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Faculty of Science

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IN VITRO EFFECTS OF FOOD CONSTITUENTS ON THE ARYL HYDROCARBON RECEPTOR-CYP1A1 SIGNALING PATHWAY

Ph.D. THESIS

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I declare that the Ph.D. thesis is based on my own research carried out at the Department of Cell Biology and Genetics, Faculty of Science, Palacky University, Olomouc in the period September 2010 – March 2014. Co-authors agree with the inclusion of published results. All sources cited in this work are listed in the "Reference" section.

Olomouc …… ……

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Abstract

A number of foreign substances that have possible harmful effects on human health, come from food intake. Compounds involved in the diet are either natural or synthetic origin, having the ability to activate the xenoreceptors, resulting in the induction of drug- and xenobiotic-metabolizing enzymes. Induction of various enzymes results in alteration of the drug metabolism (food-drug interactions), the distortion of the intermediary metabolism and activation of pro-carcinogens to carcinogens. This work is focused on the study food constituents' influence on the activity of arylhydrocarbon receptor (AhR) and subsequent cytochrome P-450 1A1 induction. Constituents of soft drinks, artificial sweeteners, anthocyanins and anthocyanidins were studied. The theoretical background summarizes findings about food-drug interactions, AhR receptor, and cytochrome P-450 1A and also describes the studied compounds. In the methodological section, the techniques of RT-PCR, immunodetection and gene reporter assay were used for determination of expression CYP1A1 mRNA, protein, and activation of AhR. The ligand binding assay and enzyme activity measurement were also done. Primary cultures of human hepatocytes, cancer cell lines HepG2 (liver) and LS174T (intestinal) were used as *in vitro* models. Our findings points to food constituents, that have not been studied till now, and their influence on the activity of the AhR receptor, leading to induction of CYP1A1 enzyme involved in drug metabolism as well as in the process of chemically induced carcinogenesis.

Keywords	AhR receptor, cytochrome P-450 1A1, food-drug interaction, food constituents
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Abstrakt

Řada cizorodých látek, se kterými člověk přijde do kontaktu, je přijímána potravou a může mít neblahé účinky na lidské zdraví. Sloučeniny obsažené ve stravě jsou buď přírodního, nebo syntetického charakteru se schopností aktivovat tzv. xenoreceptory, což vede k indukci enzymů metabolizujících cizorodé látky a léčiva. V důsledku indukce jednotlivých enzymů dochází ke změně v metabolismu léčiv (tzv. lékovým interakcím), k narušení intermediárního metabolismu a aktivaci prokarcinogenů na karcinogeny. Předkládaná práce je zaměřena na studium vlivu látek obsažených v potravě na aktivitu arylhydrokarbonového receptoru (AhR) a následnou indukci cytochromu P-450 1A1. Ve studii byly testovány obsahové složky nealkoholických nápojů, umělá sladidla, antokyaniny a antokyanidiny. Teoretická část práce shrnuje poznatky o lékových interakcích, AhR receptoru, cytochromech P-450 1A a popisuje studované sloučeniny. V praktické části byly využity techniky RT-PCR, imunodetekce a reporter gene assay pro stanovení exprese CYP1A1 mRNA, proteinů a aktivaci AhR. Byly také použity metody ligand binding assay a měření enzymové aktivity. Jako *in vitro* modely byly použity primární kultury lidských hepatocytů a nádorových buněčných linií HepG2 (jaterní) a LS174T (střevní). Nově získané poznatky objasňují vliv dosud nestudovaných složek potravy na aktivitu AhR receptoru, vedoucí k indukci enzymu CYP1A1, který se kromě metabolizace léčiv podílí také na procesu chemicky indukované karcinogeneze.

Klíčová slova	AhR receptor, cytochrom P-450 1A1, lékové interakce, složky potravy
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CONTENT

ABBREVIATIONS

3-MC	3-Methylchloranthrene
AhR	Aryl hydrocarbon receptor
AHRR	Aryl hydrocarbon receptor repressor
ARNT	Aryl hydrocarbon receptor nuclear translocator
CAR	Constitutive androstane receptor
CYP 450	Cytochrome P450
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin response element
ER	Estrogen receptor
EROD	Ethoxyresorufin-O-deethylase
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
HAA	Halogenated aromatic amines
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
Hsp90	Heat-shock protein 90 kDa
MW	Mineral waters
PAH	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins
PCDFs	Polychlorinated dibenzo-p-furans
PXR	Pregnane X receptor
RAR	Retinoic acid receptor
RDTs	Ready to drink teas
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SJW	Saint John´s Wort
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
VDR	Vitamin D receptor
XAP2	Hepatitis B-virus X-associated protein 2
XME	Xenobiotic metabolizing enzymes
XRE	Xenobiotic responsive element

1. INTRODUCTION

Human populations are daily exposed to a plethora of xenobiotics such as drugs, environmental pollutants, tobacco smoke, plant alkaloids, agrochemicals, dyes, etc. A large number of xenobiotics are absorbed in food substances (tea, spices, fruit, and vegetables). In addition to naturally occurring constituents, additives such as stabilizers, flavors, aromas, antioxidants, colorants, sweeteners, etc. are found in common foods. Both natural and artificial food ingredients can interfere with the regulatory pathways of drug metabolizing enzymes in humans. This can result in so-called food-drug interactions which is undesirable clinically as the drug pharmacokinetics is affected by another compound, i.e. xenobiotics (Mandlekar et al., 2006). Examples of serious food-drug interactions are acceleration of the metabolism of steroid hormones by hypericin (St. John's wort tea), and due to enzyme induction drugs, which finally leads to failure of oral contraceptives (Mai et al., 2004).

Another aspect of undesirable food ingredients and other substances on the human body is the activation of pro-carcinogens to ultimate carcinogens. Examples of chemically induced carcinogenesis is auto-induction of cytochrome P450 by polycyclic aromatic hydrocarbons (PAHs) contained in cigarette smoke, grilled and smoked meat, which ultimately result in the conversion of PAHs into powerful carcinogens and in colorectal tumors. The molecular mechanisms involved in heavy metal-mediated drug interactions or chemically-induced carcinogenesis include induction (increased de novo synthesis) of drug metabolizing enzymes, especially cytochrome P450 (Gonzalez and Gelboin, 1994). The most prominent and important inducible cytochrome P450 is CYP1A1, CYP1A2, CYP2B6, CYP2C9 and CYP3A4-the enzyme responsible for the metabolism of about 60% of drugs.

Xenobiotic-mediated induction of cytochrome P450 comprises activation of xenoreceptors, which are ligand-activated transcription factors that are responsible for initiation of the synthesis of cytochrome P450. The receptor for polyaromatic hydrocarbons (AhR) is involved in many cellular processes and it mediates response to endogenous signals or xenobiotics (Abel and Haarmann-Stemmann, 2010). When AhR was discovered, it was believed that it regulated discrete cell response to foreign chemicals, i.e. (i) the induction of cytochrome P450 CYP1A1 and CYP1A2, and (ii) the toxicity of dioxin-like compounds (Denison et al., 2002a). In the last two decades, however, it has become increasingly clear that in addition to its role as a receptor for xenobiotics, AhR plays a role in such vital cellular storylines as development, cell differentiation and the immune response (Haarmann-Stemmann et al., 2012). Currently, much is known about the effects of chemicals on the AhR, in particular

environmental pollutants (polychlorinated biphenyls, polyaromatic hydrocarbons, etc.), chemicals and pharmaceuticals (omeprazole, lansoprazole) (Quattrochi and Tukey, 1993). However, the effects of food additives on the AhR have not been systematically studied and the data in the literature are diverse. While the massive development and progress in research on the AhR have led to improvements in the safety/testing of new drugs, research on food safety and food quality has been neglected. Despite many indications that food additives affect the AhR receptor and the consequent metabolism of prescription drugs or result in carcinogenesis, systematic examination of these substances in food on the activity of AhR is result lacking.

2. AIMS

The main goal of the present thesis was to evaluate the effects of food constituents on the AhR-CYP1A1 signaling pathway. Ready to drink teas, mineral waters, anthocyanidins, anthocyanins and artificial sweeteners were selected for this study. The particular goals were evaluation of *in vitro* effects of food constituents on

1. AhR transcriptional activity by the means of gene reporter assays.

2. The expression of CYP1A1 and CYP1A2 mRNAs and proteins by the means of RT-PCR and western blotting, respectively, in primary cultures of human hepatocytes and in human cancer cell lines.

3. Catalytic activity of CYP1A1 and CYP1A2 by measurement of EROD assay.

4. Ligand binding to AhR.

3. THEORETICAL PART

3.1. Food-drug interactions

Food-drug interactions are the consequence of physical, chemical, or physiologic relationship between a drug and product consumed as food or a nutrient present in plant-derived food or dietary supplement (Genser, 2008; Santos and Boullata, 2005). The definition of these interactions is described as alterations of the pharmacokinetics or pharmacodynamics of a drug or nutritional element or a compromise in nutritional status as a result of the addition of a drug. Food-drug interactions occur when the consumption of a particular food influences the activity or expression of drug-metabolizing enzymes, e.g. phase I enzymes (cytochrome P450), phase II enzymes (conjugation) or transporters, resulting in an alteration of the pharmacokinetics of drugs. Oxidative biotransformation catalyzed by cytochrome P450 (CYP) is the most common reaction of the first phase of xenobiotic metabolism. Besides the metabolism of drugs, CYPs are involved in activating carcinogens, teratogens and number of toxic compounds.

Dietary habits are an important determinant of human health and can also influence aspects of disease progression, for instance, activation of a chemical to reactive species can exacerbate ongoing tissue injury in diabetes and liver disease. In these cases, altered CYP function may be a significant component of the overall disease pathogenesis. Knowledge of the mechanisms by which diet influences CYP expression can therefore provide insight into the disease process. An increasing number of drugs are commercially available and a large percentage of the population is on the regular medication. Natural products are usually mixtures of many compounds with putative active ingredients and other constituents that have the potential to cause interactions with various classes of prescription drugs. Taken together, the extent of prescription drug use and variability in individual nutrient status can result in great potential for food-drug interactions.

Drug-metabolizing enzymes and drug transporters alone or in concert with each other, play important roles in modulating drug absorption, distribution, metabolism and elimination. The interplay between drug-metabolizing enzymes and transporters is one of the confounding factors that have been recently shown to contribute to potentially complex drug interactions (Muntane, 2009). These interactions are mediated in two possible ways: (i) inhibition of drug-metabolizing enzymes or by competition for this enzyme; (ii) induction of drug-metabolizing enzymes *via* activation of nuclear receptors (e.g. vitamin D receptor – VDR, farnesoid X receptor – FXR), receptors for steroid

hormones (e.g. glucocorticoid receptor – GR, estrogen receptor – ER) or xenoreceptors (e.g. aryl hydrocarbon receptor - AhR).

Food-drug interactions can result in two main clinical effects: (i) decreased plasma concentration of a drug, predisposing to failure of treatment; (ii) higher plasma level, increasing the risk of adverse events and precipitating possible toxicities (Custodio et al., 2008; Singh, 1999; Singh and Malhotra, 2004). For this reason, identification of food constituents that may cause or contribute to food-drug interactions is. A number of these interactions have been reported. Foods containing complex mixtures of phytochemicals such as in vegetables, fruit, herbs, spices and teas, have the greatest potential to induce or inhibit the activity of drug-metabolizing enzymes (Mandlekar et al., 2006). The consumption of herbal dietary supplements or artificial food additives (e.g. sweeteners, preservatives, taste and flavor enhancers, colorings, stabilizers etc.) can also result in particularly severe interactions.

The number of potential food-drug interactions is almost unlimited and these often occur with the use of diuretics, antibiotics, anticoagulants, antihypertensive agents, thyroid and sodium compounds and alcohol (Bobroff, 1986). Other important drug classes involved in drug-food interactions are antiretroviral drugs (Gentry and Rodvold, 1995) and antidepressants (Jefferson, 1998). Heck et al. (2000) reported that more food–drug interactions have been reported for warfarin than for any other. Herbal medicines, such as St. John's wort, garlic, piperine, ginseng, and gingko, which are easily available, have given rise to serious clinical interactions in co-administration with prescription medicines (Nahrstedt and Butterweck, 2010).

3.1.2 Inhibition of drug metabolizing enzymes

Inhibitory drug interactions are associated with wide substrate specificities of CYPs and may occur by reversible or irreversible mechanisms. Reversible inhibition arises from direct competition between drug and components for binding to the active site of CYPs. In contrast, irreversible inhibition arises from the CYP-dependent conversion of certain foreign compounds or reactive metabolites that do not leave the active site of the enzyme (e.g. suicide substrate). The impact of irreversible inhibition is long–lived impairment of CYP activity and drug elimination (Murray, 2006).

The components of grapefruit juice are well known for their inhibitory effects and the interaction of grapefruit juice with certain drugs was discovered two decades ago (Flanagan, 2005). Since then, there have been numerous reports on the effects of grapefruit and its components on cytochrome P450 drug oxidation and transport (Cuciureanu et al., 2010; Hanley et al., 2011). As a well-known example, consumption of grapefruit juice has been shown to increase the plasma concentration of drugs, like

the calcium channel blocker, felodipine (Bailey et al., 1993; Bailey et al., 1998), the immunosuppressant cyclosporine (Yee et al., 1995), the hypnotic midazolam (Kupferschmidt et al., 1995), the HIV protease inhibitor saquinavir (Kupferschmidt et al., 1998), the HMG-CoA reductase inhibitors simvastatin and atorvastatin (Lilja et al., 1998, 1999) and the antibiotic erythromycin (Amory and Amory, 2005). The mechanism of this interaction is believed to involve both inhibition and inactivation of CYP3A4 in the small intestine, resulting in a significant reduction of drug pre-systemic metabolism. For such a drug interaction are responsible furanocoumarin derivatives contained in grapefruit juice (Guo and Yamazoe, 2004). The furanocoumarin bergamottin is transformed by CYP3A4 to bergamottin epoxide that covalently binds to the enzyme's active core as a 'suicide substrate' (Edwards et al., 1999). Owing to enzyme inactivation, long-term consumption of grapefruit juice may cause a greater magnitude and duration of interactions compared to a single dose. This inhibitory interaction should be kept in mind when using drugs metabolized by CYP3A4 (Fragoso et al., 2011).

Several other polyphenolic compounds are also responsible for inhibition of drug-metabolizing enzymes. Some flavonoids are known to inhibit CYP activity directly such as quercetin, known for its inhibitory effect on CYP1A2 activity (Bacon et al., 2003). Flavone derivatives such as acacetin and diosmetin were found to be quite potent against activities mediated by CYPs 1A1, 1A2 and 1B1. Constituents of St John´s Wort are most likely a complex of polyphenolic agents responsible for inhibiting a number of CYP activities, where I3, II8 – biapigenin was proven to be a potent inhibitor of CYP3A4, whereas hyperforin and hypericin were shown to inhibit CYPs 2D6, 2C9 and 3A4 (Obach, 2000).

Besides inhibiting the catalytic activity of drug-metabolizing enzymes, inhibitory effects of food constituents may occur at the receptor level, i.e. by antagonism or partial agonism of some receptor, known for quercetin, kaempferol, luteolin, resveratrol, curcumin and the synthetic flavonoid 39-methoxy-49-nitroflavone (Abel and Haarmann-Stemmann, 2010).

3.1.3 Induction of drug metabolizing enzymes

Induction occurs when the enzyme activity increases, resulting in increased level of RNA transcription leading to higher protein level compared with normal physiological values. In this situation, the rate of drug metabolism increases and affects the oral bioavailability as well as the systemic distribution. Thus, a disruption of this balance can result in significant changes in blood concentrations of the drugs (Fasinu et al., 2012).

Many inducers of drug-metabolizing enzymes are, at the same time, the substrates for these enzymes. Hence, the reason for the induction of drug-metabolizing enzymes is primarily the need for the accelerated metabolism of the chemicals being exposed to (Oinonen and Lindros, 1998). Certain herbs have been reported to alter the expression of drug-metabolizing enzymes. The most often interactions are known for *Hypericum perforatum* (St. John's Wort, SJW), a popular herbal remedy used for the treatment of depression and mood disorders (Sorensen, 2002). The crude extract is a complex mixture of several active compounds such as hypericin, quercetin, isoquercetin, bioflavonoids, hyperforin, catechins, etc. It was discovered that SJW increases activity of CYP3A4, which is involved in the metabolism of more than 50% of all drugs, and the drug transporter protein P-gp through activation of the pregnane X receptor (PXR) (Moore et al., 2000). Due to the induction of CYP3A4, the clearance of its substrates such as cyclosporin, tacrolimus, statins and hormonal contraceptives is accelerated, which eventually leads to ineffective drug treatment. Hyperforin, the constituent of SJW, has been identified as primarily responsible for the interaction with cyclosporine (Mai et al., 2004). In transplant recipients, SJW has been linked to ineffective treatment with calcineurin inhibitors and ensuing organ rejections (Ruschitzka et al., 2000). SJW has also been reported to reduce the anticoagulant effects of warfarin (Yue et al., 2000). One clinical study that examined the metabolism of omeprazole has established that in addition to the induction of CYP3A4, SJW enormously decreases the plasma concentration of the drug through the induction of CYP2C19, which is responsible for the hydroxylation of the drug (Wang et al., 2004).

Ginkgo biloba is another popular herbal medicine used as a remedy for memory loss and dementia, including Alzheimer´s disease (Weinmann et al., 2010). Many studies investigating the influence of Ginkgo biloba on drug metabolism have been recently reported: induction of CYP2C19 dependent omeprazole metabolism (Yin et al., 2004), induction of CYP3A4 metabolism as assessed by decrease in midazolam concentration (Robertson et al., 2008) and metabolic activity of CYP1A2 was reported to be increased in the case of increased clearance of theophylline (Tang et al., 2007).

Garlic is very popular herbal remedy and it is commonly used as a food or a spice. Garlic has also been reported to induce CYP3A4 in a garlic-saquanavir interaction study where a significant decrease in the HIV protease inhibitor saquanavir was reported (Piscitelli et al., 2002).

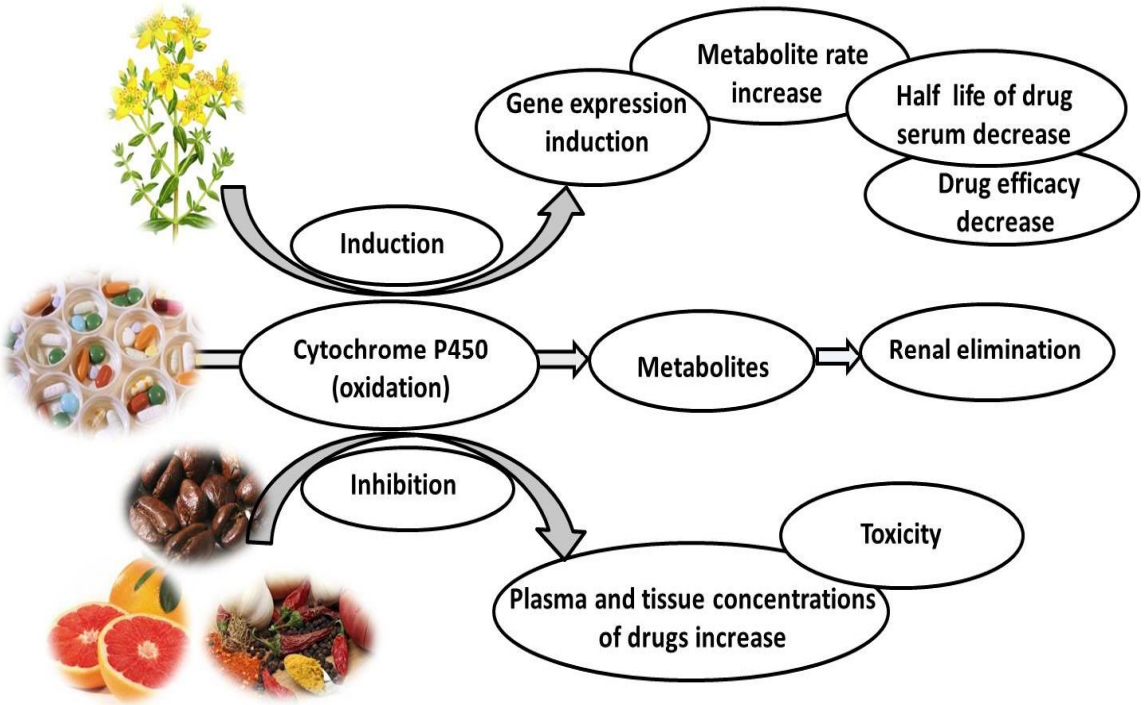

Figure 1. Food-drug interactions and their consequences for drug metabolism (Mukherjee et al., 2011).

3.2 The aryl hydrocarbon receptor – CYP1A pathway

3.2.1 The aryl hydrocarbon receptor

The aryl hydrocarbon receptor is a ligand-activated transcription factor belonging to the growing family of basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) proteins (Figure 2). Members of the bHLH-PAS family play key roles in development, neurogenesis, tracheal and salivary duct formation, adaptation to hypoxia, control of circadian rhythms, hormone receptor function and in the metabolism of xenobiotic compounds (Gu et al., 2000). The AhR was initially identified as a receptor for dioxin-like compounds (e.g. 2,3,7,8-tetrachlordibenzo-p-dioxin; TCDD) and polyaromatic hydrocarbons (PAHs), which are environmental pollutants generated by factories and waste-burning incinerators. These toxic compounds are linked to body weight loss, cancer promotion, immunosuppression, and birth defects (Denison et al., 2002b). There are also many endogenous ligands for AhR, such as indigoids, the metabolites of arachidonic acid, heme, tryptofan and dietary compounds like indole-3-carbinol derivatives and natural flavonoids (Nguyen and Bradfield, 2008).

In the un-liganded state, the AhR resides in a cytosol associated with a complex of the molecular chaperone heat shock protein 90 (hsp90), the immunophilin XAP2 and the cochaperone p23 (Petrulis and Perdew, 2002). When a ligand binds to the receptor, the complex is translocated into the nucleus. After translocation, the complex dissociates and the AhR heterodimerizes with Arnt (AhR nuclear translocator), serving as a common dimerization partner of bHLH-PAS proteins (Swanson et al., 1995). This heterodimer binds to specific xenobiotic or dioxin response elements (XREs, DREs) with a core sequence of 5'-TNGCGTG-3', located in target gene promoters with subsequent modulation of gene transcription (Fujisawa-Sehara et al., 1987). The most characterized target genes play an essential role in xenobiotic metabolism; e.g. CYP1A1, CYP1A2, CYP1B1, glutathione-S-transferase, NAD(P)H: quinone oxido-reductase 1 and UDP-glucuronosyl transferase 1 (Nebert, 2000).

The termination of AhR dependent gene transcription occurs when the AhR-Arnt complex dissociates from XRE, followed by nuclear export into cytosol mediated by its N-terminal nuclear export sequence, resulting in ubiquitin – mediated AhR proteosomal degradation (Pollenz, 2002). The activity of the AhR-Arnt complex is also attenuated by the up-regulation of a transcriptional repressor known as the aryl hydrocarbon receptor repressor (AhRR). A bHLH-PAS protein with high sequence similarity to the AhR, the AhRR, represses the transcriptional activity of AhR by competing with Arnt and binding with DRE sequence, and is thus designated the AhRR or AhR repressor. This attenuation of AhR activity by means of negative feedback loop and receptor degradation may serve to protect the organism from the consequences of

transcriptional hyperstimulation by potent agonists and to provide precise temporal control of this pathway (Mimura et al., 1999).

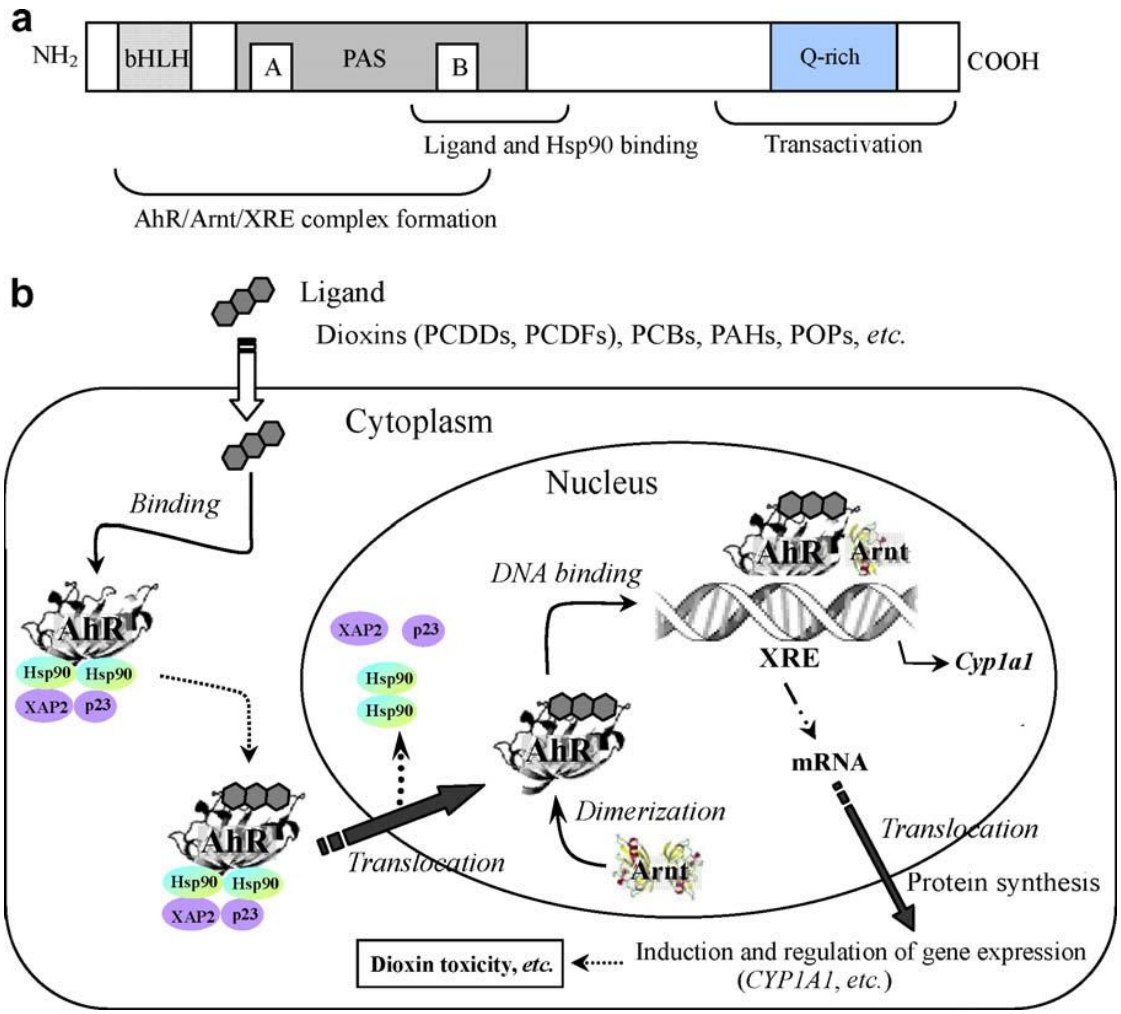

Figure 2. Structure and mechanistic model of AhR. (a) Domain structure of the aryl hydrocarbon receptor (AhR). bHLH, basic helix–loop–helix; PAS, Per-Arnt-Sim domain; Q-rich, glutamine rich region; Arnt, AhR nuclear translocator protein; XRE, xenobiotic-response element. The PAS domain contains two structural repeats (PAS A and PAS B). (b) Mechanistic model of the AhR signaling pathway (Amakura et al., 2008).

