Concerning the GSTP1 expression, it would be interesting if the effect of CYA-2 would be translated into the GSTP1 protein level especially when there is known association of the high expression of this gene in tumor tissues[28–30] and thus there would be a possible cancer preventive role in CYA-2. Nevertheless, this fact can be questioned as well since there were few observations that anthocyanins induce the GSTP1 expression on mRNA and protein levels in extra-hepatic cells[31] but on the other hand they were demonstrated to inhibit some GST activities.[32] In general, their effect is likely complex and they contribute to their protective roles by several different mechanisms.

In general, most of the anthocyanidins or anthocyanins tested in this study had no effect on the expression of major phase II metabolizing enzymes. However, some results of this study may lead to future projects which might confirm (or disprove) the effect of some anthocyanidins or anthocyanins and add new information about their protective properties on the molecular level.

Conflict of interest

The authors declare that they have no conflict of interest.

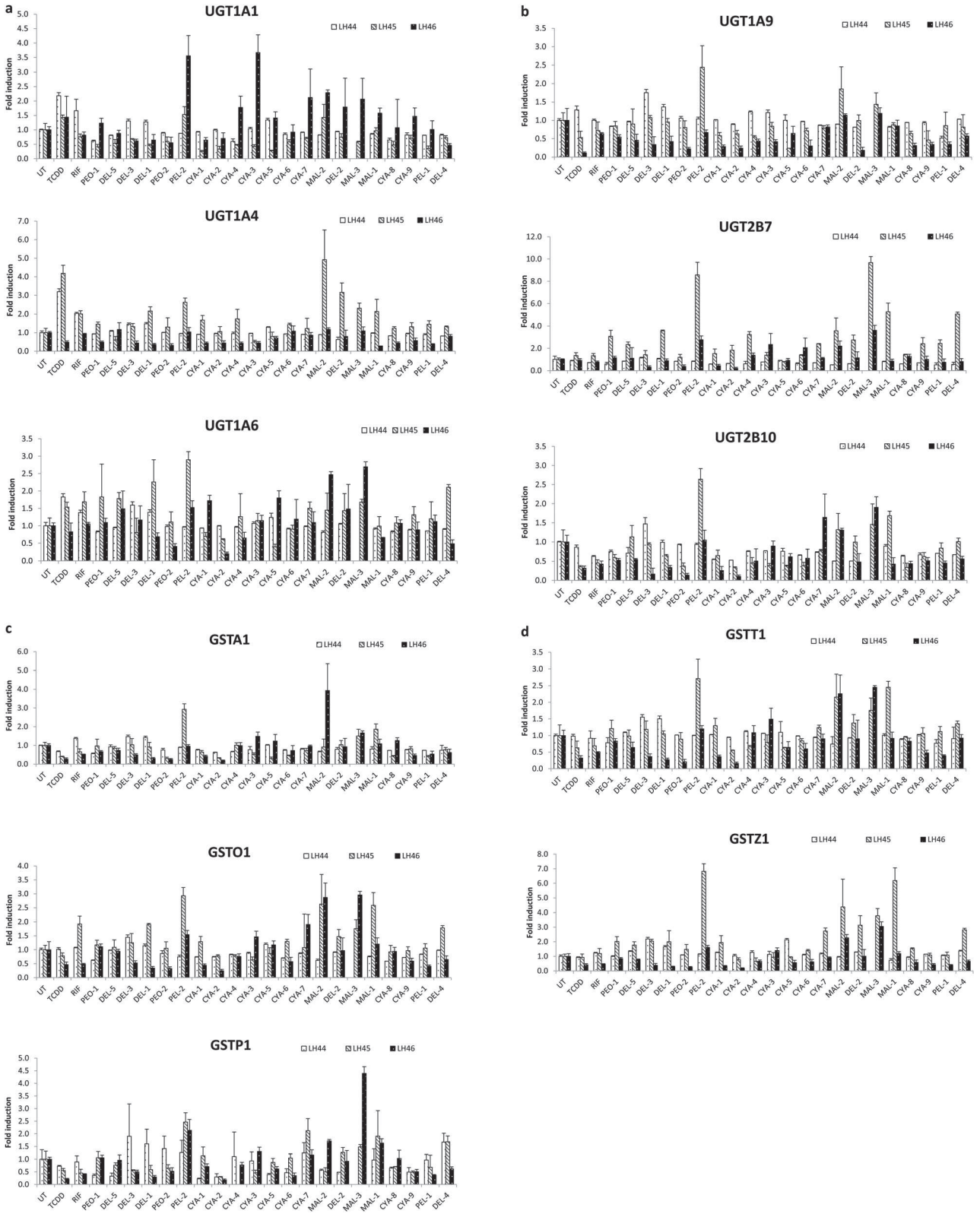


Fig. 2 Effect of anthocyanins on the mRNA expression of phase II genes in primary cultures of human hepatocytes. Primary human hepatocyte cultures (LH44, LH45, LH46) were incubated for 24 h with 21 different anthocyanins (for details see Materials and methods section), each in the concentration of 50 μM, rifampicin (10 μM), TCDD (5 nM) and DMSO (0.1% v/v; UT) as a vehicle for control. Bar graphs show RT-PCR analyses of: Panel A: UGT1A1, UGT1A4, and UGT1A6; Panel B: UGT1A9, UGT2B7, and UGT2B10; Panel C: GSTA1, GSTO1, and GSTP1; Panel D: GSTT1 and GSTZ1. The data are the mean from triplicate measurements and are expressed as a fold-induction as compared to DMSO-treated cells. The data were normalized *per* GAPDH mRNA level. Non-represented bars = not determined.

Acknowledgements

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