The role of the AhR in the human organism is ambiguous, resembling the Dr. Jekyll and Mr. Hyde story. On the one hand, AhR is essential for many physiological processes (vide infra) but on the other hand, AhR mediates the toxicity of many exogenous compounds including TCDD. The toxicity of TCDD has been studied for more than 35 years. It is an unplanned by-product of various industrial, combustion and natural processes and is detectable in air, water and soil, worldwide. This explains why the human population is exposed to dioxins daily, especially through food consumption (Huwe, 2002). TCDD exposure leads to a wide range of effects at very low concentrations. These include reproductive and developmental defects as well as tumor promotion and immune suppression. Recent data suggest that over 300 genes are potentially altered, directly or indirectly, by receptor activation, accounting for the spectrum of biological and physiological processes influenced by dioxin exposure (Puga et al., 2000). TCDD (and thereby AhR) affects multiple physiological functions.

Besides dioxin, there are many synthetic chemicals with high affinity for AhR, including polychlorinated biphenyls (PCBs), dibenzo-p-furans (PCDFs), dibenzo-p-dioxins (PCDDs) and polyaromatic hydrocarbons such as 3-methylcholantrene (3MC) (Denison and Nagy, 2003) and 7,12-dimethyl-benzanthracene, pesticides thiabendazole and carbaryl (Delescluse et al., 2001). There are also drugs that activate AhR, such as the benzimidazole proton pump inhibitors, omeprazole and lansoprazole (Quattrochi and Tukey, 1993), the non-steroidal anti-inflammatory drugs sulindac (Ciolino et al., 2006) and diclofenac (Bass et al., 2009).

Other exogenous ligands for AhR are compounds of natural origin that are synthesized by microbes, plants and animals. These natural compounds have been shown to be less toxic but still able to elicit responses through the AhR pathway (Figure 3). This large group of ligands comprises flavonoids (flavonols, flavones, isoflavones, catechins, anthocyanidins) and related polyphenols (stilbenes), alkaloids and indole derivates (Table 1). Some of these compounds inhibit TCDD-stimulated AhR signaling and therefore they act as chemopreventive agents. Well-documented AhR antagonists are quercetin, kaempferol, luteolin, resveratrol, curcumin and the synthetic flavonoid 39-methoxy-49-nitroflavone (Abel and Haarmann-Stemmann, 2010).

Table 1. Natural ligands of AhR and their sources

Compound	Source	References
Flavonols		
Quercetin	Onions, apples, back tea, red wine	
Kaempferol	Broccoli, cabbage, beans, tomato, strawberries , grapes	Ciolino et al. (1999)
Galangin	Propolis	Ciolino and Yeh (1999)
Flavones		
Diosmin	Citrus fruit	Ciolino et al. (1998c)
Tangeretin	Citrus peel	Hamada et al. (2006)
Isoflavones		
Genistein	Soybeans, coffee	Helsby et al. (1997)
Daidzein	Soybeans	Roberts et al. (2004)
Catechins		
Epigallocatechin galate	Green tea, white tea	Williams et al. (2000)
Anthocyanidins		
Pelargonidin	Berries, plums, pomegranates	Kamenickova et al. (2013)
Stilbenes		
Resveratrol	Red grapes skin	Ciolino et al. (1998b)
Alkaloids		
Berberin	*Berberis aristata, Berberis vulgaris, Hydrastis Canadensis*	Vrzal et al. (2005)
Harman	*Tribulus terrestris*, coffee, vinegar, cheese, soy sauce	El Gendy and El-Kadi (2010)
Indole derivates		
Indole-3-carbinol	Broccoli, brussels sprouts, cabbage, cauliflower	Ociepa-Zawal et al. (2007)
Others		
Curcumin	*Curcurma longa*	Ciolino et al. (1998a)
Carotenoids	Carrots, pumpkins, peppers	Yang et al. (2005)

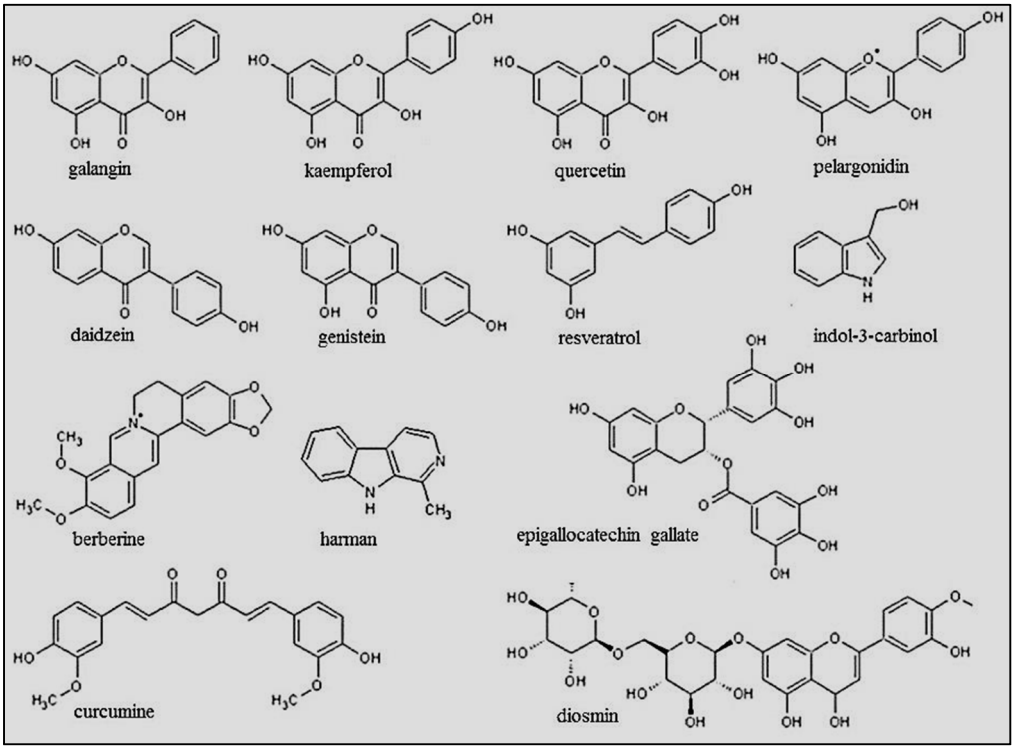

Figure 3. Structure of AhR natural ligands.

3.2.2 Cytochrome P450 enzymes

Cytochrome P450 (CYPs) enzymes belong to a superfamily of hemoproteins and play an important role in xenobiotic and drug metabolism. These enzymes catalyze the oxidative biotransformation of various exogenous substances (drugs, environmental pollutants, food additives etc.) and endogenous substrates (fatty acids, eicosanoids, prostaglandins, retinoids) (Nebert and Russell, 2002). They are also involved in the activation of chemical carcinogens and in the detoxification of numerous xenobiotics (Gonzalez and Gelboin, 1994). In addition, some CYPs are constituents of physiological processes, such as steroidogenesis and the maintenance of bile acid and cholesterol (Pikuleva, 2006). Given all these roles of CYPs together, the molecular mechanisms of their gene regulation have been intensively studied.

In humans, 57 genes and 58 pseudogenes are encoded by various gene clusters distributed over autosomal chromosomes and they are divided among 18 families and 43 subfamilies of CYPs genes. Only fifteen enzymes belonging to the families CYP1, CYP2, CYP3 and CYP4 are primarily responsible for xenobiotic metabolism (Seliskar and Rozman, 2007). The nomenclature of CYPs is based on homology in amino acid sequences. Genes encoding CYP enzymes, and the enzymes themselves, are designated with the abbreviation CYP, followed by a number indicating the gene family, a capital letter indicating the subfamily, and another numeral for the individual gene.

The regulation of xenobiotic metabolizing enzymes is under the control of ligand-activated nuclear receptors. The general scheme for this regulation involves the receptor-ligand interaction with subsequent changes in its conformation leading to binding to cognate sequence in DNA where the transcription of target gene occurs. The receptors responsible for transcriptional regulation of xenobiotic metabolizing enzymes (XME) pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR), retinoid X receptor (RXR), glucocorticoid receptor (GR), retinoic acid receptor (RAR) and vitamin D receptor (VDR) (Honkakoski and Negishi, 2000; Pascussi et al., 2003).

XME is expressed in the liver in particular but extrahepatic tissues contribute to overall xenobiotic biotransformation as well. Within the cell, CYPs are localized in the smooth endoplasmic reticulum and inner mitochondrial membrane.

3.2.3 Cytochromes P450 CYP1A1 and CYP1A2

Cytochrome P450 CYP1A1 is a member of the human CYP1 family and is located in extrahepatic tissues, such as the epithelia of the lung, skin, and gastrointestinal tract. As mentioned above, the expression of CYP1A1 is under transcriptional control of the AhR receptor. The induction of CYP1A1 is greatly enhanced after exposure to chemical inducers such as halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons and heterocyclic aromatic amines (HAA) (Gonzalez, 1988). CYP1A1 catalyses the formation of highly reactive carcinogenic metabolites through hydroxylation at a vacant position on the aromatic ring. These reactive metabolites (electrophilic reactive species such as arene oxide, diolepoxide) form DNA and protein adducts that play a role in mutagenesis, and ultimately in tumor formation and toxicity (Wogan et al., 2004). These compounds are contained in tobacco smoke, automobile exhaust, smoked and cooked food and pollutants from industrial processes. Thus, regular exposure to such substances increases the risk of cancer. CYP1A1 also catalyzes the metabolism of endogenous substrates such as the inflammatory intermediates (arachidonic acid, eicosapentoic acid) and the hormones (17β-estradiol, melatonin) (Rifkind, 2006).

CYP1A2 is a mainly hepatic, constitutively expressed enzyme and it is also induced by aromatic hydrocarbons (Goldstein et al., 1982). CYP1A2 metabolizes a variety of clinically important drugs such as caffeine, clozapine, phenacetine, theophylline, and aminopyrine (Zhou et al., 2009). CYP1A2 is involved in the metabolism of a number of natural compounds like estagole and aristocholic acid, potentially resulting in toxic metabolites. Several endogenous substrates are metabolized by CYP1A2, such as melatonin, bilirubin, arachidonic acid, uroporphytinogen, estrone and estradiol (Rendic, 2002). Together with CYP1A1, CYP1A2 also plays an important role in the bioactivation of carcinogenic polycyclic hydrocarbons. CYP1A2 is induced through AhR-mediated transactivation but compared to CYP1A1, the extent of its induction is generally lower (Nebert and Russell, 2002).

3.3 Studied compounds

3.3.1 Polyphenols

Polyphenols (in Greek *polus* means many, much and phenol refers to the chemical structure) belong to the group of chemical compounds contained in plants and characterized by the presence of multiple phenol structural units. These molecules are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. The exact definition of polyphenols was provided by scientists White, Bates, Smith, Swain and Hanslam (WBSSH definition). Polyphenols are described as water-soluble plant phenolic compounds with a molecular weight of 500-400 Da, possessing a 12-16 phenolic hydroxyl group on 5-7 aromatic rings per 1000 Da. These compounds are predicted to undergo the usual phenolic reaction and have the ability to precipitate some alkaloids, gelatin and other proteins from solution (Haslam and Cai, 1994).

Currently, polyphenols are intensively studied because of their antioxidant properties as well as their putative role in the prevention of various diseases. Furthermore, polyphenols have also been investigated for their ability to modulate the activity of a wide range of enzymes and cell receptors (Middleton et al., 2000). Polyphenols are extensively metabolized by intestinal and hepatic enzymes and by the intestinal microflora. Thus, knowledge of their bioavailability and metabolism is necessary for the evaluation of their biological activity within target tissue (Manach et al., 2005). The main classes of polyphenols are phenolic acids, flavonoids and the less common stilbenes and lignans. The pattern and number of the phenol structures defines the chemical, biological and physical properties of particular members of each class.

Two main classes of phenolic acids are characterised: derivatives of benzoic acid and derivatives of cinnamic acid. The most frequent derivatives of cinnamic acid are caffeic acid and ferulic acid. The important sources of these compounds are wheat bran, many fruits, vegetables and coffee. Gallic acid is derivative of benzoic acid and it is contained in tea (Tomas-Barberan et al., 2001).

Flavonoids are divided into six subclasses according to different types of heterocyclic structure and functions. The members of these subclasses are flavonols, flavones, isoflavones, flavonones, anthocyanidins and flavanols (catechins and proanthocyanins). Flavonoids are most abundant polyphenols in our diet, found in many fruits, vegetables and beverages (Scalbert and Williamson, 2000).

Another class of polyphenols are the stilbens, the most important member of which is resveratrol. This polyphenol is known for its antioxidant and anti-inflammatory properties and also as a chemo-preventive agent. Resveratrol has been detected in

more than 70 plant species including grapes, peanuts and berries (Baliga and Katiyar, 2006).

Lignans, the last group of polyphenols are recognized as phytoestrogens due to their estrogen agonist and antagonist properties, suggesting a potential for nutritional significance in the prevention of cardiovascular and other chronic diseases. The highest concentrations of lignans are found in flax and sesame seeds (Peterson et al., 2010).

3.3.1.1 Anthocyanidins

Anthocyanidins, belonging to the flavonoids group of phytochemicals, are naturally occurring compounds that are responsible for the typical color (bluish-red, orange-red, orange) of many fruits and vegetables. The most common anthocyanidins in higher plants are pelargonidin (12%), peonidin (12%), cyanidin (50%), malvidin (7%), petunidin (7%) and delphinidin (12%) (Kong et al., 2003). The characteristic structure of anthocyanidins is formed of an aromatic ring that is bonded to a heterocyclic ring containing oxygen bound by a carbon-carbon bond to a third aromatic ring (Castaneda-Ovando et al., 2009) (Figure 4, Table 2). Anthocyanidins are commonly found in the form of glycosides - anthocyanins by attaching to different sugars (xylose, arabinose, glucose, galactose, rhamanose) (Welch et al., 2008). Anthocyanins are present in a large amount in the nature, amounting to several hundred in total.

The biological activities of the anthocyanins and anthocyanidins have been studied for a long time. Several studies have revealed antioxidant, antitumour and antimutagenic properties *in vivo* (Kong et al., 2003). The aglycones generated from the most abundant anthocyanidins inhibited the growth of human stomach, colon, lung, breast and CNS cancer cells (Zhang et al., 2005). Anthocyanidins are also intermediate metabolites after anthocyanidin ingestion where the hydrolytic enzymes form anthocyanidins from anthocyanidin´s glycosides (Tsuda, 2012).

Considering the beneficial and healing effect of these compounds, their content in food and beverages is of value. Since flavonoids are frequently used in the form of dietary supplements, caution should be used because of their ability to interfere with xenobiotics in the organism. Several studies have shown that some flavonoids at higher concentrations act as AhR agonists leading to increasing transcription of CYP1A1 gene (Hodek et al., 2002). However, no anthocyanidin compound has been tested so far. There is evidence that co-administration of flavonoids and clinically used prescription drugs leads to flavonoid-drug interactions with altered pharmacokinetics and altered therapeutic effect of the drug, such as zoxaloamine and diclofenac (Tang

and Stearns, 2001). For this reason, the motivation of this study was flavonoid-drug interactions with respect to the AhR-CYP1A1 pathway.

3.3.1.2 Anthocyanins

Anthocyanins, another sub-group of flavonoids, are the plant pigments responsible for the red, blue and purple colors of berries, grapes, apples, purple cabbage and corn. They are water-soluble compounds occurring primarily as glycosidic polyhydroxyl and polymethoxyl derivatives of flavylium salts (Winkel-Shirley, 2001). Anthocyanins are attached with one or more sugars such as glucose, galactose, arabinose, xylose, rhamnose or glucoronic acid (Welch et al., 2008). They differ in the number of hydroxyl and methoxyl groups, in the position of attachment of sugars and in the number of aliphatic or aromatic acids attached to sugars in the molecule (Figure 4, Table 2). Thus, the chemical structures of anthocyanins vary significantly depending on the extent of glycosylation and acylation.

Anthocyanins possess a relative stabile conformation in the form of flavylium ion under highly acidic conditions at pH < 3, exhibiting a red color, but under weakly acidic or neutral pH condition anthocyanins become unstable and are converted into a colorless form (Cooke et al., 2005). Some forms exist at neutral pH and are not converted into the flavilyum form as a result of potential binding to other components in the plasma or urine leading to putative chemical reaction. In most studies, the unchanged glycosides were the only metabolites identified for anthocyanins, but in recent studies glucoronides and sulfates of anthocyanins were also identified as their metabolites (Manach et al., 2004).

Epidemiologic studies suggest that anthocyan-containing foodstuffs possess anti-oxidant, anti-obesity and anti-inflammatory effects and that they reduce the risk of diabetes, cardiovascular disease, arthritis and cancer (Prior and Wu, 2006). Considering the beneficial health effect of these compounds, their content in food and beverages is of value.

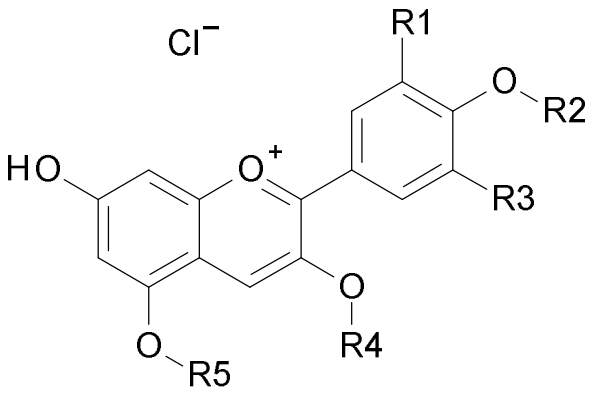

Figure 4. Structure of anthocyanins and anthocyanidins.

Table 2. Anthocyanins and anthocyanidins used in the study

Anthocyanin	R1	R2	R3	R4	R5
pelargonidin-3,5-di-O-glucoside chloride	H	H	H	glucoside	glucose
pelargonidin-3-O-rutinoside chloride	H	H	H	rutinoside	H
delphinidin-3-O-glucoside chloride	OH	H	OH	glucoside	H
delphinidin-3-O-rutinoside chloride	OH	H	OH	rutinoside	H
delphinidin-3,5-di-O-glucoside chloride	OH	H	OH	glucoside	glucose
delphinidin-3-O-sambubioside chloride	OH	H	OH	sambubioside	H
delphinidin-3-O-rhamnoside chloride	OH	H	OH	rhamnoside	H
malvidin-3-O-glucoside chloride	OCH_3	H	OCH_3	glucoside	H
malvidin-3,5-di-O-glucoside chloride	OCH_3	H	OCH_3	glucoside	glucose
malvidin-3-O-galactoside chloride	OCH_3	H	OCH_3	galactoside	H
cyanidin-3-O-glucoside chloride	OH	H	H	glucoside	H
cyanidin-3-O-rutinoside chloride	OH	H	H	rutinoside	H
cyanidin-3,5-di-O-glucoside chloride	OH	H	H	glucoside	glucose
cyanidin-3-O-sophoroside chloride	OH	H	H	sophoroside	H
cyanidin-3-O-arabinoside chloride	OH	H	H	arabinoside	H
cyanidin-3-O-rhamnoside chloride	OH	H	H	rhamnoside	H
cyanidin-3-O-galactoside chloride	OH	H	H	galactoside	H
cyanidin-3-O-sambubioside chloride	OH	H	H	sambubioside	H
cyanidin-3-O-lathyroside chloride	OH	H	H	lathyroside	H
Anthocyanidin	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

3.3.2 Artificial Sweeteners

Artificial sweeteners are non-caloric substances that are hundreds of times sweeter than sugar compounds. The main five artificial sweeteners are regulated as direct food additives by different international authorities; they comprise aspartame, acesulfame K, cyclamate, saccharin and sucralose (Figure 5). The main reason for the use of artificial sweeteners is the reduction of sugar consumption and replacement of sugar for diabetic and obese patients. In the current food supply, artificial sweeteners are widely used in thousands of products including diet soft drinks, yogurts, desserts and gum (Gardner, 2014).

The first artificial sweetener was saccharin that was synthetized in 1879 and it became widespread during World Wars I and II because of sugar shortage and low production costs. After World War II, saccharin has shifted from low cost product category to low calorie product, which was important for diabetics. However, saccharin was also known for its bitter aftertaste. Several safety studies on rodents and monkeys found that saccharin increases the risk for bladder cancer (Cohen et al., 1998; Fukushima et al., 1983; Taylor et al., 1980; Whysner and Williams, 1996). A few epidemiological studies also found some association between saccharin and bladder cancer in humans (Andreatta et al., 2008; Armstrong and Doll, 1975; Howe et al., 1977), nevertheless, most epidemiological studies did not support the association between saccharin and cancer in humans because the mechanism of its carcinogenicity was shown to be specific to rats (Bandyopadhyay et al., 2008; Hoover and Strasser, 1980; Sturgeon et al., 1994; Wynder and Stellman, 1980). According to these studies, the U.S. Environmental Protection Agency (EPA) removed saccharin and its salts from the agency's list of hazardous substances in December 2010. Saccharin is no longer considered a potential hazard to human health. In the European Union, saccharin is known under the code E954.

Sodium cyclamate was the second artificial sweetener invented in 1950, providing a better taste than saccharin. Some unpleasant aftertaste was found as well, but not as bad as saccharin. Cyclamate is 30-50 times sweeter than sugar and it is often used in a mixture with other sweeteners, in particular with saccharin. Cyclamate undergoes conversion by the intestinal bacteria to cyclohexylamine, a compound reported to have chronic toxicity in animals (Renwick, 1986). A study in 1969 found that a mixture of cyclamate and saccharin caused cancer in laboratory rats (Price et al., 1970). Other animal studies revealed that large amounts of cyclamate can cause liver damage, bladder cancer, birth mutations and defects (Takayama et al., 2008; Weihrauch and Diehl, 2004). Cyclamate has been banned in the USA since 1969, but it is approved in over 100 countries, including all the states of the EU.

Aspartame is another artificial sweetener discovered in 1965. Aspartame is about 200 times sweeter than sucrose, consisting of two amino acid, phenylalanine and aspartame, linked to a methanol backbone. Aspartame was approved in 1981 by the U.S. Food and Drug Administration (FDA) and it is marketed under the trade name 'Nutra-Sweet'. The safety of aspartame has been the subject of several political and medical controversies, but the overall medical opinion is that aspartame is a safe non-nutritive sweetener at current levels of consumption. However, people with the metabolic disease phenylketonuria must avoid consumption of aspartame because of its breakdown product phenylalanine. Aspartame is used in soft drinks and other low-calorie and sugar-free food throughout the world and it is also referred to as E951.

The artificial sweetener acesulfame K (acesulfame potassium) is 200 times sweeter than sugar and it has been used in numerous foods since 1988. This sweetener is usually used in combination with other low-calorie sweeteners because it enhances the sweet taste in food and beverages. The safety of acesulfame K has been also controversial, but the United States Food and Drug Administration and equivalent authorities in European Union have approved its general use. In the European Union it is known by the code E950.

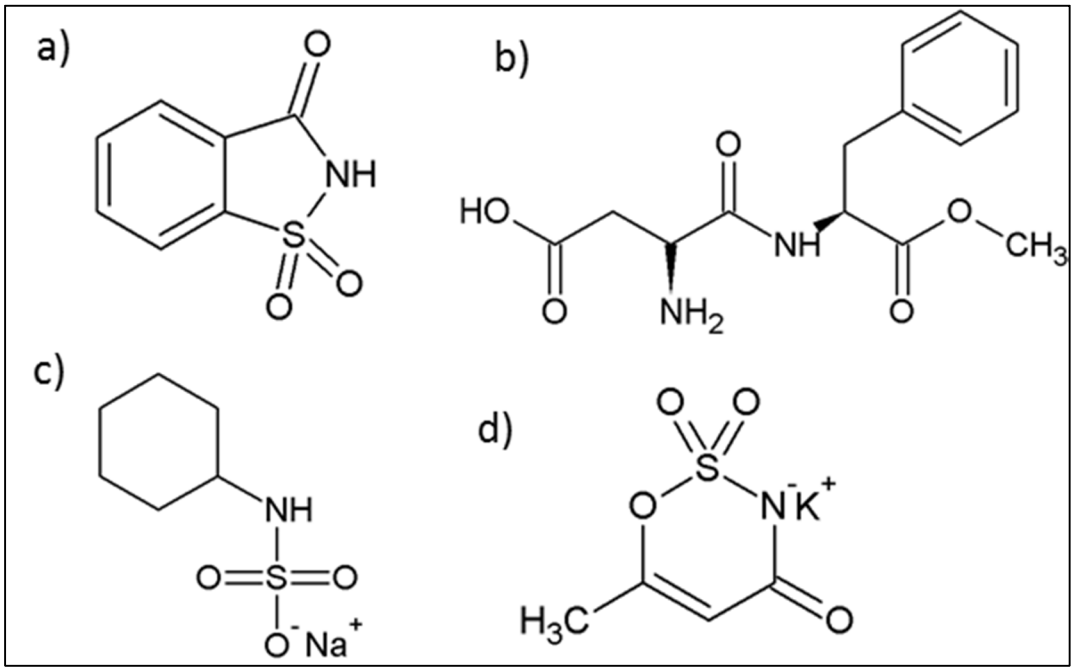

Figure 5. Structures of artificial sweeteners. a) saccharin, b) aspartame, c) cyclamate sodium, d) acesuflam K.

3.3.3 Ready to drink teas and flavored mineral waters

Tea is one of the oldest beverages in the world. Today, ready to drink teas (RDTs), also known as iced teas are very popular non-alcoholic beverages. Iced tea is made from green, black, white or red tea and it is mixed with different flavored syrups like lemon, peach, apple, apricot, pear, mango etc. RDTs can be produced either from an extract of tea or from freshly brewed tea, in the both cases, the leaves of the plant *Camelia Sinesis* are used, the same leaves from which regular teas are made. The manufacturing process starts with the brewing of dried tea leaves with hot water. The tea infusion is then filtered, chilled followed by differential centrifugation to remove leaf residues and by the concentration of the tea. The obtained extract is dissolved in water and other ingredients, such as sugar and/or non-nutritive sweeteners, flavorings, juices, acidity regulators and antioxidants are added. RDTs do not contain fat, protein, fiber, but different levels of sugars and calories are added according to the type of RDT. Iced teas also contain compounds typically found in brewed leaf tea products such as flavonoids, caffeine and theanine. Since RDTs contain many natural and synthetic xenobiotic, these beverages have the potential for interaction with drug-metabolizing enzymes.

Flavored mineral waters (MWs) are another popular non-alcoholic beverage. The consumption in the Czech Republic is very high and the trend is stagnant. These flavored mineral waters contain water, carbon dioxide, stabilizers, non-nutritive sweeteners, synthetic aromas etc. A wide range of different extracts are added, such as fruit extracts (lemon, grapefruit, orange, pear, mango, etc.) and herb extracts (saliva, mellisa, chestnut, linden, wild-thyme, dog-rose, etc.). There are many companies producing mineral waters and the content of each differs. Flavored mineral waters contain a large number of artificial and natural compounds, there should be awareness of their potential harmful side-effects on human health.

4. MATERIALS AND METHODS

4.1 Biological material

4.1.1 Human cancer cell lines

Human Caucasian colon adenocarcinoma cells LS174T (ECAC No. 87060401) and human Caucasian hepatocellular carcinoma cells HepG2 (ECAC No. 85011430) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 µg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acid, and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

4.1.2 Human hepatocytes

Human hepatocytes were obtained from two sources: (i) Hepatocytes were prepared from liver tissue, resected from multiorgan donors. The tissue acquisition protocol was in accordance with requirements issued by the local ethics commission of the Czech Republic. Human liver tissue used in the study was obtained from nine multiorgan donors: LH36 (male, 74 years), LH37 (male, 28 years), LH38 (male, 58 years), LH44 (female, 57 years), LH45 (male, 46 years), LH46 (male, 37 years), LH47 (male, 47 years), LH49 (male, 38 years), LH50 (female, 55 years). Culture medium was enriched for plating with 2% fetal calf serum (v/v). The medium was exchanged for serum-free medium the day after the culture was allowed to stabilize for an additional 24 h. (ii) Long-term human hepatocytes in monolayer batch were purchased from Biopredic International, France: HEP220633 (male, 73 years), HEP220670 (female, 64 years) and HEP220708 (female, 64 years). Hepatocytes were treated for 24 h or 48 h with tested compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5 nM) and vehicle (DMSO, ethanol; 0,1% V/V). Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator.

4.2 Compounds and reagents

Dimethylsulfoxide (DMSO), resveratrol, hygromycin B, aspartame, acesulfane, cyclamate, saccharin, Dulbecco´s modified Eagle´s medium, fetal bovine serum, streptomycin, penicillin, L-glutamine, sodium pyruvate, non-essential amino acids were purchased from Sigma-Aldrich (Czech Republic); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (USA). The anthocyanins, peonidin-3-O-glucoside chloride (PEO-1; ref.#0929S; purity ≥95%), peonidin-3-O-rutinoside chloride (PEO-2; ref.#0945S; purity ≥95%), pelargonidin-3,5-di-O-glucoside chloride (PEL-1; syn. pelargonin; ref.#0903S; purity ≥90%), pelargonidin-3-O-rutinoside chloride (PEL-2;

ref.#0943S; purity ≥90%), delphinidin-3-O-glucoside chloride (DEL-1; syn. myrtillin; ref.#0938S; purity ≥95%), delphinidin-3-O-rutinoside chloride (DEL-2; syn. tulipanin; ref.#0901S; purity ≥95%), delphinidin-3,5-di-O-glucoside chloride (DEL-3; syn. delphin; ref.#0941S; purity ≥97%), delphinidin-3-O-sambubioside chloride (DEL-4; ref.#0948S; purity ≥90%), delphinidin-3-O-rhamnoside chloride (DEL-5; ref.#0940S; purity ≥90%), malvidin-3-O-glucoside chloride (MAL-1; syn. oenin; ref.#0911S; purity ≥95%), malvidin-3,5-di-O-glucoside chloride (MAL-2; syn. malvin; ref.#0930S; purity ≥95%), malvidin-3-O-galactoside chloride (MAL-3; syn. primulin; ref.#0931S; purity ≥97.5%), cyanidin-3-O-glucoside chloride (CYA-1; syn. kuromanin, asterin, chrysanthemin; ref.#0915S; purity ≥96%), cyanidin-3-O-rutinoside chloride (CYA-2; syn. keracyanin; ref.#0914S; purity ≥96%), cyanidin-3,5-di-O-glucoside chloride (CYA-3; cyanin; ref.#0932S; purity ≥97%), cyanidin-3-O-sophoroside chloride (CYA-4; ref.#0937S; purity ≥95%), cyanidin-3-O-arabinoside chloride (CYA-5; ref.#0908S; purity ≥97%), cyanidin-3-O-rhamnoside chloride (CYA-6; ref.#0939S; purity ≥90%), cyanidin-3-O-galactoside chloride (CYA-7; syn. ideain; ref.#0923S; purity ≥97%), cyanidin-3-O-sambubioside chloride (CYA-8; ref.#0949S; purity ≥95%) and cyanidin-3-O-lathyroside chloride (CYA-9; ref.#0936S; purity ≥97%) were purchased from Extrasynthese (France). The anthocyanidins, cyanidin chloride (ref.#0909S; purity ≥96%), delphinidin chloride (ref.#0904S; purity ≥97%), malvidin chloride (ref.#0913S; purity ≥97%), peonidin chloride (ref.#0906S; purity ≥ 97%), petunidin chloride (ref.#0942S; purity ≥95%) and pelargonidin chloride (ref.#0912S; purity ≥97%) were also purchased from Extrasynthese (France). Luciferase lysis buffer and P450-Glo CYP1A1 assay were purchased from Promega (USA). TRI Reagent, M-MLV Reverse Transcriptase, Random hexamers and oligonucleotide primers were purchased from Invitrogen (USA). LightCycler FastStart DNA Master^{PLUS} SYBR Green I and protease and phosphatase inhibitor coctails were purchased from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). Primary antibodies – actin goat polyclonal (sc-1616), CYP1A1 goat polyclonal (sc-9828), CYP3A4 mouse monoclonal (sc-53850), and secondary horseradish peroxidase conjugated antibody and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (USA). The following dietary supplements were purchased from the public pharmacy store Antistax® (Boehringer Ingelheim International GmbH, Germany), Urinal Akut® (Walmark, Czech Republic), Ostrovid (Swiss Herbal Remedies LTD., Canada). All other chemicals were of the highest quality commercially available.

4.3 Methods

4.3.1 Preparation of samples from ready to drink teas and mineral waters

Ready to drink teas (RDTs) and flavored mineral waters (MWs) were purchased in various supermarkets in Olomouc City, Czech Republic (Table 3 and Table 4, Figure 6). The extracts from 17 different RDTs and 28 different MW were prepared as follows: Aliquots of 250 ml were taken from four different bottles containing RDTs and MWs. The aliquots were mixed together, to a total sample volume of 1000 ml. The entire sample (1000 ml) was concentrated using vacuum evaporation down-to volume of 200 ml. Concentrated (acidic) sample was extracted twice with 2 x 200 ml of diethyl ether. The sample was then alkalized using concentrated sodium hydroxide to pH approximately 9-10. The extraction with 2 x 200 ml of diethyl ether was performed again. All the extracts, both acidic and alkaline, were pooled and evaporated to dryness. Dried extracts were dissolved in 1 ml of ethanol. The resulting ethanolic solutions were 1000x concentrated RDTs and MWs. In cell experiments, ethanolic samples were diluted 1000 times in the culture medium; hence the concentrations of RDTs' and MWs' constituents in the cell cultures were identical to those in RDTs and MWs.

Figure 6. Photo of tested flavored mineral waters and ready to drink teas.

Table 3. List of flavored mineral waters

Code	Trade name	Type	Added substances
MWDV1	DOBRA VODA	Raspberry	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV2	DOBRA VODA	Mango	Vit C 92 mg/L, sugar, citric acid, aroma
MWDV3	DOBRA VODA	Elderberry	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV4	DOBRA VODA	Lemon	Vit C 92 mg/L, sugar, citric acid, aroma, acidity regulator
MWDV5	DOBRA VODA		-
MWDV6	DOBRA VODA	Orange	Vit C 92 mg/L, sugar, citric acid, aroma
MWDV7	DOBRA VODA	Grapefruit	Vit C 92 mg/L, sugar, citric acid, aroma
MWDV8	DOBRA VODA	White Grapes	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV9	DOBRA VODA	Pear	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV10	DOBRA VODA	Lemon, Green Tea, Passion-flower	Sugar, aroma, herb extract 2.5 g, sodium benzoate, aspartam, acesulpham K, citric acid, ascorbic acid
MWDV11	DOBRA VODA	Orange, Violet,Howthorn, Blueberry	Sugar, aroma, herb extract 4 g, sodium benzoate, aspartam, acesulpham K, citric acid, ascorbic acid
MWDV12	DOBRA VODA	Grapefruit, Ginseng, Guarana	Sugar, aroma, herb extract 1 g, sodium benzoate, aspartam, acesulpham K, citric acid, ascorbic acid
MWPO1	PODEBRADKA	Lemon	Citric acid, aroma, acesulphame A, aspartam
MWPO2	PODEBRADKA	Grapefruit	Citric acid, aroma, acesulphame A, aspartam
MWPO3	PODEBRADKA	Plum & Elderberry	Citric acid, sodium citrate, L-carnitin 100 mg, acesulpham A, aspartam, fiber 1 g
MWPO4	PODEBRADKA	Sour cherry	Citric acid, aroma, acesulphame A, aspartam
MWPO5	PODEBRADKA	Pomelo & Cranberry	Citric acid, sodium citrate, L-carnitin 100 mg, acesulphame A, aspartam, fiber 1 g
MWPO6	PODEBRADKA	Lime	Citric acid, aroma, acesulphame A, aspartam
MWPO7	PODEBRADKA	Orange	Citric acid, aroma, acesulphame A, aspartam
MWPO8	PODEBRADKA	Plum	Citric acid, sodium benzoate, aroma, glucose-fructose syrup
MWPO9	PODEBRADKA	Passion-flower	Citric acid, sodium benzoate, aroma, glucose-fructose syrup
MWRA1	RAJEC	Dandelion	Glucose syrup, citric acid, herb extract from damdelion 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA2	RAJEC	Dog-rose	Glucose syrup, citric acid, herb extract from dog-rose 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA3	RAJEC	Wild-thyme	Glucose syrup citric acid, herb extract from wild-thyme 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA4	RAJEC	Salvia	Glucose syrup, citric acid, herb extract from salvia 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA5	RAJEC	Mellisa	Glucose syrup, citric acid, herb extract from mellisa 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA6	RAJEC	Chestnut	Fructose, citric acid, herb extract from chestnut 0.1 g, aroma, sodium benzoate
MWRA7	RAJEC	Linden	Fructose, citric acid, herb extract from linden 0.1 g, aroma, sodium benzoate, potasium sorbate

Table 4. List of ready to drink teas

Code	Brand	Type	Added substances
TDV1	DOBRA VODA	Green Tea + Lemon	Sugar, tea extract 5 g, natural aroma, citric acid, sodium benzoate, potasium sorbate, dimethyldicarbonate
TDV2	DOBRA VODA	White Tea + Apricot	Sugar, tea extract 4 g, natural aroma, citric acid, sodium benzoate, potasium sorbate, dimethyldicarbonate
TDV3	DOBRA VODA	Tea Citron	Sugar, tea extract 2.1 g, natural aroma, citric acid, sodium citrate, sodium benzoate, potasium sorbate, dimethyldicarbonate
TDV4	DOBRA VODA	Tea Peach	Sugar, tea extract 2.1 g, natural aroma, citric acid, sodium citrate, sodium benzoate, potasium sorbate, dimethyldicarbonate
TAT1	AQUILA TEAM	Green Tea + Lemon	Sugar, green tea extract, natural aroma green tea + lemon, natural lemon juice, sodium benzoate
TAT2	AQUILA TEAM	Black Tea + Lemon	Sugar, citric acid, caramel dye, black tea extract, phosphoric acid, sodium benzoate, natural lemon aroma, vitamin C
TAT3	AQUILA TEAM	Yellow tea + apple	Sugar, yellow tea extract, citric acid, ascorbic acid, natural apple aroma, apple juice, sodium citrate, sodium benzoate
TAT4	AQUILA TEAM	White tea + pomegranate	Sugar, white tea extract, citric acid, ascorbic acid, natural pomegranate aroma, pomegranate juice, sodium citrate, sodium benzoate
TAT5	AQUILA TEAM	Red tea + pear	Sugar, red tea extract, citric acid, ascorbic acid, natural pear aroma, pear juice, sodium citrate, sodium benzoate
TAT6	AQUILA TEAM	Black tea + peach	Sugar, citric acid, caramel dye, black tea extract, phosphoric acid, sodium benzoate, natural peach aroma, vitamin C
TAT7	AQUILA TEAM	Black tea + forest fruits	Sugar, natural forest fruits aroma, citric acid, caramel dye, black tea extract, sodium benzoate, vitamin C
TNE1	NESTEA VITAO	White tea + apricot	Sugar, citric acid, sodium citrate, white tea extract 0.2%, apricot juice 0.1% of concentrate, aroma, ascorbic acid, polyphenols 700 mg
TNE2	NESTEA VITAO	Red tea + pear	Sugar, citric acid, sodium citrate, roibois extract 0.1%, pear juice 0.1% of concentrate, aroma, ascorbic acid, polyphenols 440 mg
TNE3	NESTEA VITAO	Green Tea + aloe vera + strawberry	Sugar, malic acid, sodium citrate, strawberry aroma, aloe vera aroma, green tea extract 0.15%, strawberry juice 0.1% of concentrate, ascorbic acid, polyphenols 570 mg
TNE4	NESTEA	Peach	Sugar, citric acid, sodium citrate, tea extract 0.1%, aroma, peach juice 0.1% of concentrate,
TNE5	NESTEA	Lemon	Sugar, citric acid, sodium citrate, tea extract 0.1%, aroma, lemon juice 0.1% of concentrate, ascorbic acid
TNE6	NESTEA VITAO	Green Tea + lemon	Sugar, citric acid, sodium citrate, green tea extract 0.2%, aroma, orange and lemon juice 0.2% of concentrate, ascorbic acid, polyphenols 800 mg

4.3.2 RNA isolation and qRT-PCR

HepG2 and LS174T cells were seeded on six-well plates in a density of $1x10^6$ cells/well using culture media enriched with fetal bovine serum (10% v/v). After 16 hours of cultivation, cells were treated for 24 h with tested compounds, extracts, TCDD (5 nM) and/or vehicle (DMSO; 0.1% v/v). Cells were washed by PBS buffer (pH 7.4; 137 mM NaCl; 10 mM Na_2HPO_4; 10 mM KH_2PO_4; 2.7 mM KCl) and lysed by adding 1 ml of TRI Reagent® onto each well. The cells were scraped into 1.5 ml tubes and 200 µl chloroform was added, followed by shaking the samples for 60 seconds and incubation at room temperature for 7 minutes. Then the samples were centrifuged at 13 000 x g for 15 minutes at 4°C. After the centrifugation, the upper aqueous phase was transferred into a new tube. The precipitation of RNA was carried out by adding 0.5 ml of isopropyl alcohol and then the samples were incubated at room temperature for 10 minutes followed by centrifugation at 13 000 x g for 15 minutes at 4°C. The supernatant was then discarded and the pellet was washed twice with 1 ml of 75% ethanol. The pellet was left to dry and dissolved in RNase-free water. The concentration of RNA was quantified by spectrometry at 260 nm and purity was assessed from the ratio of absorbances A_{260nm}/A_{280nm}. cDNA was synthetized from 1000 ng of total RNA according to a common protocol, using M-MLV Reverse Transcriptase and random hexamers. One tenth of new synthetized cDNA was used for qRT-PCR using a Light Cycler apparatus 480 II (Roche Diagnostic Corporation, Prague, Czech Republic). The following program was used: an activation step at 95°C for 10 min was followed by 40 cycles of PCR (denaturation at 95°C for 10 seconds; annealing for 7 seconds at 65 °C for CYP1A1 and CYP3A4 or 68°C for GAPDH; elongation for 17 seconds at 72°C). The measurements were performed in triplicate. Gene expression was normalized *per* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The data were processed according to the delta-delta method.

Table 5. Oligonucleotides used for quantitative RT-PCR

Oligonucleotide name		Oligonucleotide sequence	Tm [°C]	Number of cycles
GAPDH	Forward	5′-TTCAGCAAGAAGAACAAGGACAA-3′	68	30
GAPDH	Reverse	5′-GGTTGAAGAAGTCCTCCTAAGC-3′		
CYP1A1	Forward	5′-CAAAGTTGTCATGGATGACC-3′	65	35
CYP1A1	Reverse	5′-GGTCGGAGTCAACGGATTTGGTCG-3′		
CYP3A4	Forward	5′-TCCGGGACATCACAGACAGC-3′	65	40
CYP3A4	Reverse	5′-ACCCTGGGGTTCATCACCAA-3′		

4.3.3 Preparation of protein extracts

HepG2 and LS174T cells were seeded on six-well plates in a density of $1x10^6$ cells/well using culture media enriched with fetal bovine serum (10% v/v). After 16 hours of incubation, cells were treated for 48 h with tested compound extracts, TCDD (5nM) and/or vehicle (DMSO; 0.1% v/v). Total protein extracts were prepared as follows: cells were washed twice with 1 ml of ice-cold PBS and scraped into 1,5 ml tubes in 1 ml of PBS. The suspension was centrifuged (4500 x g/5 min/4°C), the supernatant was aspirated and the pellet was re-suspended in 150 µl of ice-cold lysis buffer (10 mM Hepes pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; anti-protease/phosphatase cocktail; 0.2% (v/v) sodium dodecylsulfate). The mixture was vortexed for 5 min and then centrifuged at 13 000 x g for 15 min at 4°C. The supernatant was collected and the protein concentration was determined by the Bradford method.

4.3.4 Western blotting and protein detection

Total protein extracts (30 µg/well) were analyzed on SDS-PAGE gels (10%) using a Bio-Rad apparatus according to the standard procedure. Protein transfer onto polyvinylidine difluoride (PVDF) membrane was carried out. After the transfer, the membrane was stained with Ponceau S red dye for control of transfer and then saturated with 5% non-fat dried milk dissolved in a TBS-T buffer for 1 hour at room temperature, followed by incubation with primary antibody overnight at 4°C. The dilution of antibodies against CYP1A1 and CYP3A4 was 1/500 and against actin 1/2000. Subsequent incubation with an appropriate secondary antibody was carried out for 1 hour at room temperature. Chemiluminiscence detection was performed using horseradish peroxidase-conjugated secondary antibody and ECL kit.

4.3.5 Plasmid, transfection and gene reporter assay

HepG2 cells were transiently transfected by lipofection (Fugene 6) in 24-well plates with 300 ng/well of reporter *p1A1-luc* plasmid containing 5´flanking region (-1566-1999) of human CYP1A1 gene subcloned into the *KpnI-HindIII* double digested pGL3-Basic vector upstream of the firefly luciferase reporter gene. Following 16 h stabilization, the cells were treated for 24 h with tested extracts. After treatments, cells were lysed and luciferase activity was measured and standardized per milligram of protein. Experiments were performed in stably transfected gene reporter cell line AZ-AHR, which was derived from HepG2 cells transfected with a construct containing several binding sites upstream of luciferase reporter gene (Novotna et al., 2011). The

cells were seeded on a 96-well plate in a density of 25 000 cells per well. After plating, cells were stabilized for 16 hours and then incubated for 24 hours with tested compounds, extracts, TCDD (5nM) and/or vehicle (DMSO; 0.1% v/v). After the treatments, cells were lysed using Reporter Lysis Buffer according to the manufacturers´ instruction and luciferase activity was measured in 96-well plate format, using Infinite M200 luminometer (Tecan, Schoeller, Prague).

4.3.6 7-Ethoxyresorufin-O-deethylase activity of CYP1A1/2 in cell cultures

HepG2 cells and/or LS174T cells were seeded in 96-well plates at a density of 2.4×10^4 cells/cm^2 in DMEM supplemented with 10% FBS and stabilized for 24 h. Cells were then incubated for 48 h with test compounds, TCDD (5 nM) and/or vehicle (DMSO; 0.1%, v/v). The 7-ethoxyresorufin-O-deethylase (EROD) activity in cell cultures was measured as described elsewhere (Donato et al., 1993). Briefly, monolayers were washed with PBS and the serum-free medium containing 8 μM 7-ethoxyresorufin and 10 μM dicumarol was applied to cells. Following 30 min of incubation at 37 ^oC, an aliquot of 75 μl of the medium was mixed with 125 μl of methanol and fluorescence was measured in a 96-well plate with 530 nm excitation and 590 nm emission filters. The data were expressed as the ratio of treated over control values (DMSO-treated cells).

4.3.7 Enzyme activity of CYP1A1 and CYP1A2 in human liver microsomes

Pooled human liver microsomes were obtained from Biopredic (Rennes, France) in accordance with the ethical regulations of the country of origin (France). They were from twenty-six donors (twenty males and six females) with a protein content of 25 mg/ml; the CYP1A1/2 and CYP3A4/5 enzyme activities were verified before the experiment.

The activity of CYP1A1/2 was measured either as EROD (see above) with 7-ethoxyresorufin as substrate according to an established method (Leclercq et al., 1996) or by a luciferase based assay with CYP1A1 specific substrate Luciferin-CEE (www.promega.com, Promega, Hercules, CA). The quantity of product of the reaction was determined by HPLC (Shimadzu Class VP, Tokyo, Japan) with fluorescence detection or by measuring the luminescence using Infinite M 200 spectrophotometer/spectrofluorometer/luminometer (TECAN Austria, Vienna). Tested compounds were dissolved in an aqueous solution of pH 3.5 to concentration of 1 mM. Incubation mixture contained 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (0.8 mM NADP+, 5.8 mM isocitrate, 0.3 U/ml of isocitrate dehydrogenase and 8 mM $MgCl_2$), 35 pmol human liver microsomes and 2.6 μM 7-ethoxyresorufin. The final

incubation mixture volume was 100 µl. Inhibition experiments were performed with five concentrations of tested compounds (10, 20, 40, 80, 100 µM). Experimental conditions were the same as the above. Reaction mixtures were pre-incubated with inhibitors at 37°C for 30 min. With luciferin-CEE luminogenic substrate, microsomes with 22 pmol total CYP were preincubated with 60 µM luciferin-CEE and tested compounds (same final concentrations as above) for 30 minutes at 37°C; the NADPH-generating system was then added (same as above) and the system was incubated for 10 min. Detection reagent was then added and the reaction mixture was incubated for 20 min according to the recommended protocol (www.promega.com). Two independent experiments were done in duplicate which did not differ by more than 10%. Inhibition of CYP1A activities was in all cases evaluated by plotting the respective remaining activity versus the inhibitor concentration.

4.3.8 AhR ligand binding assay

[^{3}H]TCDD was kindly provided by Dr Steven Safe (Texas A&M university) and 2,3,7,8- tetrachlorodibenzofuran (TCDF) was from Accustandard (New Haven, CT USA). The competitive displacement of [^{3}H]TCDD from guinea pig hepatic cytosol was as previously described (Korashy et al. 2011). Briefly, hepatic guinea pig cytosol diluted to 8 mg/mL protein in MEDG (25 mM MOPS-NaOH, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% v/v glycerol) was incubated with different concentrations of tested compound or 200 nM TCDF for 30 min at room temperature or on ice, and further incubated for 1 h at room temperature in the presence of 2 nM [^{3}H]TCDD. The amount of [^{3}H]TCDD specific binding was determined by hydroxyapatite protocol, and specific binding was determined as the difference between the ‘no competitor’ and TCDF reaction (Denison et al., 2002a).

5. RESULTS

5.1 Effects of ready to drink teas (RDTs) on AhR-CYP1A1 signaling pathway

5.1.1 Effect of RDTs on CYP1A1 expression in HepG2 and LS174T cells

In the first series of experiments, the effects of RDTs on the expression of CYP1A1 mRNA and protein were examined in human hepatocarcinoma cells (HepG2) and human colon cancer cells (LS174T). These cells were chosen because the liver and intestine are primary organs for contact with RDTs. Cells were treated for 24 h and 48 h with ethanolic solution obtained from RDTs (dilution 1000x), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5nM) and vehicle (ethanol; 0,1% v/v). TCDD, the prototypical inducer of CYP1A1 genes, strongly induced CYP1A1 mRNA in both cell lines (fold induction - HepG2 approx. 165 x; LS174T approx. 443 x). The expression of CYP1A1 mRNA after treatment with RDTs was only negligibly elevated in several samples in both cell lines (Figure 7). The increase in CYP1A1 mRNA expression exceeded 2% compared to the fold induction attained by TCDD. The effects of RDTs on the expression of CYP1A1 protein in both HepG2 and LS174T were consistent with the data obtained at mRNA level (Figure 7). The data imply low toxicological and food-drug interaction potential of RDTs in terms of CYP1A1 expression in hepatic and intestinal cancer cell lines. Noteworthy, the induction of both CYP1A1 mRNA and protein by TCDD was much stronger in LS174T cells compared to HepG2 cells (Figure 7). Hence, intestinal LS174T cells seem to be a very appropriate model for studying CYP1A1 expression by food constituents. Finally, the use of HepG2 and LS174T cells implies: (i) Cell lines do not express the standard spectrum of biotransformation enzymes – i.e. the effects of RDTs' constituents but not metabolites, are assessed. (ii) The expression of CYP1A1 is regulated mainly by AhR. Hence, interactions between RDTs constituents and other receptors may occur.

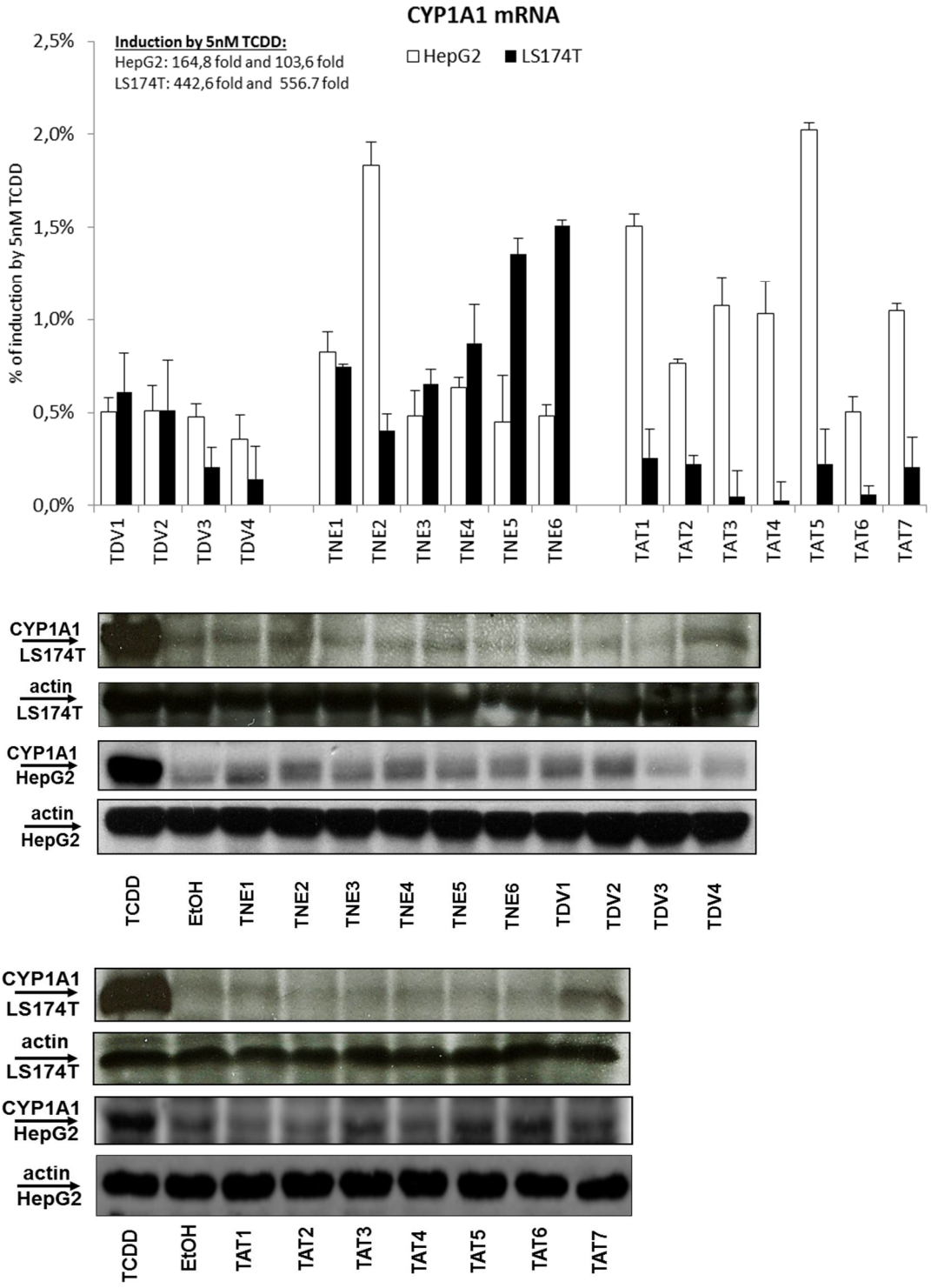

Figure 7. Effects of RDTs on CYP1A1 expression in HepG2 and LS174T cells. Cell lines were treated with ethanolic solutions obtained from 17 different RDTs (dilution 1000 x), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% V/V). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNA from HepG2 and LS174T cells. Treatment duration was 24 h. The data are means ± SD from duplicate measurements and are expressed as percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels. Western blots show representative analysis of CYP1A1 protein from HepG2 and LS174T cells. Treatment duration was 48 h. Similar profiles were observed in three independent experiments. As a loading control, the blots were probed to actin.

5.1.2 Effects of RDTs on CYP1A1 expression in primary cultures of human hepatocytes

In the following series of experiments, we studied the effects of RDTs on the expression of CYP1A1 mRNA and protein in primary human hepatocytes (LH36, LH37). Experiments were performed in two different human hepatocytes cultures (for detail see Materials and Methods section). Hepatocytes were treated for 24 h and 48 h with ethanolic solutions obtained from RDTs (dilution 1 000x), TCDD (5 nM) and vehicle (ethanol; 0.1% v/v).

TCDD induced CYP1A1 mRNA and protein strongly in LH36, but in LH37 the induction of TCDD was very weak – only 11 fold. There was also a problem in protein detection using LH37 in two samples (TNE1 and TNE3) We observed mild increase in CYP1A1 mRNA by TAT1 and TAT5; tested RDTs displayed similar effects on CYP1A1 mRNA expression in HepG2 cells (Figure 7) and human hepatocytes (Figure 8). The levels of CYP1A proteins were slightly elevated by TAT1 (Figure 9). The data from human hepatocytes allow assessment of the effects of RDTs and also their metabolites on the expression of CYP1A1 enzyme.

Finally, unlike cancer cell lines, primary human hepatocytes are non-transformed cells and this is a model much closer to the physiological situation in man.

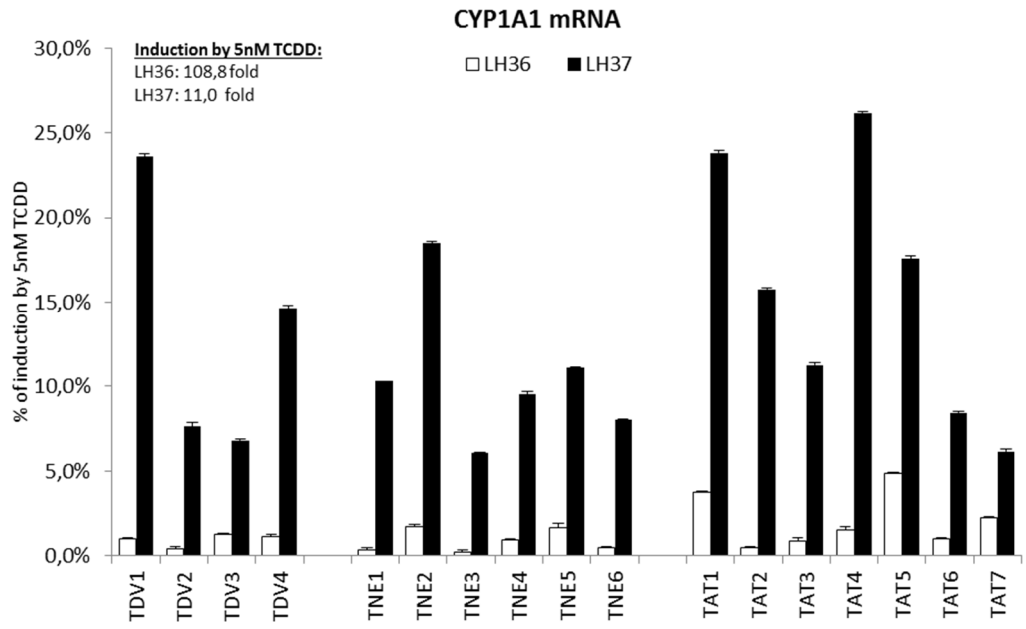

Figure 8. Effects of RDTs on mRNA CYP1A1 expression in primary cultures of human hepatocytes LH36 and LH37. Cells were treated with ethanolic solutions obtained from 17 different RDTs (dilution 1000x), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% V/V). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNAs from two human hepatocytes cultures. Treatment duration was 24 h. The data are mean ± SD from duplicate measurements are expressed as percentage of maximal induction attained in TCDD-treated cells. The data were normalised per GAPDH mRNA levels.

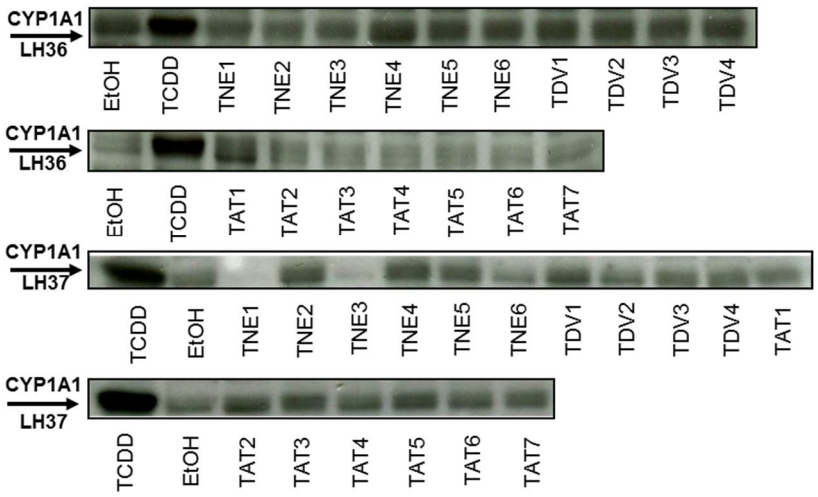

Figure 9. Effects of RDTs on CYP1A1 expression at the protein level in human hepatocytes. Cells were treated with ethanolic solutions obtained from 17 different RDTs (dilution 1000x), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% V/V). Western blots show representative analysis of CYP1A1 protein from two human hepatocytes cultures. Treatment duration was 48 h. As a loading control, the blots were probed to actin (data not shown).

5.1.3 Effects of RDTs on AhR transcriptional activity in HepG2 cells

In the next series of experiments, we examined the effects of RDTs on transcriptional activitiy of AhR. The gene reporter assay was performed in HepG2 cells transiently transfected with *p1A1-luc* (AhR-responsive) plasmid, as described in the Materials and Methods section. Cells were treated for 24 h with ethanolic solutions obtained from RDTs (dilution 1 000x and 10 000x), TCDD (5 nM) and vehicle (ethanol; 0.1% V/V).

AhR-dependent luciferase activity in HepG2 transfected with *p1A1-luc* was strongly induced by TCDD, and the fold induction ranged from 12 fold to 65 fold. Dose-dependent activation of *p1A1-luc* was observed for samples TDV1, TDV2, TAT1, TNE1, TNE2, TNE3, TNE4, TNE5 and TNE6 (Figure 10). These effects are probably caused by various polyphenols that are known as AhR partial agonists.

The data obtained from cancer cell line transiently transfected with *p1A1-luc* reveal the ability of RDTs to activate the AhR xenoreceptor. These data are of importance not only on up-regulation of drug-metabolizing enzymes but also dysregulation of intermediary metabolism.

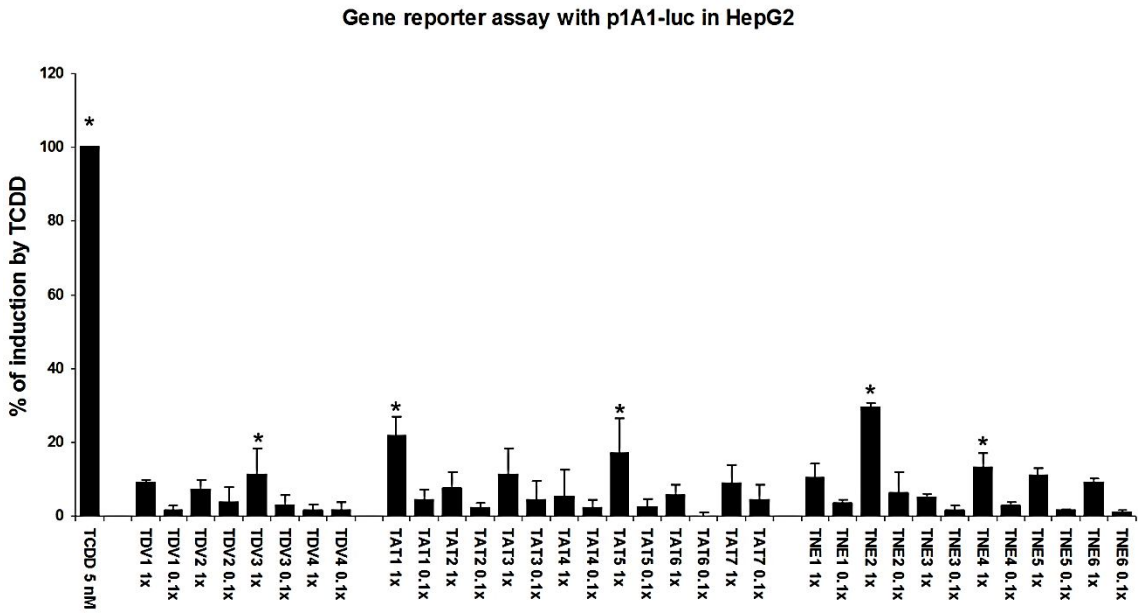

Figure 10. Effects of RDTs on AhR transcriptional activity in HepG2 cells. HepG2 cells were transiently transfected by lipofection (FuGENE 6) with 300 ng/per well of *p1A1-luc* reporter plasmid. Following 16 h of stabilization, the cells were treated for 24 h with ethanolic solutions obtained from 17 different RDTs (dilution 1000x and 10,000x), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and/or vehicle (EtOH = UT; 0.1% V/V). After the treatments, cells were lysed and luciferase activity was measured and standardised per milligram of protein. The data are represented as means ± SD from triplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). * - value is significantly different from EtOH-treated cells ($p < 0.05$).

5.2 Effects of flavored mineral waters (MWs) on the AhR-CYP1A1 signaling pathway

5.2.1 Effect of MWs on CYP1A1 expression in HepG2 and LS174T cells

Since intestinal cells are the primary target of food constituents and the liver is the main organ of drug metabolism in the human body, we examined the effects of MWs on CYP1A1 expression in intestinal cell line LS174T and in hepatoma cell line HepG2. We treated cells for 24 h (mRNA expression) and 48 h (protein expression) with ethanolic solutions of extracts from 28 different MWs (dilution 1.000 ×), TCDD (5 nM) and vehicle (EtOH; 0.1 % v/v). Dioxin (TCDD), a model activator of AhR a inducer of CYP1A1, increased the expression of CYP1A1 mRNA approx. 350 fold in LS174T and 800 fold in HepG2 as compared to vehicle-treated cells. Of the mineral waters tested, significant induction of CYP1A1 mRNA was achieved with samples MWDV10 (12 fold) and MWDV12 (20 fold) (Figure 11) in LS174T cells, but no significantly induction was detected in HepG2. The level of CYP1A1 protein in both cell lines treated for 48 h with TCDD was drastically increased. However, none of the MWs tested caused significant elevation of CYP1A1 protein level (Figure 11).

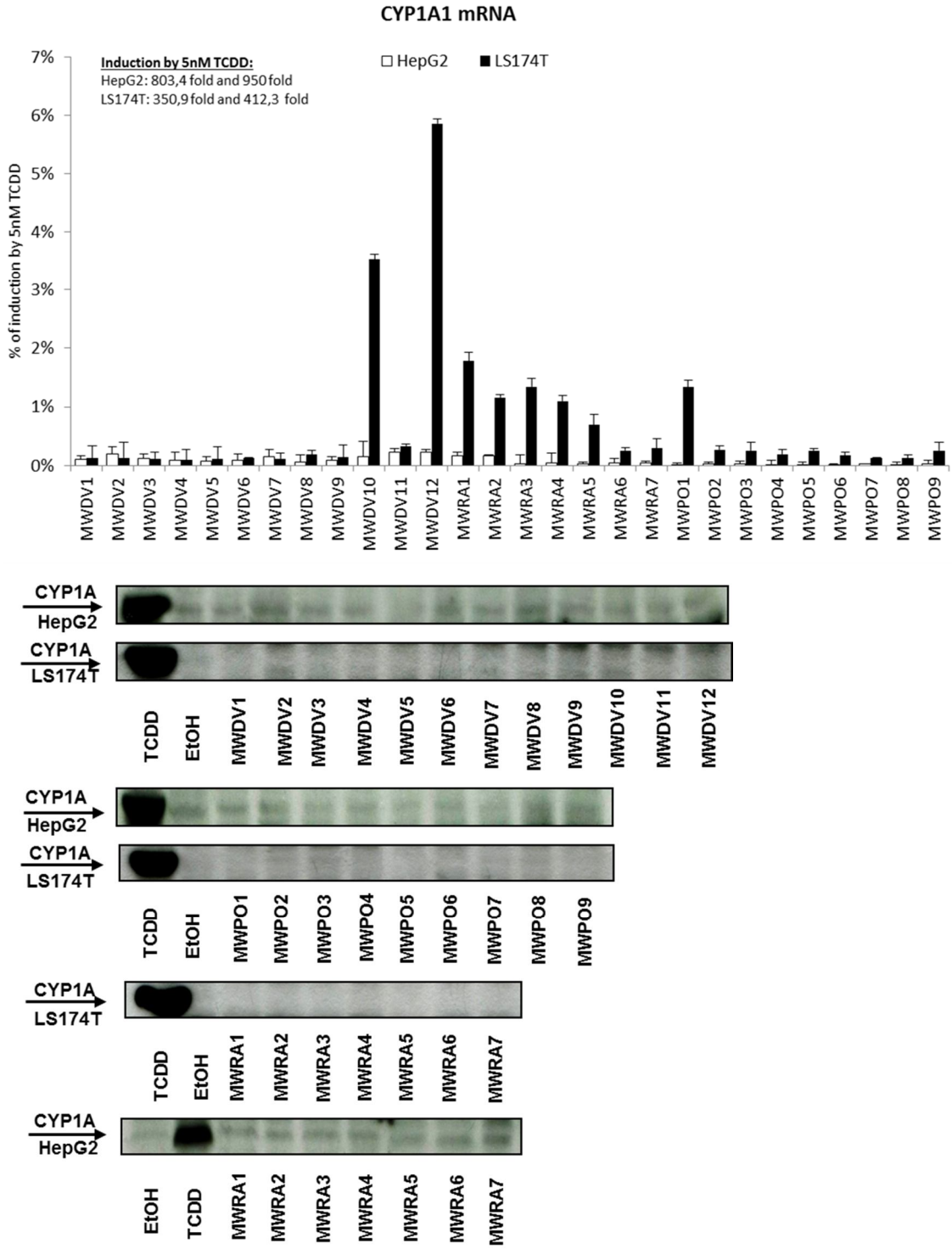

Figure 11. Effects of MWs on CYP1A1 expression in HepG2 and LS174T cell lines. Cells were treated with ethanolic solutions obtained from 28 different MWs (dilution 1000x), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% V/V). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNAs after 24 h treatment. The data are represented as means ± SD from duplicate measurements and are expressed as percentage of maximal induction attained in TCDD-treated cells. The data were normalised *per* GAPDH mRNA levels. Western blots show representative analysis of CYP1A1 protein from two cancer cell lines. Treatment duration was 48 h. Similar profiles were observed in three independent experiments. As a loading control, the blots were probed to actin (data not shown).

5.2.2 Effects of MWs on CYP1A1 and CYP1A2 expression in primary cultures of human hepatocytes

In the next series of experiments, the effects of MWs in two different primary cultures of human hepatocytes were examined. In addition to cell lines, primary human hepatocytes allow to assess the effects of parent compounds and their metabolites. Dioxin induced CYP1A1 mRNAs were 320 fold and 121 fold, in cultures Hep220633 and LH36, respectively. The level of CYP1A1mRNA was increased by samples MWPO1 (22 fold) and MWPO9 (11 fold) in culture Hep220633 (Figure 12). Some of the tested samples (MWPO1, MWWPO8, MWRA5 and MWRA7) slightly induced CYP1A proteins in Hep220633 culture, while TCDD strongly up-regulated CYP1A proteins (Figure 13). The expression of CYP1A1 mRNA was significantly increased by MWDV10 (7 fold) in culture LH36 (Figure 12). Besides of TCDD, the level of CYP1A proteins was elevated in LH36 cells treated with MWPO1 and MWDV5 (Figure 13)

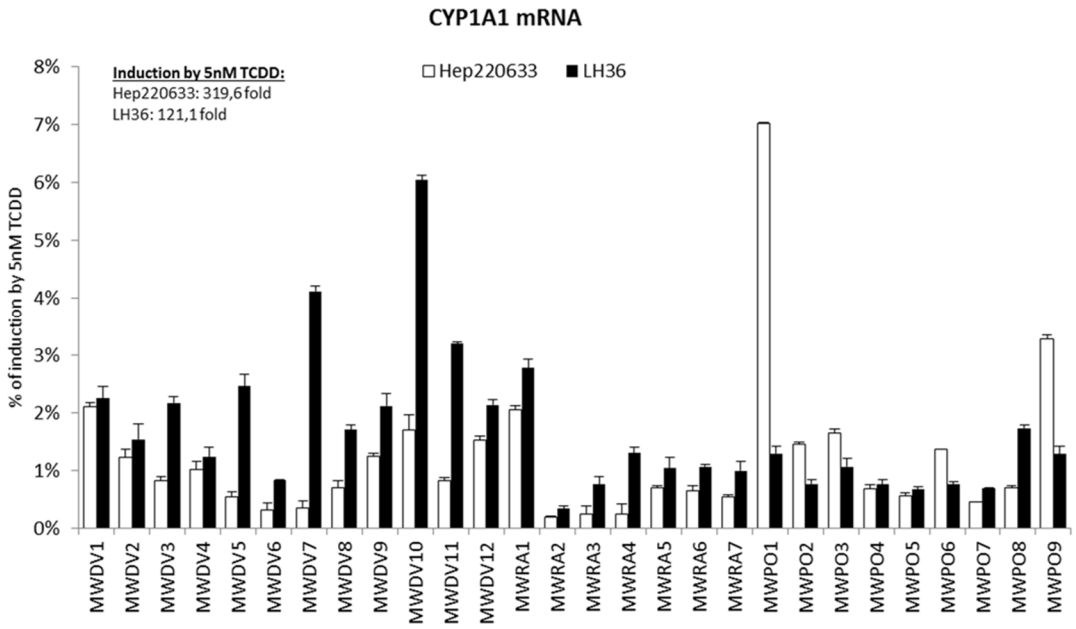

Figure 12. Effects of MWs on mRNA CYP1A1 expression in primary cultures in human hepatocytes. Cells were treated with ethanolic solutions obtained from 28 different MWs (dilution 1000 x), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% V/V). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNA from two human hepatocytes cultures HEP22063 and LH36. Treatment duration was 24 h. The data are mean ± SD from duplicate measurements and are expressed as percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels.

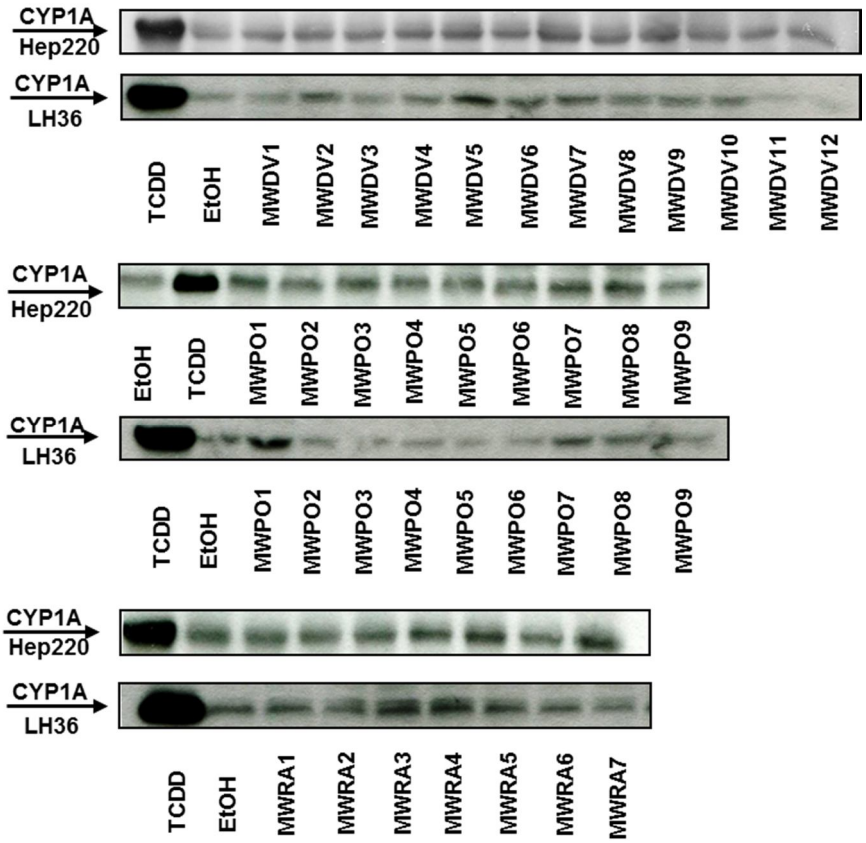

Figure 13. Effects of MWs on CYP1A1 protein expression in primary cultures in human hepatocytes. Cells were treated with ethanolic solutions obtained from 28 different MWs (dilution 1000 x), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% V/V).. Western blots show representative analysis of CYP1A1 protein from HEP220633 and LH36 cells. Treatment duration was 48 h. As a loading control, the blots were probed to actin (data not shown).

5.2.3 Effects of MWs on transcriptional activity of AhR in AZ-AHR reporter cell line

The expression of CYP1A1 is transcriptionally regulated by AhR. Therefore, inducers of CYP1A1 are mostly ligands of AhR. For this reason, we examined the effects of tested MWs in the AZ-AHR stable reporter cell line derived from HepG2 cells, containing several dioxin-response elements up-stream of luciferase gene. This cell line is well characterized and it allows screening for AhR activators in high-throughput format. Treatment of AZ-AHR cells with TCDD for 24 h resulted in approx. 157 fold increase in luciferase activity, compared to vehicle-treated cells. Of 28 MWs tested, the samples MWDV10 (21 fold) and MWDV12 (23 fold) significantly increased luciferase activity (Figure 14), which is consistent with the data for CYP1A1 mRNA expression in LS174T cells (Figure 11).

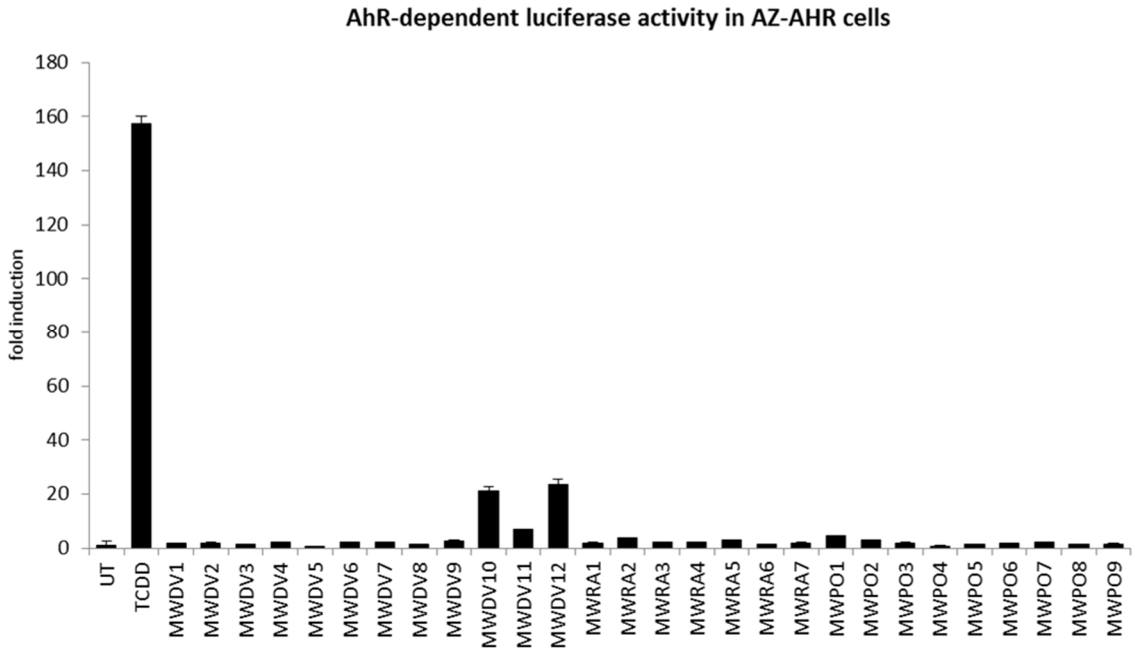

Figure 14. Effects of mineral waters on transcriptional activity of AhR in AZ-AHR reporter cell line. AZ-AHR cells were seeded on 96-well plates, and allowed to attach and stabilize for 16 h. Thereafter, the cells were treated for 24 h with ethanolic solutions obtained from 28 different MWs (dilution 1 000×), 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (EtOH = UT; 0.1% V/V). After the treatments, cells were lysed and luciferase activity was measured. The data are means ± SD from triplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). Experiments were performed in three independent cell passages.

5.3 Effects of anthocyanidins on AhR-CYP1A1 signaling pathway

5.3.1 Effect of anthocyanidins on CYP1A1 expression in HepG2 and LS174T cells

A typical target gene of ligand- and AhR-dependent signal transduction is CYP1A1. For this reason, we studied the effects of anthocyanidins on the expression of CYP1A1 mRNA, protein and enzyme catalytic activity in hepatic HepG2 and intestinal LS174T human cancer cells. Cells were incubated with anthocyanidins, TCDD (5 nM) and DMSO (0.1%, v/v) for 24 h and 48 h. TCDD strongly induced CYP1A1 mRNA after 24 h of incubation in HepG2 cells (588-fold and 611-fold) and in LS174T cells (1319-fold and 7940-fold). While cyanidin, delphinidin, malvidin, peonidin and petunidin did not influence CYP1A1 mRNA, protein and enzyme activity levels, pelargonidin increased CYP1A1 mRNA expression in a concentration-dependent manner in both cell lines. However, pelargonidin was a relatively weak inducer of CYP1A1 mRNA in HepG2 and LS174T cells increasing CYP1A1 mRNA levels by only about 2% and 4% of that observed with a maximal inducing concentration of TCDD, respectively (Figure 15 A). Pelargonidin (50 µM) incubation did increase CYP1A1 protein levels in HepG2 and LS174T cell lines after 48 h incubation (Figure 15 B). EROD activity in HepG2 and LS174T cells was induced 36-fold and 17-fold by TCDD after 24 h of incubation, respectively. Like its effect on CYP1A1 mRNA and protein levels, pelargonidin increased EROD activity ~3-fold in a concentration-dependent manner in both cell lines (Figure 15 C). Collectively, these results further support the conclusion that pelargonidin is an AhR agonist, albeit relatively weak compared to TCDD.

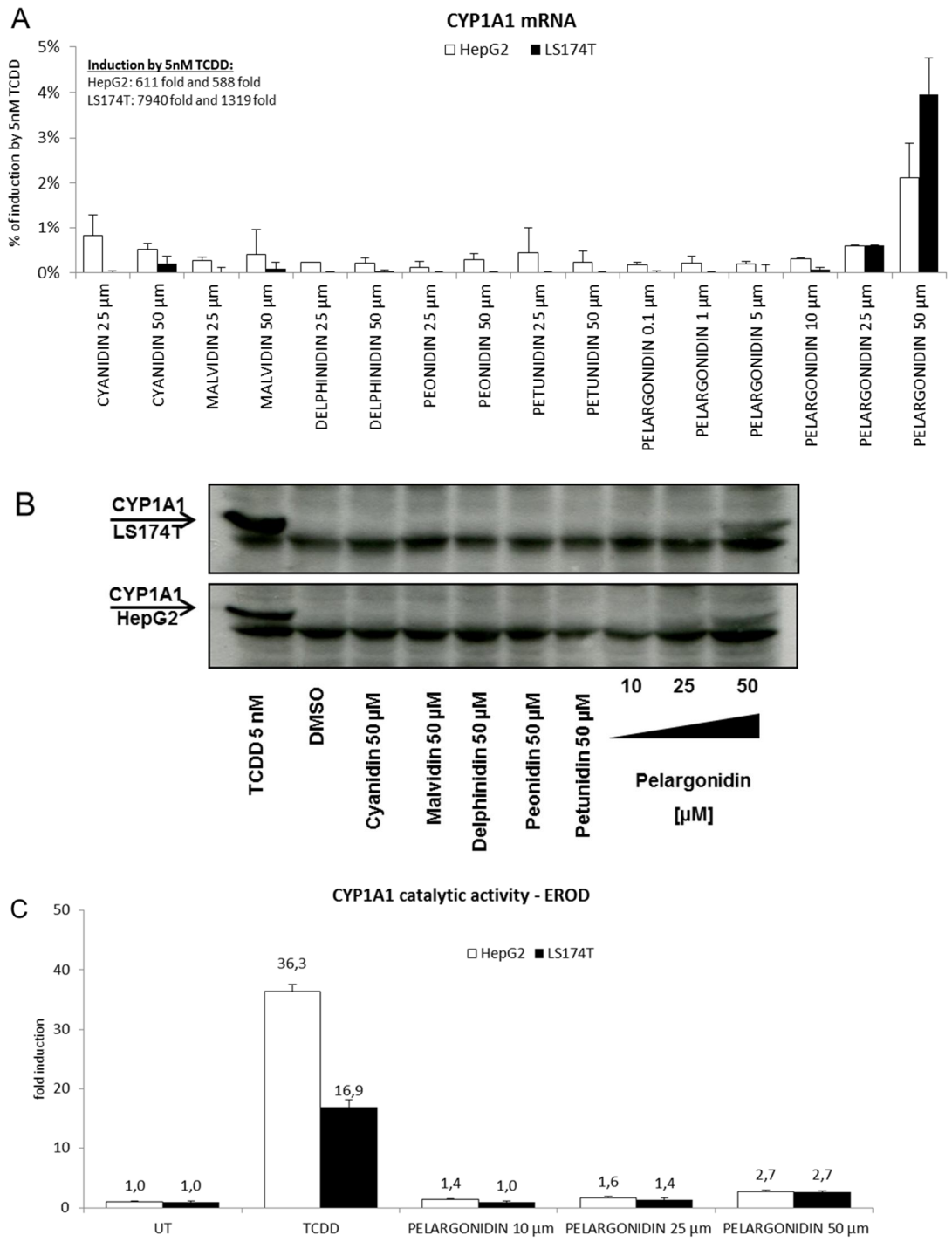

Figure 15. Effects of anthocyanidins on CYP1A1 expression in HepG2 and LS174T cells. Panel A: cells were treated with anthocyanidins, TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v). Bar graph shows representative RT-PCR analyses (2 independent experiments) of CYP1A1 mRNA after 24 h treatment. The data are the means of duplicate measurements and are expressed as percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels. Panel B: cells were treated with anthocyanidins, TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v) for 48 h. Western blots show a representative analysis of CYP1A1 protein. Similar profiles were observed in three independent experiments. As a loading control, the blots were probed to actin (data not shown). Panel C: cells were treated with pelargonidin (10 µM, 25 µM and 50 µM), TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v) for 48 h. Catalytic activity of CYP1A1 (EROD) was measured as described in detail in Section 2. The data are means of triplicate measurements and are expressed as fold induction over DMSO-treated cells.

5.3.2 Effects of anthocyanidins on CYP1A1 and CYP1A2 expression in primary cultures of human hepatocytes

In the next series of experiments, we tested the effects of anthocyanidins on the expression of CYP1A genes in primary cultures of human hepatocytes. In contrast to the cell lines, human hepatocytes express a full panel of drug-metabolizing enzymes, hence, the mixed effects of the parent compounds and their metabolites were examined. Three different primary human hepatocytes cultures were used (HH44, HH45 and HEP220670). Dioxin strongly induced the expression of CYP1A1 and CYP1A2 mRNAs in all human hepatocyte cultures at 24 h, and the magnitude of induction ranged from 160 and 331 fold and 52 and 144 fold, respectively. Pelargonidin induced CYP1A1 mRNAs in a concentration dependent manner in all of the primary hepatocyte cultures (Figure 16). While slight induction of CYP1A1 mRNAs was observed for other tested anthocyanidins, these increases occurred in a culture dependent manner, i.e. they were not systematic. The levels of CYP1A proteins in two human hepatocyte cultures were moderately increased only by incubation with 50 μM pelargonidin for 48 h, compared to the vehicle-treated cells (Figure 17).

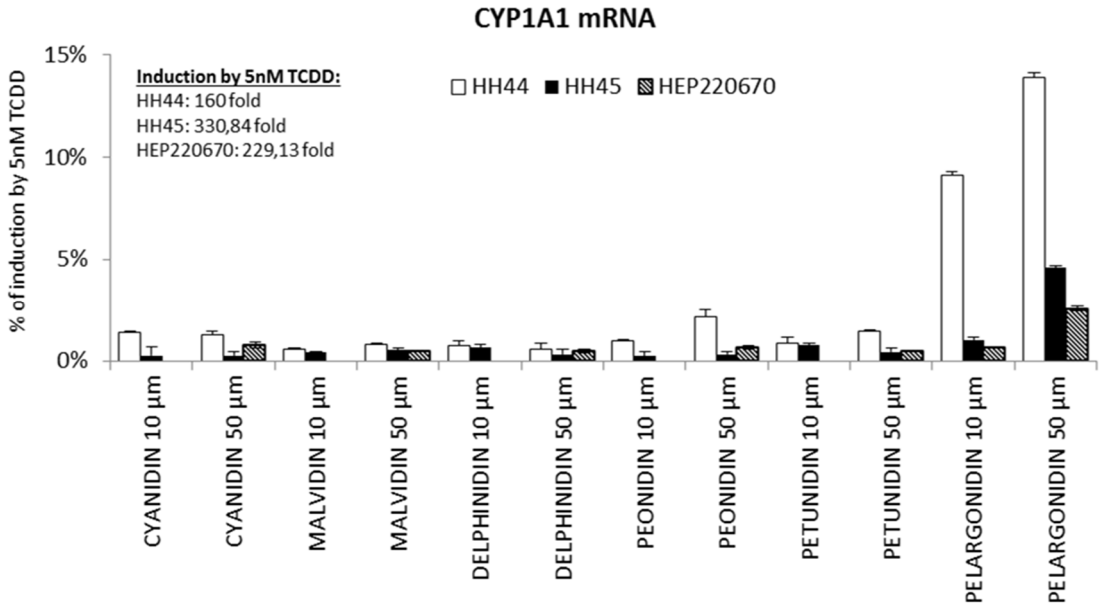

Figure 16. Effects of anthocyanidins on CYP1A1 expression in primary human hepatocytes. Cells were treated with anthocyanidins, TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v). Bar graph shows representative RT-PCR analyses of CYP1A1 mRNA from three cultures of human hepatocytes – HH44, HH45 and HEP220670 after 24 h treatment. The data are the mean of duplicate measurements and are expressed as percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels.

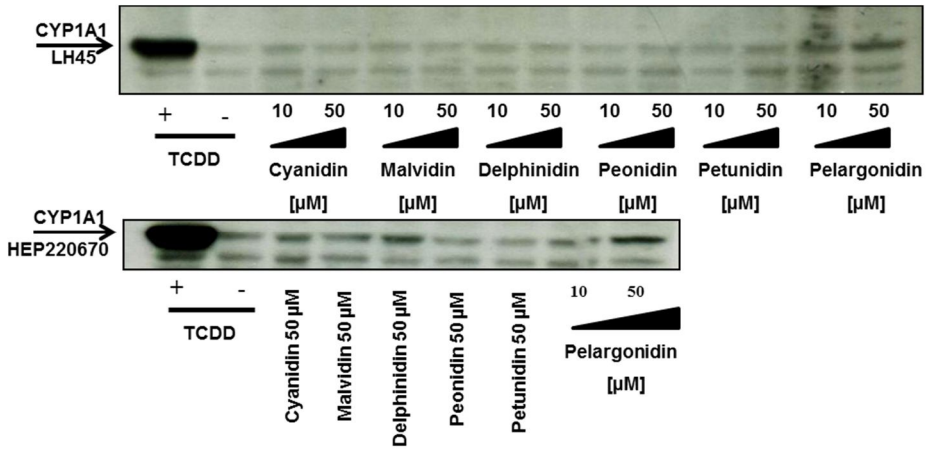

Figure 17. Effects of anthocyanidins on CYP1A protein expression in primary human hepatocytes. Cells were treated with cyanidin (10 µM and 50 µM), delphinidin (10 µM and 50 µM), malvidin (10 µM and 50 µM), peonidin (10 µM and 50 µM), petunidin (10 µM and 50 µM), pelargonidin (10 µM and 50 µM), TCDD (5 nM) and vehicle (DMSO; 0.1%, V/V) for 48 h. Western blots show analyses of CYP1A proteins from two different primary human hepatocytes cultures. As a loading control, the blots were probed to actin (data not shown).

5.3.3 Effects of anthocyanidins on transcriptional activity of AhR in AZ-AHR reporter cell line

In the last series of experiments, we examined the effects of the anthocyanidins on the transcriptional activity of AhR. Experiments were performed in recombinant AZ-AHR cells. In agonist experiments, the cells were incubated for 24 h with increasing concentrations (10 nM – 50 µM) of anthocyanidins (cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin), TCDD (5 nM) or vehicle (0.1%, v/v DMSO). Luciferase activity was increased 1253-fold by TCDD. Pelargonidin produced a concentration dependent induction of luciferase activity, which was significantly different from vehicle at concentrations of 10 µM and higher (Figure 18, upper panel). Although the magnitude of induction by 50 µM pelargonidin was 9-fold greater than that of the vehicle (DMSO) control, the magnitude of the induction response was very low compared to TCDD. While a very low, but significant level of induction of luciferase activity was observed for 50 µM cyanidin (Figure 18, upper panel), no significant induction of luciferase activity was observed with other anthocyanidins. In antagonist experiments, the cells were incubated with increasing concentrations of anthocyanidins and/or resveratrol in the presence of TCDD. While the induction of luciferase activity was not significantly altered by any of the anthocyanidins tested, resveratrol, a known antagonist of AhR, produced a concentration-dependent decrease of TCDD-induced luciferase activity to a maximum of 95% inhibition at 50 µM resveratrol (Figure 18, lower panel). Taken together, these results indicate that pelargonidin is a weak AhR agonist with no apparent antagonist activity.

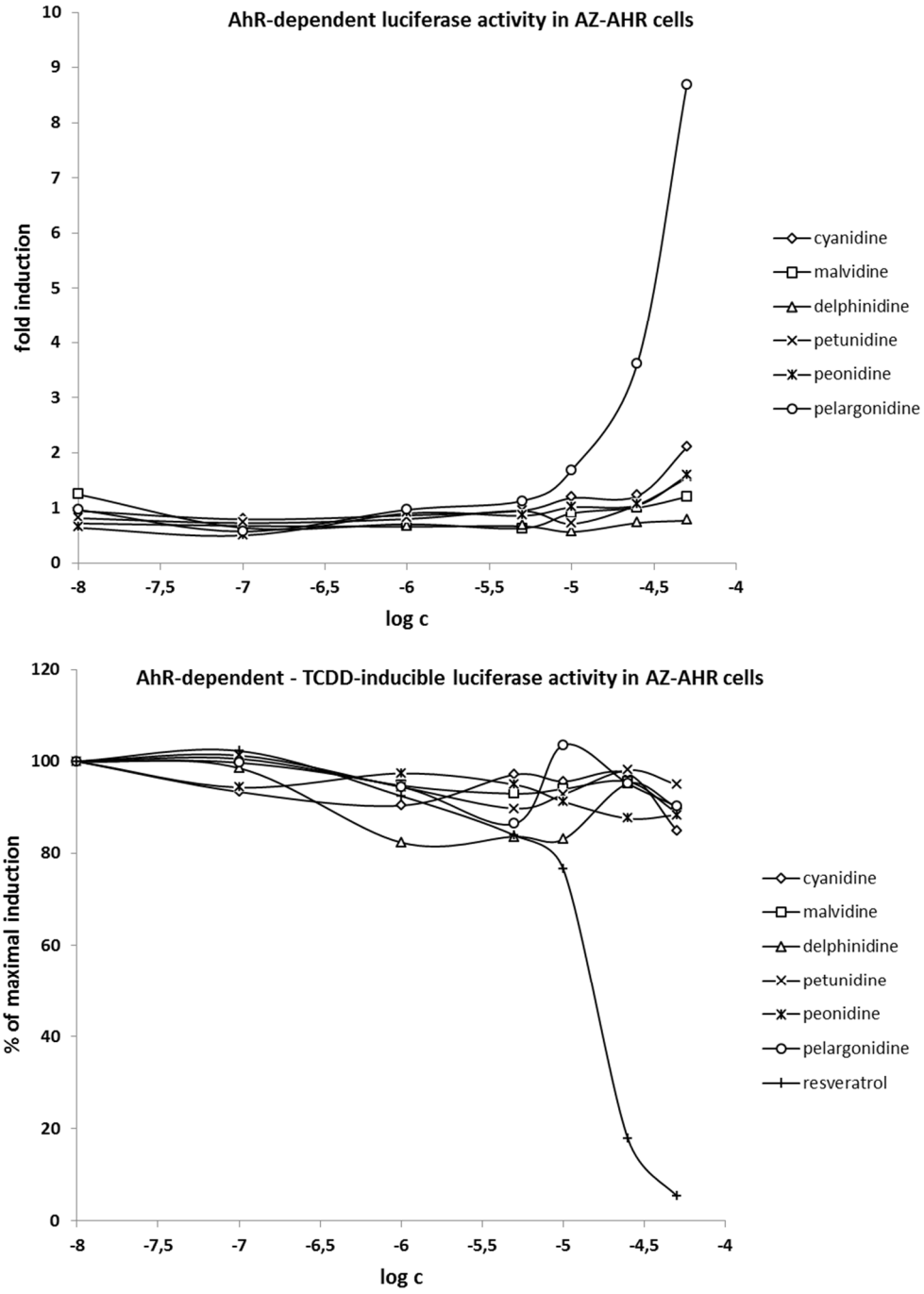

Figure 18. Effects of anthocyanidins on transcriptional activity of AhR in AZ-AHR reporter cell line. AZ-AHR cells were seeded in 96-well plates and stabilized for 16 h. Thereafter, the cells were treated for 24 h with anthocyanidins (10 nM–50 μM; i.e. cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1%, v/v). Upper panel: Agonist mode, i.e. incubation with individual compounds; lower panel: antagonist mode, i.e. combined incubation of 5 nM TCDD plus tested compounds and/or resveratrol in increasing concentrations. After the treatments, cells were lysed and luciferase activity was measured. The data are the means of triplicate measurements and are expressed either as fold induction over DMSO-treated cells (agonist mode) or as percentage of the induction by TCDD (antagonist mode). Experiments were performed in three different passages of AZ-AHR cells. The differences between individual measurements were lower that 5%.

5.3.4 Ligand binding assay

Activation of AhR may occur by ligand-dependent or ligand independent mechanisms. For this reason, we tested whether the effects of pelargonidin on the AhR-CYP1A1 signaling pathway involved binding of pelargonidin to the AhR. For this purpose, we performed AhR ligand binding assays using guinea pig hepatic cytosol. Pelargonidin competitively inhibited [^{3}H]-TCDD binding to the AhR when present in the binding incubation at 50 μM (13–16% inhibition) or 100 μM (25% inhibition), when cytosol was pre-incubated with pelargonidin on ice or at room temperature; there were no consistent differences between pre-incubation conditions (Figure 19). These data are consistent with the previous results which suggest that pelargonidin is a weak ligand/agonist of the AhR and that the effects of pelargonidin on AhR-CYP1A1 signaling pathway likely occur via a ligand-dependent mechanism.

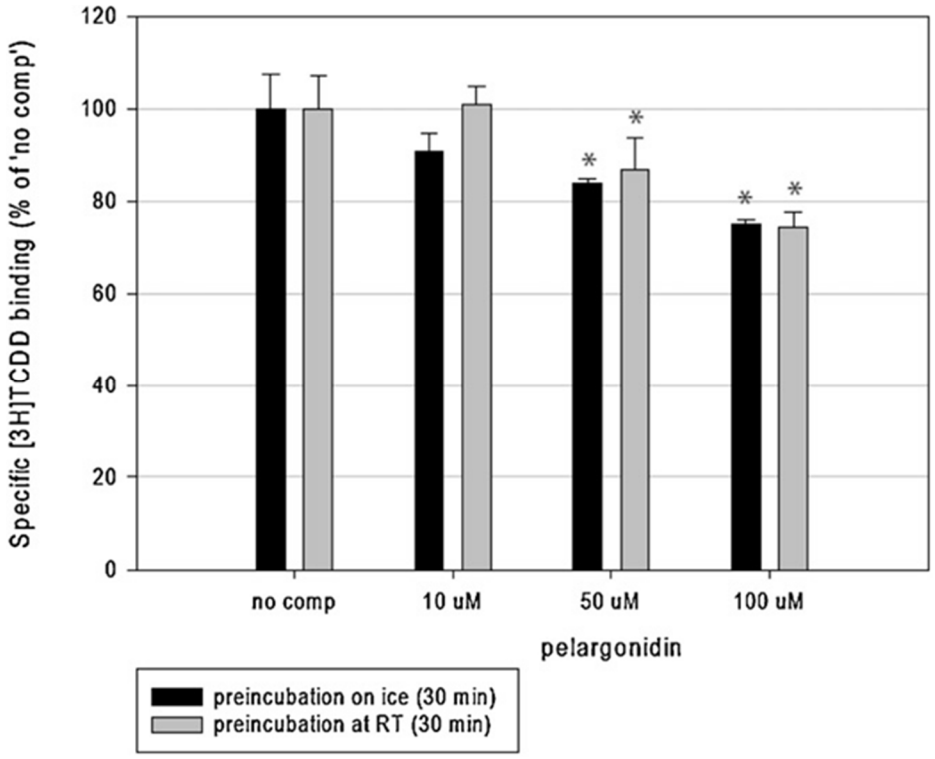

Figure 19. Ligand binding assay. Pelargonidin chloride is a weak AhR ligand. Guinea pig hepatic cytosol was pre-incubated with the indicated concentrations of pelargonidin chloride or TCDF for 30 min at room temperature or on ice, followed by addition of 2 nM [^{3}H]TCDD and further incubation for 1 h at room temperature. Ligand binding to the cytosolic proteins was determined by the hydroxyapatite binding protocol and scintillation counting. Specific binding was determined as the difference between total and non-specific (TCDF) reactions. The values are presented as means ± SD of three independent reactions. * – values significantly different from the 'no competitor' reaction at $p < 0.05$ as determined by the Student's t-test. The results are representative of two independent experiments.

5.3.5 Effects of anthocyanidins on CYP1A1 and CYP1A2 catalytic activity in human liver microsomes

Given the ability of many AhR ligands to also bind to and be metabolized by the coordinately induced CYP1A1/2, the effects of anthocyanidins were examined on the activities of two drug metabolizing enzymes by determining their effects on CYP1A1/2 catalytic (EROD) and to CYP1A1-specific luciferin activating activity using human microsomes. The results of these competitive kinetic analyses revealed a weak concentration-dependent inhibition of CYP1A1/2 EROD activity (the EROD deethylating activity is not specific either to CYP1A1 or CYP1A2 as both enzymes share this activity with CYP1A1 being more active) following the addition of petunidin, cyanidin, peonidin and malvidin, albeit to a relatively low extent (to 80–95% of the original activity at the highest concentration (i.e. 100 µM) of the respective anthocyanidin). In contrast, delphinidin inhibited CYP1A1/2 EROD activity down to 28% and pelargonidin to 75% of the initial activity (data not shown). This was why luciferin-based activity specific for CYP1A1 was tested with these two anthocyanidins. CYP1A1 was chosen for detailed study on the basis of the results with AhR activation and CYP1A1 expression in cancer cells (see the respective paragraphs). The data (Figure 20) clearly show the inhibition of CYP1A1 activity down to 22% and 40% of the initial activity (i.e. without anthocyanidin added) corresponding to IC_{50} values of 33 µM and 77 µM for pelargonidin and delphinidin, respectively. These results thus document the ability of at least two anthocyanidins to interact with the drug metabolizing system of CYP1A1/2.

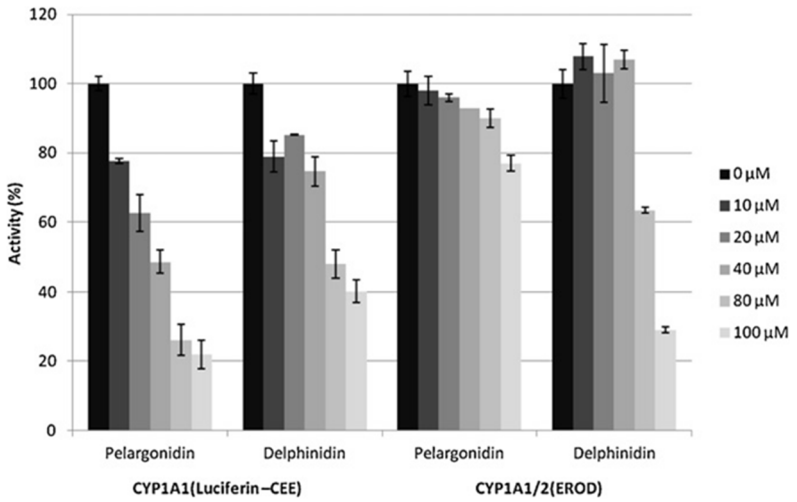

Figure 20. Effects of anthocyanidins on CYP1A1 catalytic activity in human liver microsomes. Inhibition of human microsomal CYP1A1 catalytic activity by pelargonidin and delphinidin expressed as the amount of activity remaining, relative to control (without anthocyanidin) in percent. Concentration of respective anthocyanidins in the reaction mixture was 0, 10, 20, 40, 80 and 100 µM.

5.4 Effects of anthocyanins on AhR-CYP1A1 signaling pathway

5.4.1 Effect of anthocyanins on CYP1A1 expression in HepG2 and LS174T cells

The effects of anthocyanins were studied on the expression of CYP1A1, a typical target gene of ligand- and AhR-dependent signal transduction. The levels of CYP1A1 mRNA and protein were determined in hepatic HepG2 and intestinal LS174T human cancer cells, which were incubated with 21 different anthocyanins (chemical structures and abbreviations are explained in Section 2.3, Fig. 3). The concentration of anthocyanins was 50 µM, TCDD (5 nM) and DMSO (0.1% v/v) and the treatment lasted for 24 h (mRNA) and 48 h (protein). In two independent experiments, TCDD strongly induced CYP1A1 mRNA after 24 h of incubation in HepG2 cells (322-fold and 216-fold) and in LS174T cells (1003-fold and 724-fold). The level of CYP1A1 mRNA was significantly increased by PEL-2 and CYA-3 in HepG2 cells, and by CYA-3 in LS174T cells, while 19 other anthocyanins had no significant effects on CYP1A1 mRNA in either cell line. However, PEL-2 and CYA-3 were relatively weak inducers of CYP1A1 mRNA in both cell lines, increasing CYP1A1 mRNA levels by only about 0.5% - 2% of that observed with a maximal inducing concentration of TCDD (Figure 21). In contrast, none of the 21 anthocyanins tested increased levels of CYP1A1 protein in HepG2 and LS174T cells after 48 h incubation, whereas TCDD strongly induced CYP1A1 protein in both cell lines (Figure 22). This discrepancy is probably due to weak induction of mRNA and hence not detectable change in protein level.

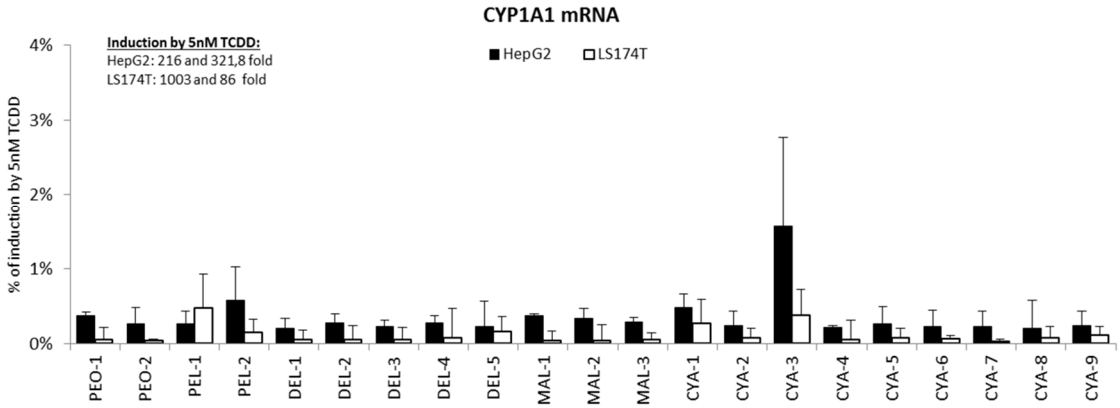

Figure 21. Effects of anthocyanins on the expression of CYP1A1 mRNAs in HepG2 and LS174T cells. Cells were incubated for 24 h with 21 anthocyanins (50 µM; i.e. PEO-1, PEO-2, PEL-1, PEL-2, DEL-1, DEL-2, DEL-3, DEL-4, DEL-5, MAL-1, MAL-2, MAL-3, CYA-1, CYA-2, CYA-3, CYA-4, CYA-5, CYA-6, CYA-7, CYA-8, CYA-9), TCDD (5 nM) and vehicle (DMSO; 0.1% v/v). Bar graph shows RT-PCR analyses (2 independent experiments) of CYP1A1 mRNA. The data are the means of triplicate measurements and are expressed as a percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels.

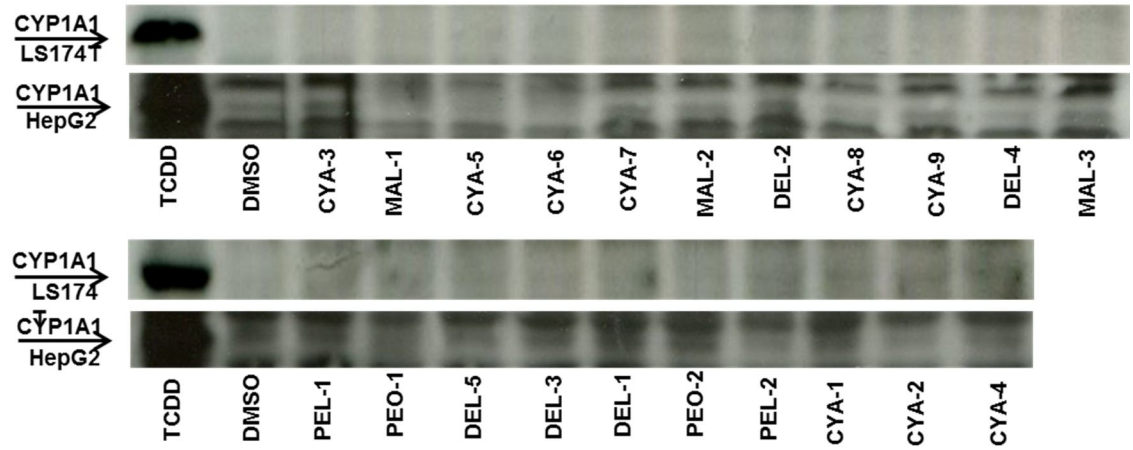

Figure 22. Effects of anthocyanins on the protein expression of CYP1A1 in HepG2 and LS174T cells. Cells were incubated for 48 h with 21 anthocyanins (50 µM; i.e. PEO-1, PEO-2, PEL-1, PEL-2, DEL-1, DEL-2, DEL-3, DEL-4, DEL-5, MAL-1, MAL-2, MAL-3, CYA-1, CYA-2, CYA-3, CYA-4, CYA-5, CYA-6, CYA-7, CYA-8, CYA-9), TCDD (5 nM) and vehicle (DMSO; 0.1% v/v). Western blots show a representative analysis of CYP1A1 protein. Similar profiles were observed in two independent experiments. As a loading control, the blots were probed to actin (data not shown).

5.4.2 Effects of anthocyanins on CYP1A1 expression in primary cultures of human hepatocytes

In the next series of experiments, we tested the effects of anthocyanins on the expression of CYP1A1 mRNA and protein in primary human hepatocytes. In contrast to the cell lines, human hepatocytes express a full panel of drug-metabolizing enzymes, hence, the mixed effects of the parent compounds and their metabolites were examined. Therefore, we also tested the effects of food supplements. Four different primary human hepatocytes cultures were used (LH45, LH46, LH47, LH49). Dioxin strongly induced the expression of CYP1A1 mRNAs in all human hepatocytes cultures at 24 h, and the magnitude of induction in cultures LH45, LH46, LH47 and LH49 was 125-fold, 344-fold, 105-fold and 71-fold, respectively. The levels of CYP1A1 protein were strongly induced after 48 h of incubation with 5 nM TCDD in four human hepatocytes cultures; data shown for two cultures (Figure 21). While slight induction of CYP1A1 mRNA and protein was observed for some of the tested anthocyanins, these increases occurred in a culture dependent manner, i.e. they were not systematic (Figure 23).

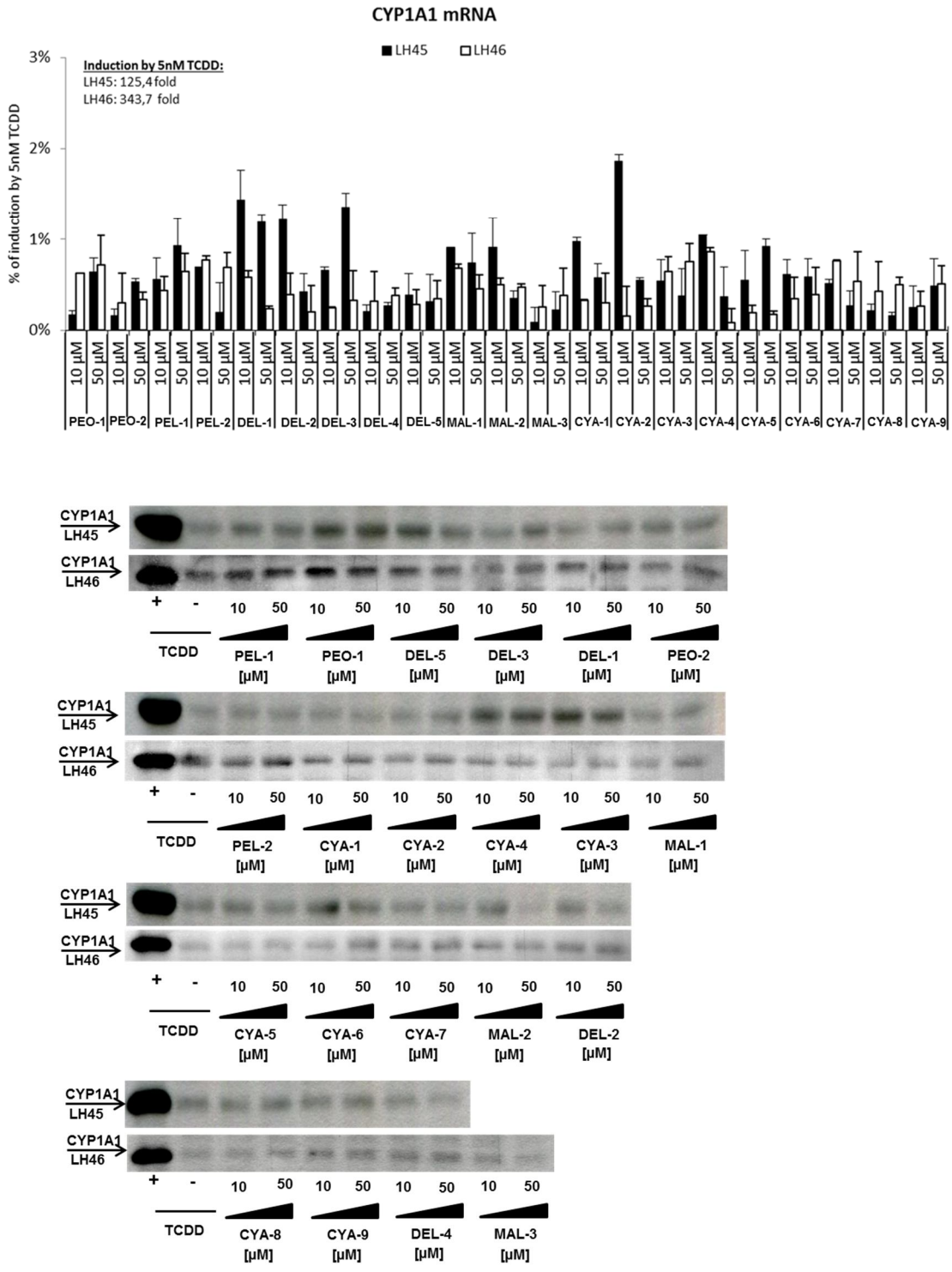

Figure 23. Effects of anthocyanins on the expression of CYP1A1 in primary human hepatocytes. Cells were incubated for 24 h with 21 anthocyanins (10 µM and 50 µM), TCDD (5 nM) and vehicle (DMSO; 0.1% v/v). Bar graph shows RT-PCR analyses (2 independent experiments) of CYP1A1 mRNA. The data are the means of duplicate measurements and are expressed as a percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels. Western blots show a representative analysis of CYP1A1 protein after 48 h treatment with anthocyanins (10 µM and 50 µM), TCDD (5 nM) and vehicle (DMSO; 0.1% v/v). Results are obtained from two cultures of human hepatocytes (LH45 and LH46). As a loading control, the blots were probed to actin (data not shown).

5.4.3 Effects of anthocyanins on transcriptional activity of AhR in AZ-AHR reporter cell line

In the next series of experiments, the effects of 21 anthocyanins were examined on the transcriptional activity of AhR in recombinant AZ-AHR cells. Cells were incubated for 24 h with 21 different anthocyanins (10 µM and 50 µM), proanthocyanidin A2 (10 µM and 50 µM), extracts from 3 dietary supplements (1000× dilution), TCDD (5 nM) or vehicle (0.1%, v/v DMSO). In nine independent experiments, induction of luciferase activity by 5 nM TCDD varied from 87-fold to 1023-fold. Anthocyanins PEL-2 (pelargonidin-3-O-rutinoside) and CYA-3 (cyaniding-3,5-di-O-glucoside) produced a concentration dependent induction of luciferase activity, which was significantly different from vehicle at a concentration of 50 µM (Figure 24). Although the magnitude of induction by 50 µM PEL-2 and CYA-3 was 10-fold and 17-fold greater than that of the vehicle (DMSO) control, respectively, the magnitude of the induction response was very low compared to TCDD (i.e. between 2% and 3% of the maximal level of induction by TCDD). These results are consistent with those obtained from RT-PCR, and further support the conclusion that PEL-2 and CYA-3 are AhR agonists, albeit relatively weak compared to TCDD. No significant induction of luciferase activity was observed with 19 other anthocyanins, proanthocyanidin A2 (data not shown) and extracts from food supplements (dilution 1000×; data not shown). Taken together, these results indicate that PEL-2 and CYA-3 are weak AhR agonists. Based on the data, we did not further test extracts from food supplements and proanthocyanidin A2 for their effects on the AhR–CYP1A1 pathway in cell lines.

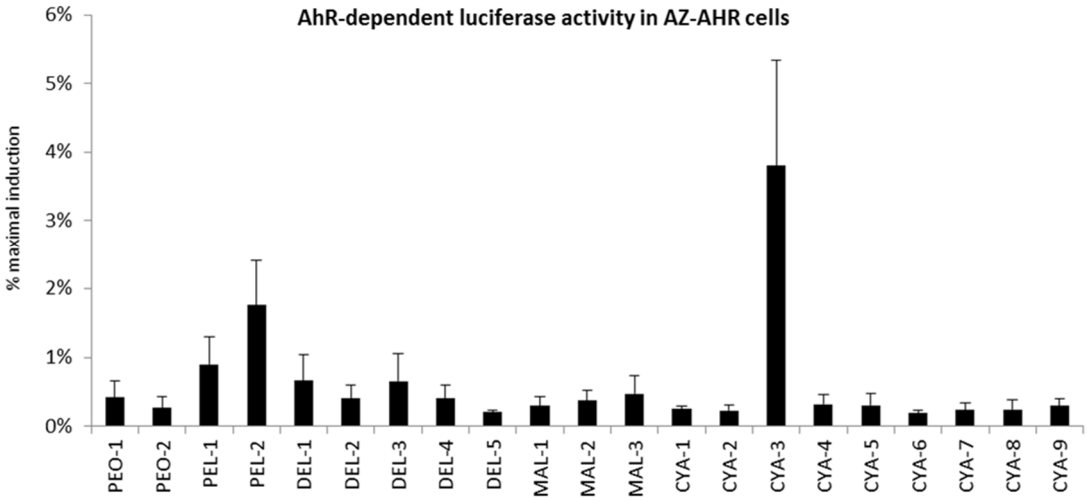

Figure 24. Effects of anthocyanins on transcriptional activity of AhR in AZ-AHR transgenic cell line. AZ-AHR cells were plated at 96-well plates and stabilized for 16 h. Thereafter, cells were treated for 24 h with 21 anthocyanins (50 µM), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1%, v/v). After the treatments, cells were lysed and luciferase activity was measured. The data are the means of triplicate measurements and are expressed as a percentage of the induction attained by TCDD.

5.4.4 Ligand binding assay

Since the activation of AhR may occur by ligand-dependent or ligand-independent mechanisms, we tested whether the effects of PEL-2 and CYA-3 on AhR–CYP1A1 signaling pathway involved binding of PEL-2 and CYA-3 to the AhR. We performed AhR ligand binding assays using guinea pig hepatic cytosol. PEL-2 competitively inhibited [^{3}H]-TCDD binding to the AhR when present in the binding incubation at 50 μM (61–71% inhibition) (Fig. 5). These data are consistent with the previous results which suggest that PEL-2 is a weak ligand/agonist of the AhR, and that the effects of PEL-2 on AhR–CYP1A1 signaling pathway likely occur via a ligand dependent mechanism. On the other hand, CYA-3 did not displace radiolabeled TCDD from AhR in any tested concentration (Figure 25). Therefore, the effects of CYA-3 on AhR–CYP1A1 signaling pathway are probably ligand-independent.

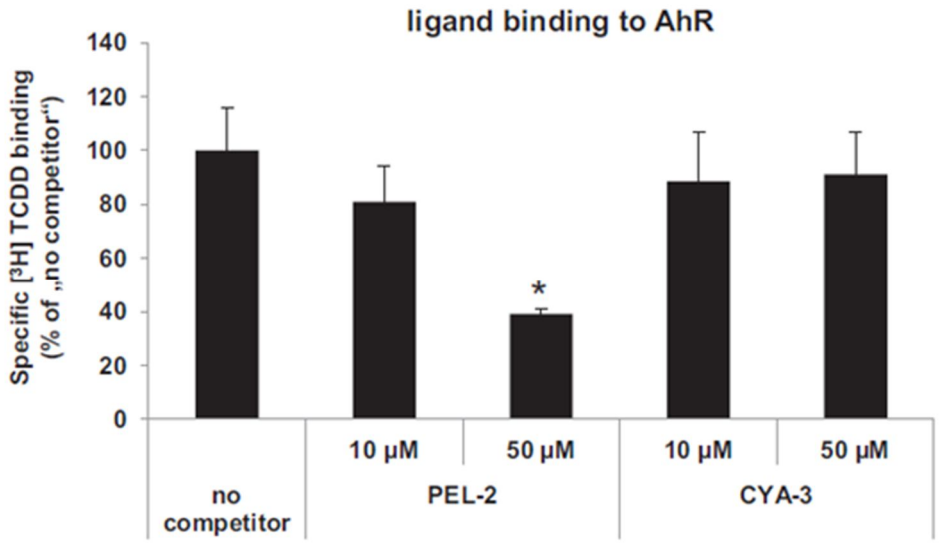

Figure 25. Ligand binding assay. Guinea pig hepatic cytosol was incubated with indicated concentrations of PEL-2 (pelargonidin-3-O-rutinoside chloride), CYA-3 (cyanidin-3,5-di-O-glucoside chloride) or 200 nM TCDF for 1 h at room temperature in the presence of 2 nM [3H]TCDD. Ligand binding to the cytosolic proteins was determined by the hydroxyapatite binding protocol and scintillation counting. Specific binding was determined as a difference between total and non-specific (TCDF) reactions. The values are presented as mean ± SD of three independent reactions. *Values significantly different from the 'no competitor' reaction at $p < 0.05$ as determined by the Student's t-test. The results are representative of two independent experiments.

3.4.5 Effects of anthocyanins on CYP1A1 and CYP1A2 catalytic activity in human liver microsomes

The effects of anthocyanins were directly examined on the activity of the CYP1A1 by following a specific luciferin activating activity using human microsomes. Tested compounds were selected based on the composition of food supplements tested in the current study. The results of competitive kinetic analyses revealed a weak concentration-dependent inhibition of CYP1A1 after the addition of anthocyanins CYA-1, CYA-5, CYA-7, MAL-1, PEO-1, PEO-2, DEL-1, CYA-3, albeit to a relatively low extent (to 80–65% of the initial activity, i.e. without anthocyanin added) at the highest concentration of the respective anthocyanin (i.e. 100 μM). The data (Figure 26) clearly document the course of the inhibition of CYP1A1 activity by various anthocyanins with maximum extent down to 62% with 100 μM of malvidine 3- glucoside (MAL-1). These results thus show the relatively weak ability of anthocyanins to interact with the drug metabolizing system of CYP1A1.

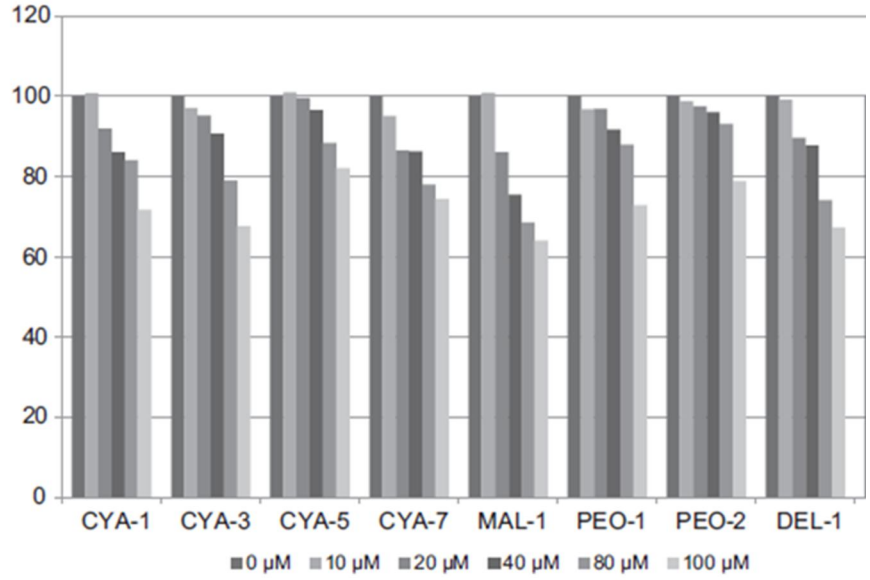

Figure 26. Effects of anthocyanins on CYP1A1 catalytic activity in human liver microsomes. Inhibition of human microsomal CYP1A1 catalytic activity by eight anthocyanins expressed as the amount of activity remaining relative to control (without anthocyanin) in percent. Concentration of respective anthocyanins in the reaction mixture was 0, 10, 20, 40, 80 and 100 μM.

5.5 Effects of artificial sweeteners on AhR-CYP1A1 signaling pathway

5.5.1 Effect of artificial sweeteners on CYP1A1 expression in HepG2 and LS174T cells

The effects of artificial sweeteners were studied on the expression of CYP1A1 in hepatic HepG2 and intestinal LS174T human cancer cells at the levels of protein and mRNA expression. Sweeteners were tested in concentrations up to those occurring in non-alcoholic beverages (Bergamo et al., 2011). Cells were incubated with artificial sweeteners – aspartame (0.4 µM and 400 µM), acesulfame (0.745 µM and 745 µM), cyclamate (3.48 µM and 3480 µM), saccharin (0.44 µM and 440 µM), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1% v/v) for 24 h and 48 h. TCDD strongly induced CYP1A1 mRNA and protein after 24 h and 48 h of incubation in both types of cells, respectively. None of the tested artificial sweeteners significantly induced the expression of CYP1A1 mRNA or protein in LS174T and HepG2 cells (Figure 27).

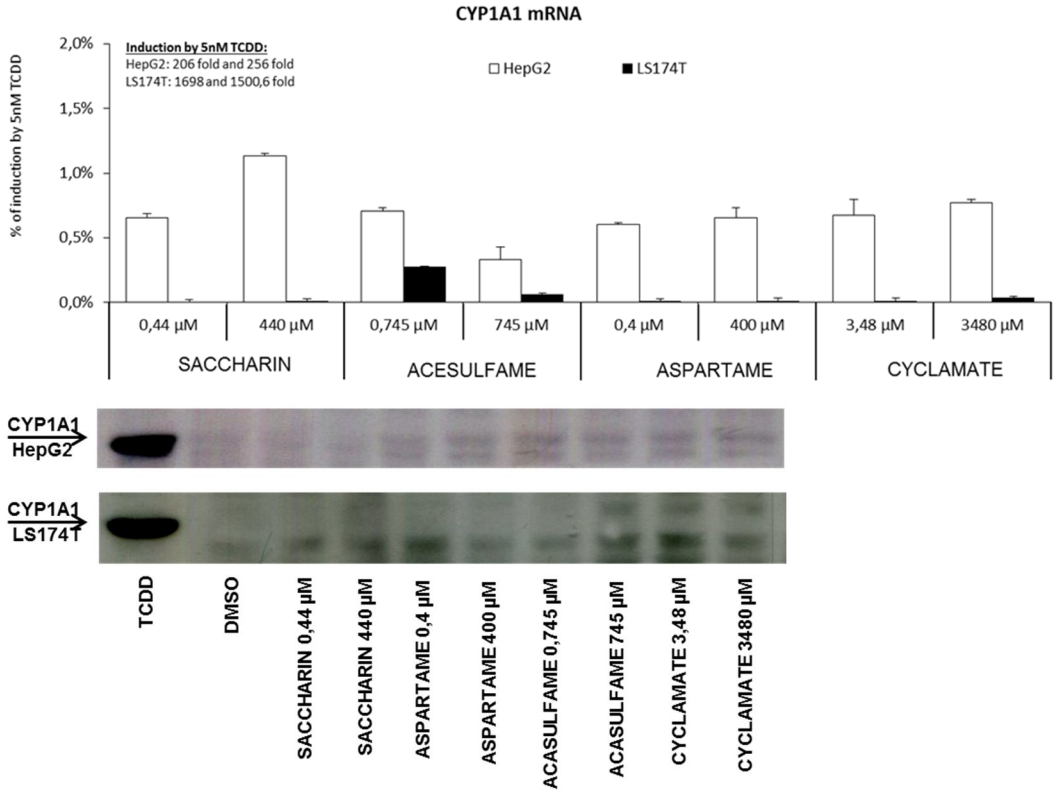

Figure 27. Effects of sweeteners on CYP1A1 expression in LS174T intestinal and HepG2 hepatic cancer cells. LS174T cells and HepG2 cells were treated for 24 h and 48 h with artificial sweeteners at different concentrations, 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1% v/v). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNA after 24 h of incubation (3 independent experiments were performed). The data are the means of duplicate measurements and are expressed as a percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels. Western blot shows a representative analysis of CYP1A1 protein after 48 h of incubation. Similar profiles were observed in two independent experiments. As a loading control, the blots were probed to actin (data not shown).

5.5.2 Effects of artificial sweeteners on CYP1A1 expression in primary cultures of human hepatocytes

In the next series of experiments, the effects of sweeteners were tested on the expression of CYP1A1 mRNA and protein in primary cultures of human hepatocytes. Four different primary human hepatocytes cultures were used (LH47, LH49, LH50 and HEP220708). Dioxin strongly induced the expression of CYP1A1 mRNA in all human hepatocytes cultures after 24 h of incubation (105-fold, 152-fold, 221-fold and 882-fold). Accordingly, TCDD caused drastic induction of CYP1A1 protein after 48 h of incubation in all human hepatocytes cultures. The levels of CYP1A1 mRNA and protein in four human hepatocytes cultures were not significantly altered by tested sweeteners, also implying the inactivity of metabolites originating from sweeteners (Figure 28). Given no induction of protein of mRNA by artificial sweeteners, representative results show only two cultures of human hepatocytes (LH47 and LH50).

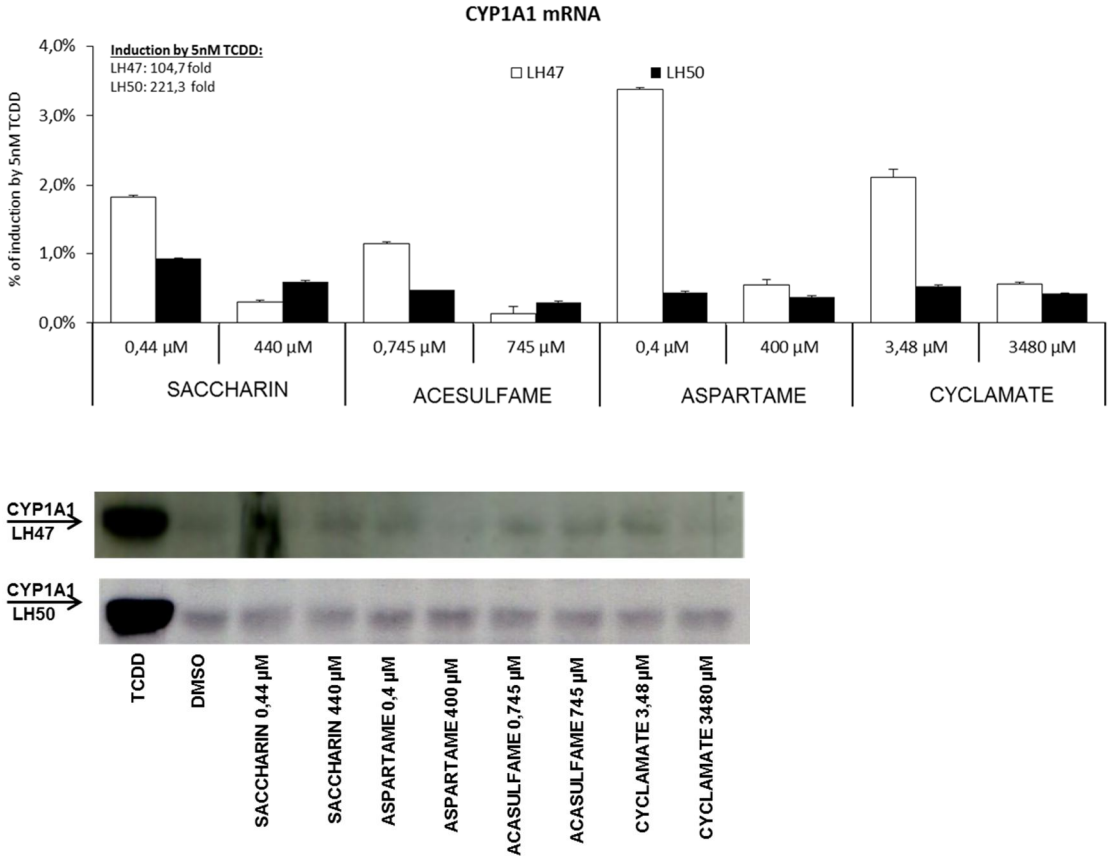

Figure 28. Effects of sweeteners on CYP1A1 expression in human hepatocytes. Hepatocytes were treated for 24 h and 48 h with artificial sweeteners at different concentration, 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1% v/v). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNA after 24 h of incubation in LH47 and LH50 cultures of hepatocytes (similar profile was observed in four independent cultures). The data are the means of duplicate measurements and are expressed as a percentage of maximal induction attained in TCDD-treated cells. The data were normalized per GAPDH mRNA levels. Western blot shows a representative analysis of CYP1A1 protein after 48 h of incubation. Similar profiles were observed in four independent cultures. As a loading control, the blots were probed to actin (data not shown).

5.5.3 Effects of artificial sweeteners on transcriptional activity of AhR in AZ-AHR reporter cell line

The effects of artificial sweeteners were examined on transcriptional activity of the AhR in recombinant AZ-AHR cells. The cells were incubated for 24 h with sweeteners (aspartame, acesulfame, cyclamate, saccharin) or vehicle (0.1% v/v DMSO), in the presence of ligand (5 nM TCDD). In three independent experiments, luciferase activity in AZ-AHR cells was increased 1011-fold, 181-fold and 153-fold by TCDD (5 nM), whereas no significant induction of luciferase activity was observed with saccharin, aspartame, acesulfame or cyclamate (Figure 29).

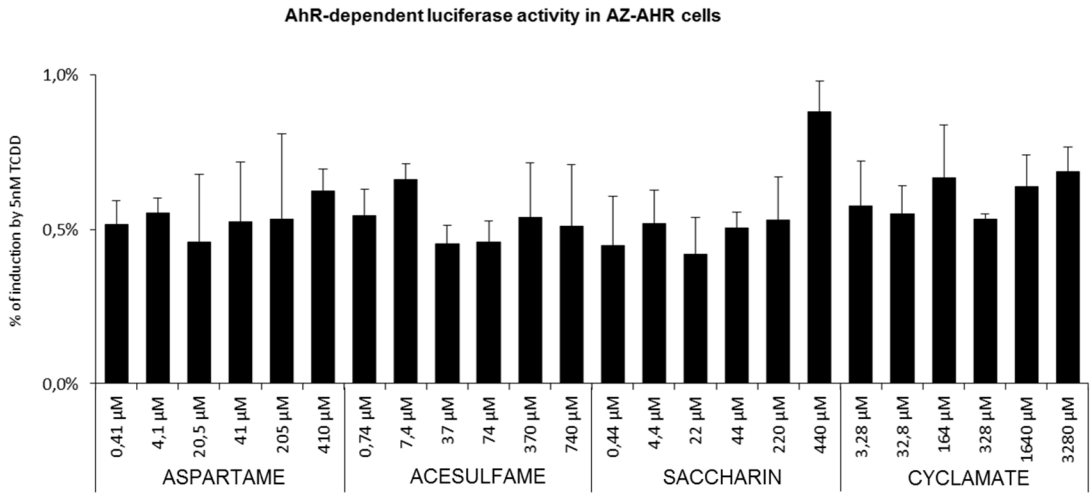

Figure 29. Effects of sweeteners on transcriptional activity of AhR in AZ-AHR transgenic cell line. AZ-AHR cells were plated at 96-well plates and stabilized for 16 h. Thereafter, cells were treated for 24 h with four artificial sweeteners at different concentration, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1%, v/v). After the treatments, cells were lysed and luciferase activity was measured. The data are the means of triplicate measurements and are expressed as a percentage of the induction attained by TCDD. Experiments were performed in three different passages of AZ-AHR cells. The differences between individual measurements were lower that 5%.

6. DISCUSSION

The phenomenon of food-drug interactions has emerged in the last few years. These interactions occur when the consumption of a particular food modulates the activity of drug-metabolizing enzymes, resulting in an alteration of the pharmacokinetics of drugs. While drug-drug interactions may be effectively managed by a physician, the food-drug interactions are not subject to such control. Oral prescription drugs taken concurrently with a meal alter the physicochemical conditions within the gastrointestinal tract, and may affect the rate/or extent of drug absorption with clinical significance, because drug bioavailability varies with drug properties and meal characteristics (Davit et al., 2008). As the majority of xenobiotics that enter the body are food components rather than pharmaceutical agents, it is reasonable to predict that some dietary constituents will also be capable of modulating drug-metabolism. Just as metabolic drug-drug interactions can have dramatic clinical consequences, resulting in severe adverse events or treatment failure (Honig et al., 1993; Raschetti et al., 1999; Szoka and Edgren, 1988), metabolic food-drug interaction also have thewhypotential for such effects.

Food that contains complex mixtures of phytochemicals, such as fruits, vegetables, herbs, spices and teas, have the greatest potential to induce or inhibit the activity of drug-metabolizing enzymes. Induction of drug-metabolizing enzymes occurs via activation of nuclear receptors (e.g. vitamin D receptor-VDR, farnesoid X receptor-FXR), receptors for steroid hormones (e.g. glucocorticoid receptor-GR, estrogen receptor-ER) (Monostory and Dvorak, 2011) or xenoreceptors (e.g aryl hydrocarbon receptor-AhR or pregnane X receptor-PXR) (Pavek and Dvorak, 2008). Activation of nuclear, steroid and/or xeno-receptors by xenobiotics, such as prescription drugs and food constituents can have a plethora of physiological and/or pathophysiological consequences other than induction of drug-metabolizing enzymes. For instance, AhR is a key transcriptional regulator of phase I enzymes (CYP1A1, CYP1A2, CYP1B1) and phase II enzymes (GSTA1, UGT1A2). On the other hand, AhR plays various physiological roles (e.g. in immune response, in cellular proliferation and differentiation) (Haarmann-Stemmann et al., 2012) and pathophysiological roles (e.g. in chemically induced carcinogenesis) (Abel and Haarmann-Stemmann, 2010). Given the complex role of AhR in the toxicological response and in human physiology and pathophysiology, it is of topical interest to examine the effects of xenobiotics, including food constituents, on the activity of AhR.

In the present study, we examined the effects of 17 ready to drink teas (RDTs), 28 flavored mineral waters (MWs), 6 major anthocyanidins, 21 anthocyanins and 4

artificial sweeteners on the AhR-CYP1A1 signaling pathway. *In vitro* models of primary cultures of human hepatocytes and cancer cell lines (HepG2 and LS174T) were chosen. Three main experimental approaches were used: (i) gene reporter assays in stably or transiently transfected cancer cell lines, allowing the examination of xenobiotic effects on AhR transcriptional activity; (ii) measurement of CYP1A1 mRNA and protein levels in cell lines, allowing assessment of the functional consequences of AhR activation with regard to drug metabolism; (iii) measurement of CYP1A1 mRNA and protein levels in primary cultures of human hepatocytes, allowing evaluation of the xenobiotics and their metabolites - metabolic activation/inactivation. The foregoing implies that while the data from each model may differ, the combination of these approaches increases the probability of fair evaluation of the xenobiotic effects.

The results reported in *Food Chemistry* in 2012 (appendix I.) firstly showed that some ready to drink teas were able to increase CYP1A1 activity. Consistent data were obtained for two samples, TAT1 (Green tea + lemon) and TAT5 (Red tea + pear), both from the brand Aquila. These RDTs induced CYP1A1 mRNA in HepG2 cells and human hepatocytes, and activated AhR in HepG2 cells transfected with *p1A1-luc* plasmid. The protein levels of CYP1A1 were not significantly altered. We found no significant changes in CYP1A1 induction by the remaining 15 samples of RDTs, suggesting that the elevated level of mRNA and increased activity of AhR by TAT1 and TAT5 were due to the specific content of these products. Several *in vitro* studies have shown the effects of food polyphenols (citrus fruits, isoflavones and flavones) on AhR, resulting in its activation (agonist/antagonist) mode (Amakura et al., 2008) and which could be the case for the RTDs.

In the study published in *Food and Chemical Toxicology* in 2012 (appendix II.), we found that the following four samples of flavored mineral waters (MWs) activated AhR and/or induced CYP1A1: MWDV10 (DOBRA VODA; lemon, green tea, passion-flower), MWDV12 (DOBRA VODA; grapefruit, ginseng, guarana), MWPO1 (PODEBRADKA; lemon) and MWPO9 (PODEBRADKA; passion-flower). There were some discrepancies between the data from human cancer cell lines and human hepatocytes, probably due to the extensive metabolism of mineral water constituents in hepatocytes. These results suggest, as well as those from RDTs´, that some active constituents of MWs´ have the ability to activate the AhR receptor with subsequent induction of CYP1A1 at the levels of mRNA and protein. We observed no significantly consistent changes caused by the other 24 MWs and their interference with the AhR-CYP1A1 signaling pathway.

The study published in *Toxicology Letters* in 2013 (appendix III.) by our research team with Professor Michael Denison from University of California in Davis, USA, was

focused on the effects of anthocyanidins on AhR-dependent gene expression and on CYP1A1/2 expression, production and enzymatic activity. Analysis of the inhibition of CYP1A1/2 activity revealed that the effects of the majority of these compounds on the metabolic pathways mediated by CYP1A1/2 enzymes are unlikely. However, pelargonidin and delphinidin were shown to interfere with the CYP1A1/2 drug metabolizing system (Figure 20), although this effect occurred at concentrations higher than those corresponding to the average values reached in human plasma after fruit or juice consumption, i.e. 274 nmol/L and 1220 ng/L for pelargonidin (Mullen et al., 2008) and delphinidin glycosides (Frank et al., 2005), respectively. Since CYP1A enzymes are responsible for activation of many pro-carcinogens (e.g. polycyclic aromatic hydrocarbons) and detoxification of many others (Anzenbacher and Anzenbacherova, 2001; Monostory et al., 2009), interference by anthocyanidins present in food with these enzymes may be even beneficial. The major finding of this study is that pelargonidin is a weak ligand/agonist of the AhR receptor, as revealed by ligand binding assay in guinea pig cytosols (Figure 19) and gene reporter assays in recombinant human AZ-AHR cells (Figure 18), respectively. Pelargonidin also induced CYP1A1 mRNA, protein and catalytic activity in human hepatic HepG2 and human intestinal LS174T cancer cells (Figure 15) and also induced CYP1A1 mRNA in three different primary human hepatocytes cultures (Figure 16). The levels of CYP1A proteins were slightly elevated by 50 μM pelargonidin in human hepatocytes (Figure 17). While we also observed slight induction of CYP1A1/2 mRNAs in human hepatocytes by 50 μM cyanidin, these effects were not consistent, possibly due to the metabolic transformation of cyanidin in human hepatocytes. Other anthocyanidins did not display significant or systematic effects on AhR transcriptional activity or CYP1A1 expression. Regarding structure–activity relationship, pelargonidin is the only anthocyanidin mono-substituted (monohydroxylated) at the phenyl group bound at 2-position of the chromenylium backbone. Speculatively, this feature may be one of the explanations for the unique activity of pelargonidin towards the AhR because other anthocyanidins are either di-substituted (cyanidin, peonidin) or tri-substituted (malvidin, petunidin, delphinidin) (Figure 4, Table2). Taken together, pelargonidin activates AhR through a ligand dependent mechanism and induces CYP1A and AhR-dependent reporter gene expression in human cancer cell lines and human hepatocytes, which may be of toxicological significance given the multiple roles of the AhR in the human organism.

An ongoing study, published in *Toxicology Letters* in 2013 (appendix IV.), with Professor Denison investigated the effects of 21 major anthocyanins and 3 extracts from food supplements containing anthocyanins. The amounts of anthocyanins and

other phenolics in food supplements are sometimes so high that their consumption results in intestinal or plasma concentrations an order of magnitude much higher than those attained by consumption of the fresh fruits. Indeed, one food supplement SUP-2 (Urinal Akut®, Tablets as 36 mg of proanthocyanidins Walmark a.s., Czech Republic), used in the current study, contains in one tablet, an amount of extract equivalent to 338,400 mg of fresh cranberries, as declared by the manufacturer. The data show that only two anthocyanins of the 21 tested, displayed any activity toward the AhR–CYP1A1 signaling pathway. This was the case of pelargonidin-3-O-rutinoside (PEL-2) and cyanidin-3,5-O-diglucoside (CYA-3), which dose-dependently activated the AhR, as revealed by gene reporter assay. In addition, these two compounds induced CYP1A1 mRNA but not protein in HepG2 and LS174T cells. The effects of PEL-2 and CYA-3 on the AhR occurred by ligand-dependent and ligand-independent mechanisms, respectively, as demonstrated by ligand binding assay. The effects of PEL-2 are analogical to those observed for pelargonidin. Neither compound nor extract from food supplements induced CYP1A1 mRNA or protein in four different primary human hepatocytes cultures. This is an important finding given that examination in primary human hepatocytes comprises both maternal compounds and metabolites (Vanzo et al., 2011). In line with these results, CYP1A1 enzyme activity was not prominently influenced by tested anthocyanins. Inhibition of CYP1A1 was concentration-dependent; however, it did not reach 50% even at the highest anthocyanin concentration, i.e. 100 μM. Conjugation with sugar part, however, lowered the inhibition of enzyme activity as shown in this study and led to less prominent activation of the AhR. Collectively, tested anthocyanins and the extracts from food supplements that contain anthocyanins possessed very low, if any, potential for food–drug interactions with respect to the AhR–CYP1A1 pathway. This result is – taken from the consumer's point of view – important for the safety of their use.

In the study published in *Toxicology In Vitro* in 2013 (appendix V.), the most common artificial sweeteners (saccharin, aspartame, cyclamate and acesulfame K) were tested for their potential effects on AhR-CYP1A1 signaling pathway. The studied sweeteners are components of regularly used food and beverages and chosen for analysis for this reason. We found that the tested sweeteners did not influence the basal and ligand-inducible transcriptional activity of AhR, as was revealed by gene reporter assay. The expression of CYP1A1 mRNA and protein was not induced by any of the sweeteners tested in primary human hepatocytes or in human intestinal LS174T and hepatic HepG2 cancer cells. These results suggest that the use of aspartame, acesulfame, cyclamate and saccharin in foods may be considered safe, with regard to effects on CYP1A1 induction and activation of the AhR receptor.

Activation of AhR and induction of CYP1A1 and CYP1A2 by xenobiotics may have several physiological and/or clinical consequences: (i) food–drug interaction, i.e. induction of CYP1A enzymes alter the pharmacokinetic of the drug(s) taken by a patient. This may decrease/increase the efficacy of the drug as well as its side effects (toxicity). Hence, an undesired or even dangerous clinical situation may occur (Sergent et al., 2009). (ii) Chemically-induced carcinogenesis, i.e. active AhR and elevated activities of CYP1A1 and CYP1A2 increase the risk of chemically-induced cancers (Vondracek et al., 2011). (iii) Perturbation of intermediary metabolism; i.e. drug-metabolising enzymes CYP1A1 and CYP1A2 are also involved in intermediary metabolism, in particular in the metabolism of eicosanoids and retinoids (Benisek et al., 2011). (iv) Others; e.g. AhR, controls the expression of phase II enzymes and transporters. AhR is involved in a variety of cell functions, including the immune response, differentiation and apoptosis. Hence, the activation of AhR may have effects on all the cell functions controlled by AhR (Abel and Haarmann-Stemmann, 2010). On the other hand, the majority of drug–drug interactions, and probably also food–drug interactions are caused by an inhibition of the enzyme activity by a drug or food constituent, respectively. Therefore, the AhR–CYP1A signaling pathway may be influenced by constituents from the studied compounds at the level of drug-metabolizing enzymes activities, as well.

Overall, with increasing consumption of prescribed drugs, and with increasing use of over-the-counter products containing natural and synthetic xenobiotics, the phenomenon of food-drug interactions is of growing importance. For this reason, drinking flavored mineral waters and RDTs as well as using dietary supplements in excessive amounts or on a daily basis should be considered with prudence by people on regular medication. This study also emphasizes the need for careful investigation of the potential benefits and adverse effects before making recommendations regarding dietary supplements and the importance of educating the consumer on potential drug interactions, as reported by Rapaka and Coates (2006).

7. CONCLUSION

The present thesis evaluated the effects on the AhR-CYP1A1 signaling pathway of 17 ready to drink teas, 28 mineral waters, 6 anthocyanidins, 21 anthocyanins and 4 artificial sweeteners. *In vitro* models were used for studying the potential interaction of food constituents with the AhR receptor and CYP1A1/2 induction. The activity of AhR was increased by two ready to drink teas, four mineral waters, pelargonidin, pelargonidin-3-O-rutinosid, cyanidin-3,5-diglucoside which was revealed by reporter gene assay. The RT-PCR analysis revealed increased induction of CYP1A/2 genes at the mRNA level by the same compounds and immunoblotting method confirmed the elevated level of CYP1A1 protein after treatment by these substances as well.

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9. CURRICULUM VITAE

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Education

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Postgraduate studies of molecular and cell biology in the Department of Cell Biology and Genetics, Faculty of Science, Palacky University in Olomouc

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Thesis topic: Target identification of potential antitumor drugs inducing changes in the cell cycle.

Completed study 2005-2008:

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Thesis topic: Screening of potential antitumor drugs inducing changes in the cell cycle.

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Practical courses from cell biology II

Practical courses from modern cellular studies

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Short-term research fellowship in Barcelona, Institute of Research in Biomedicine, Laboratory of molecular medicine (3 months).

November-December 2013-January 2014
Short-term research fellowship in Granada, Biomedical Research Center, University of Granada (3 months).

Participation in projects (principal investigator)

FRVŠ 80/2012/G3 – Implementation of Practical courses from modern cellular studies

Participation in projects (researcher)

P303/12/G163 – Center of Excellence: Centre of Drug-Dietary Supplements Interactions and Nutrigenetics. Czech Scientific Foundation (2012 – 2018)

OPVK CZ 1.07/2.2.00/07.0354 – Innovation studies of molecular and cellular biology (2009 – 2012)

13-01809S – Enantiospecific interactions between clinically used drugs and regulatory pathways of human cytochromes P450 (2013-2017)

GACR 503/10/0579 – Study interaction of content elements in soft drinks with regulatory pathway of drug metabolism and carcinogenesis (2010-2012)

OPVK CZ.1.07/2.3.00/20.0062 – Antabuse (disulfiram) as a pilot case of nonprofit drug, (2011 – 2014)

Publications

Kamenickova A., Vrzal R., Dvorak Z. (2012) Effects of ready to drink teas on AhR- and PXR-mediated expression of cytochromes P450 CYP1A1 and CYP3A4 in human cancer cell lines and primary human hepatocytes. *Food Chem.* 131(4):1201–1206 **[IF_{2012} 3.34]**

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Novotna A., **Kamenickova A.**, Dvorak Z.: Identification of enantiospecific interactions between clinically used chiral drugs and AHR using in vitro stable transfected luciferase reporter gene cell line AZ-AHR. Abstracts of the 49th Congress of the European Societies of Toxicology (EUROTOX), Interlaken, Switzerland, 28.-31.8. 2011. *Toxicology Letters* 221(S1):p. S232

APPENDIX I

Kamenickova A., Vrzal R., Dvorak Z. (2012) Effects of ready to drink teas on AhR- and PXR-mediated expression of cytochromes P450 CYP1A1 and CYP3A4 in human cancer cell lines and primary human hepatocytes. *Food Chem.* 131(4):1201–1206

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Effects of ready to drink teas on AhR- and PXR-mediated expression of cytochromes P450 CYP1A1 and CYP3A4 in human cancer cell lines and primary human hepatocytes

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ABSTRACT

A variety of xenobiotics are taken in the diet and they can interfere with regulatory pathways of drug metabolizing enzymes in humans. This can result in food–drug interactions, which is undesirable clinical situation where drug pharmacokinetics are influenced by dietary compounds. Xenobiotics-mediated food–drug interactions include the induction of drug metabolizing cytochromes P450. The expression of the most important inducible cytochromes CYP1A and CYP3A4 are regulated by xenoreceptors PXR and AhR.

We examined extracts from 17 different flavoured ready to drink teas (RDTs) for their capabilities to activate PXR and AhR receptors and to induce CYP3A4 and CYP1A genes. Primary cultures of human hepatocytes and cancer cell lines HepG2 and LS174T were used as *in vitro* models. Gene reporter assays, RT-PCR and Western blots were performed.

We identified three RDTs that induced CYP3A4 mRNA and protein, implying a potential for food–drug interactions. Several RDTs slightly elevated CYP1A1 expression or activated AhR.

1. Introduction

Inhibition of catalytic activities and induction of gene expression of drug-metabolizing cytochromes P450 (CYP) is the main cause of drug–drug interactions. For these reasons, each new drug is tested for its capability to inhibit or to induce CYP, prior to its introduction to the market. On the other hand, food (fruits, vegetables, spices, etc.), beverages (teas, sodas, etc.) and OTC products (food supplements, para-pharmaceuticals) contain a variety of xenobiotics (natural or synthetic), which may interfere with drug-metabolizing enzymes, causing both inhibitions and inductions. Striking examples of food constituents causing inhibition and induction of P450 with clinical consequences are furocoumarins from grapefruit juice (Girennavar, Jayaprakasha, & Patil, 2007) and hypericin from St. John's Wort preparations (Kober, Pohl, & Efferth, 2008), respectively. However, the attention to the food, beverages or OTCs consumed by patients is not systematically paid, as compared to drugs. In last few years, new phenomenon of food–drug or drug–food interactions is seriously considered (Berginc & Kristl, 2011; Genser, 2008; Mandlekar, Hong, & Kong, 2006).

Iced teas, also called ready to drink teas (RDTs), are popularly consumed non-alcoholic beverages. There are marketed different types of RDTs, (e.g. green, white, black, red) that are enriched with extracts from various fruits (e.g. lemon, apricot, lemon, peach, apple, pear, mango) and supplemented with stabilizers, synthetic aromas, flavours, etc. Therefore, this is clear that RDTs contain many natural and synthetic xenobiotics.

Drug–drug and food–drug interactions involving induction of CYP enzymes are mediated mainly via xenoreceptors pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR). Upon the activation by a ligand (drug, food constituent), xenoreceptors AhR and PXR transcriptionally up-regulate the expression of CYPs. The induction of CYP1 genes (CYP1A1, CYP1A2, CYP1B1) is regulated by AhR, while the induction of CYP2 (CYP2A6, CYP2B6, CYP2C8/9) and CYP3A4 is regulated by PXR (Pavek & Dvorak, 2008).

In the current paper, we have prepared the extracts from 17 different RDTs (three different brands) marketed in the Czech Republic. We have examined the effects of these extracts on AhR- and PXR-mediated expression of CYP1A1 and CYP3A4 enzymes in human hepatic HepG2 cells, human intestinal LS174T cells and in primary cultures of human hepatocytes. The methods of RT-PCR

Abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; GR, glucocorticoid receptor; HepG2, human Caucasian hepatocellular carcinoma cell line; LS174T, human colon tumour cell line; PXR, pregnane X receptor; RDT, ready to drink tea; RIF, rifampicin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; VDR, vitamin D receptor.

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(mRNA expression), Western immunoblotting (protein analyses) and gene reporter assays were employed. We have identified two RDTs that strongly induced CYP3A4 mRNA and protein in primary human hepatocytes, and several RDTs that transactivated CYP1A1 promoter in HepG2 cells. We conclude that based on *in vitro* data, RDTs may be a cause of food–drug interactions, with clinical significance equipotent to drug–drug interactions.

2. Materials and methods

2.1. Materials

Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCycler FastStart DNA Master^{PLUS} SYBR Green I and FuGENE HD transfection reagent were from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). Rifampicin and DMSO were from Sigma–Aldrich (Prague, Czech Republic). All other chemicals were of the highest quality commercially available.

2.2. Preparation of samples from ready to drink teas

Ready to drink teas were purchased in various supermarkets in Olomouc city, Czech Republic. The extracts from 17 different RDTs were prepared as follows (for details of RDTs see Table 1): Aliquots of 250 ml were taken from four different bottles containing RDTs. The aliquots were mixed together, to get total sample volume of 1000 ml. The entire sample (1000 ml) was concentrated using vacuum evaporation down-to volume of 200 ml. Concentrated (acidic) sample was extracted twice with 2×200 ml of diethyl ether. Thereafter, sample was alkalized using concentrated sodium hydroxide to pH approximately 9–10. The extraction with 2×200 ml of diethyl ether was performed again. All the extracts, both acidic and alkaline, were pooled and evaporated to dryness. Dried extracts were dissolved in 1 ml of ethanol. The resulting ethanolic solutions were 1000× concentrated RDTs. In cell experiments, ethanolic samples were diluted 1000 times in the culture medium; hence the concentrations of RDTs' constituents in the cell cultures were identical to those in RDTs.

2.3. Human cancer cell lines

Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430) and human Caucasian colon adenocarcinoma cells LS174T (ECACC No. 87060401) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of foetal calf serum, 100 U/ml streptomycin, 100 μg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Human hepatocytes

Hepatocytes were prepared from liver tissue, resected from multiorgan donors. Tissue acquisition protocol was in accordance with the requirements issued by local ethical commission in the Czech Republic. Human liver tissue used in this study was obtained from two multiorgan donors: LH36 (male; 74 years) and LH37 (male, 28 years). In addition, Long-term human hepatocytes in monolayer Batch HEP220586 (Biopredic International, Rennes, France), were used. Culture medium was enriched for plating with 2% foetal calf serum (V/V). The medium was exchanged for a serum-free medium the day after and the culture was allowed to

Table 1
List of tested ready to drink teas (RDTs).

Abbreviation	Brand	Type	Carbohydrate	Fat	Protein	Others
TDV1	Dobra Voda	Green tea + lemon	80 g	0.2 g	0.2 g	Sugar, tea extract 5 g, natural aroma, citric acid, sodium benzoate, potassium sorbate, dimethyldicarbonate
TDV2	Dobra Voda	White tea + apricot	80 g	0.2 g	0.2 g	Sugar, tea extract 4 g, natural aroma, citric acid, sodium benzoate, potassium sorbate, dimethyldicarbonate
TDV3	Dobra Voda	Tea citron	72 g	0.1 g	0.1 g	Sugar, tea extract 2.1 g, natural aroma, citric acid, sodium citrate, sodium benzoate, potassium sorbate, dimethyldicarbonate
TDV4	Dobra Voda	Tea peach	72 g	0.1 g	0.1 g	Sugar, tea extract 2.1 g, natural aroma, citric acid, sodium citrate, sodium benzoate, potasium sorbate, dimethyldicarbonate
TAT1	Aquila Team	Green tea + lemon	n.a.	n.a.	n.a.	Sugar, green tea extract, natural aroma green tea + lemon, natural lemon juice, sodium benzoate
TAT2	Aquila Team	Black tea + lemon	n.a.	n.a.	n.a.	Sugar, citric acid, caramel dye, black tea extract, phosphoric acid, sodium benzoate, natural lemon aroma, vitamin C
TAT3	Aquila Team	Yellow tea + apple	n.a.	n.a.	n.a.	Sugar, yellow tea extract, citric acid, ascorbic acid, natural apple aroma, apple juice, sodium citrate, sodium benzoate
TAT4	Aquila Team	White tea + pomegranate	n.a.	n.a.	n.a.	Sugar, white tea extract, citric acid, ascorbic acid, natural pomegranate aroma, pomegranate juice, sodium citrate, sodium benzoate
TAT5	Aquila Team	Red tea + pear	n.a.	n.a.	n.a.	Sugar, red tea extract, citric acid, ascorbic acid, natural pear aroma, pear juice, sodium citrate, sodium benzoate
TAT6	Aquila Team	Black tea + peach	n.a.	n.a.	n.a.	Sugar, citric acid, caramel dye, black tea extract, phosphoric acid, sodium benzoate, natural peach aroma, vitamin C
TAT7	Aquila Team	Black tea + forest fruits	n.a.	n.a.	n.a.	sugar, natural forest fruits aroma, citric acid, caramel dye, black tea extract, sodium benzoate, vitamin C
TNE1	Nestea Vitao	White tea + apricot	63 g	0 g	0.3 g	Sugar, citric acid, sodium citrate, white tea extract 0.2%, apricot juice 0.1% of concentrate, aroma, ascorbic acid, polyphenols 700 mg
TNE2	Nestea Vitao	Red tea + pear	48 g	0 g	0.1 g	Sugar, citric acid, sodium citrate, roibois extract 0.1%, pear juice 0.1% of concentrate, aroma, ascorbic acid, polyphenols 440 mg
TNE3	Nestea Vitao	Green tea + aloe vera + strawberry	74 g	0 g	0.3 g	Sugar, malic acid, sodium citrate, strawberry aroma, aloe vera aroma, green tea extract 0.15%, strawberry juice 0.1% of conc., ascorbic acid, polyphenols 570 mg
TNE4	Nestea	Peach	69 g	0 g	0 g	Sugar, citric acid, sodium citrate, tea extract 0.1%, aroma, peach juice 0.1% of concentrate
TNE5	Nestea	Lemon	75 g	0 g	0 g	Sugar, citric acid, sodium citrate, tea extract 0.1%, aroma, lemon juice 0.1% of concentrate, ascorbic acid
TNE6	Nestea Vitao	Green tea + lemon	73 g	0 g	0.4 g	Sugar, citric acid, sodium citrate, green tea extract 0.2%, aroma, orange and lemon juice 0.2% of concentrate, ascorbic acid, polyphenols 800 mg

stabilize for an additional 24 h. Hepatocytes were treated for 24 h or 48 h with tested extracts, rifampicin (10 μM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5 nM) and/or vehicle (DMSO; 0.1% V/V). Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.5. Plasmids, transfections and gene reporter assays

A chimeric *p3A4-luc* reporter construct containing the basal promoter (−362/+53) with proximal PXR response element and the distal xenobiotic responsive enhancer module (−7836/−7208) of the CYP3A4 gene 5′-flanking region inserted to pGL3-Basic reporter vector was described (Goodwin, Hodgson, & Liddle, 1999). *p1A1-luc* plasmid containing 5′-flanking region (−1566–+73) of human CYP1A1 gene subcloned into the *Kpn*I–*Hind*III double-digested pGL3-Basic vector (Promega, Madison, WI) upstream of the firefly luciferase reporter gene was a generous gift from Dr. Robert Barouki (INSERM U490, Paris, France).

HepG2 and LS174T cells were transiently transfected by lipofection (FuGENE 6) with 300 ng/well of reporters (*p1A1-luc* or *p3A4-luc*) in 24-well plates, as we described elsewhere (Dvorak et al., 2008). Following 16 h of stabilization, the cells were treated for 24 h with tested extracts, rifampicin (10 μM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (EtOH; 0.1% V/V). After the treatments, cells were lysed and luciferase activity was measured and standardised per milligram of protein.

2.6. Protein detection and Western blotting

Total protein extracts were prepared as described elsewhere (Dvorak et al., 2007). SDS–PAGE gels (8%) were run on a Hoefer apparatus according to the general procedure. Protein transfer onto PVDF membrane was carried out. The membrane was saturated with 5% non-fat dried milk for 2 h at room temperature. Blots were probed with antibody against CYP1A1 (goat polyclonal; sc-1616), CYP3A4 (mouse monoclonal; sc-53850, HL3) and actin (goat polyclonal; sc-1616, 1–19), all purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit.

2.7. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent®. cDNA was synthesized according to common protocol, using M-MLV Reverse Transcriptase F-572 (Finnzymes) and random hexamers 3801 (Takara). qRT-PCR was carried out on Light Cycler apparatus (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP1A1, CYP1A2, CYP3A4 and GAPDH mRNAs were determined, as described elsewhere (Dvorak et al., 2008; Pavek et al., 2007). The measurements were performed in duplicates. Gene expression was normalised per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by delta–delta method.

2.8. Statistical analyses

Results were expressed as mean ± standard deviation. Pair Student's pair test was applied to all analyses.

3. Results

3.1. Effects of RDTs on CYP1A1 and CYP3A4 expression in HepG2 and LS174T cells

In the first series of experiments, we have examined the effects of RDTs on the expression of CYP1A1 and CYP3A4 mRNAs and proteins in human hepatocarcinoma cells (HepG2) and human colon cancer cells (LS174T). The cell models of hepatic and intestinal cells were chosen, because liver and intestine are primary organs with which RDTs come in contact. Cells were treated for 24 h and 48 h with ethanolic solutions obtained from RDTs (dilution 1000×), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and vehicle (ethanol; 0.1% V/V) as described in Section 2. Prototypic inducer of CYP1A genes TCDD strongly induced CYP1A1 mRNA in both HepG2 (fold induction approx. 165×) and LS174T (fold induction approx. 443×) cell lines. The expression of CYP1A1 mRNA in HepG2 and LS174T cells was only negligibly elevated in several samples from the panel of 17 different RDTs tested (Fig. 1). The increase in CYP1A1 mRNA expression did not exceed 2% as compared to the fold induction attained by TCDD (100%). The effects of RDTs on the expression of CYP1A1 protein in both HepG2 and LS174T were consistent with the data obtained at mRNA level (Fig. 1). The data imply low toxicological and food–drug interaction potential of RDTs in terms of CYP1A1 expression in hepatic and intestinal cancer cell lines. Noteworthy, the induction of both CYP1A1 mRNA and protein by TCDD was much stronger in LS174T cells as compared to HepG2 cells (Fig. 1). Hence, intestinal LS174T cells seem to be highly competent model for studying CYP1A1 expression by food constituents.

Since CYP3A4 is not inducible in HepG2 cells, we tested the effects of RDTs on CYP3A4 expression only in LS174T cells. Rifampicin (RIF), a model inducer of CYP3A4, increased the expression of CYP3A4 mRNA in LS174T cells approximately 8-fold. Slight increase of CYP3A4 mRNA was observed for TNE1, TNE5 and TNE6, while other RDTs had no effect (Fig. 2). We have detected CYP3A4 protein in LS174T cells, but we did not observe induction by RIF (Fig. 2). Therefore, the effects of RDTs were not tested at protein level.

Finally, the use of HepG2 and LS174T cells implies: (i) Cell lines do not express standard spectrum of biotransformation enzymes – i.e. the effects of RDTs' constituents, but not metabolites, are assessed. (ii) The expression of CYP1A1 and CYP3A4 is regulated mainly (but not exclusively) by AhR and PXR receptors, respectively. Hence, the interactions between RDTs constituents and other receptors may occur.

3.2. Effects of RDTs on PXR and AhR transcriptional activity in HepG2 and LS174T cells

In next series of experiments, we examined the effects of RDTs on transcriptional activities of AhR and PXR. We performed gene reporter assays in HepG2 and LS174T cells transiently transfected with *p1A1-luc* (AhR-responsive) and *p3A4-luc* (PXR-responsive) plasmids, as described in Section 2. Cells were treated for 24 h with ethanolic solutions obtained from RDTs (dilution 1000× and 10,000×), TCDD (5 nM), RIF (10 μM) and vehicle (ethanol; 0.1% V/V).

AhR-dependent luciferase activity in HepG2 transfected with *p1A1-luc* was strongly induced by TCDD, and the fold induction ranged between 12-fold and 65-fold. Dose-dependent activation of *p1A1-luc* was observed for samples TDV1, TDV2, TAT1, TNE1, TNE2, TNE3, TNE4, TNE5 and TNE6 (Fig. 3A). These effects are probably caused by various polyphenols, that are known as AhR partial agonists. PXR-dependent luciferase activity in LS174T cells

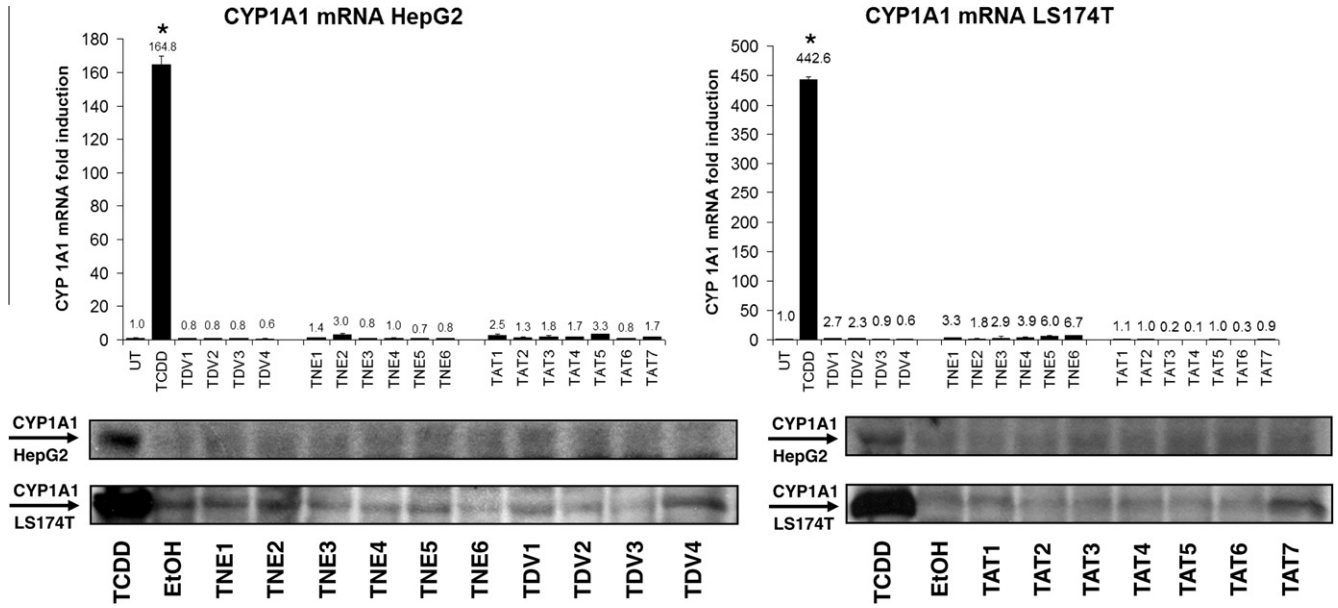

Fig. 1. Effects of RDTs on CYP1A1 expression in HepG2 and LS174T cells. Cell lines were treated with ethanolic solutions obtained from 17 different RDTs (dilution 1000×), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% V/V). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNA from HepG2 (left) and LS174T (right) cells. Treatment duration was 24 h. The data are mean ± SD from duplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). The data were normalised per GAPDH mRNA levels. * – value is significantly different from EtOH-treated cells ($p < 0.05$). Western blots show representative analysis of CYP1A1 protein from HepG2 (upper strips) and LS174T (lower strips) cells. Treatment duration was 48 h. Similar profiles were observed in three independent experiments (cell passages).

transfected with *p3A4-luc* was induced by RIF, and the fold induction ranged between 11-fold and 21-fold. Dose-dependent activation of *p3A4-luc* was observed for samples TDV1, TDV2, TDV3, TAT7, TNE1 and TNE4 (Fig. 3B).

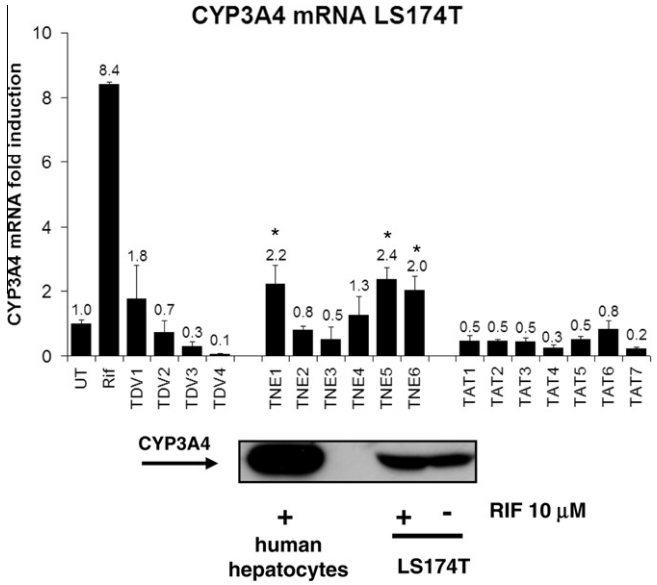

Fig. 2. Effects of RDTs on CYP3A4 expression in LS174T cells. Cells were treated with ethanolic solutions obtained from 17 different RDTs (dilution 1000×), rifampicin (RIF; 10 μM) and vehicle (ethanol = UT; 0.1% V/V). Bar graph shows representative RT-PCR analyses of CYP3A4 mRNA. Treatment duration was 24 h. The data are mean ± SD from duplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). The data were normalised per GAPDH mRNA levels. * – value is significantly different from EtOH-treated cells ($p < 0.05$). Similar profiles were observed in three independent experiments (cell passages). Western blot shows analysis of CYP3A4 protein from LS174T cells treated for 48 h with EtOH and RIF; positive control of protein from RIF-treated human hepatocytes was loaded for control (left band).

The data obtained from cancer cell lines transiently transfected with *p1A1-luc* and *p3A4-luc* reveal about the capability of RDTs to activate xenoreceptors AhR and PXR, respectively. These data are of importance not only concerning up-regulation of drug-metabolizing enzymes, but also dysregulation of intermediary metabolism.

3.3. Effects of RDTs on CYP1A1 and CYP3A4 expression in primary cultures of human hepatocytes

In final series of experiments, we studied the effects of RDTs on the expression of CYP1A1 and CYP3A4 mRNAs and proteins in primary human hepatocytes. Experiments were performed in three different human hepatocytes cultures (for details see Section 2). Hepatocytes were treated for 24 h and 48 h with ethanolic solutions obtained from RDTs (dilution 1000×), TCDD (5 nM), RIF (10 μM) and vehicle (ethanol; 0.1% V/V).

TCDD strongly induced CYP1A1 mRNA and protein in all primary human hepatocytes cultures used (note: at Western blots, both CYP1A1 and CYP1A2 proteins are recognised by antibody in one huge band). We observed mild increase of CYP1A1 mRNA by TAT1 and TAT5; tested RDTs displayed similar effects on CYP1A1 mRNA expression in HepG2 cells (Fig. 1) and human hepatocytes (Fig. 4). The levels of CYP1A proteins were slightly elevated by TAT1 (Fig. 4).

Out of 17 RDTs tested, samples TNE2, TNE4 and TAT2 strongly induced CYP3A4 mRNA and protein. The strongest induction was attained by TNE2 (red tea with pear flavour), with potency of about 30% of RIF-treated hepatocytes (Fig. 4).

The data from human hepatocytes allow assessing the effects of RDTs, but also their metabolites on the expression of CYP1A1 and CYP3A4 enzymes. In addition, the involvement of AhR and PXR xenoreceptors and other CYP regulators (e.g. GR, VDR) can be assessed. Finally, unlike cancer cell lines, primary human hepatocytes are non-transformed cells that is a model much closer to physiological situation in man.

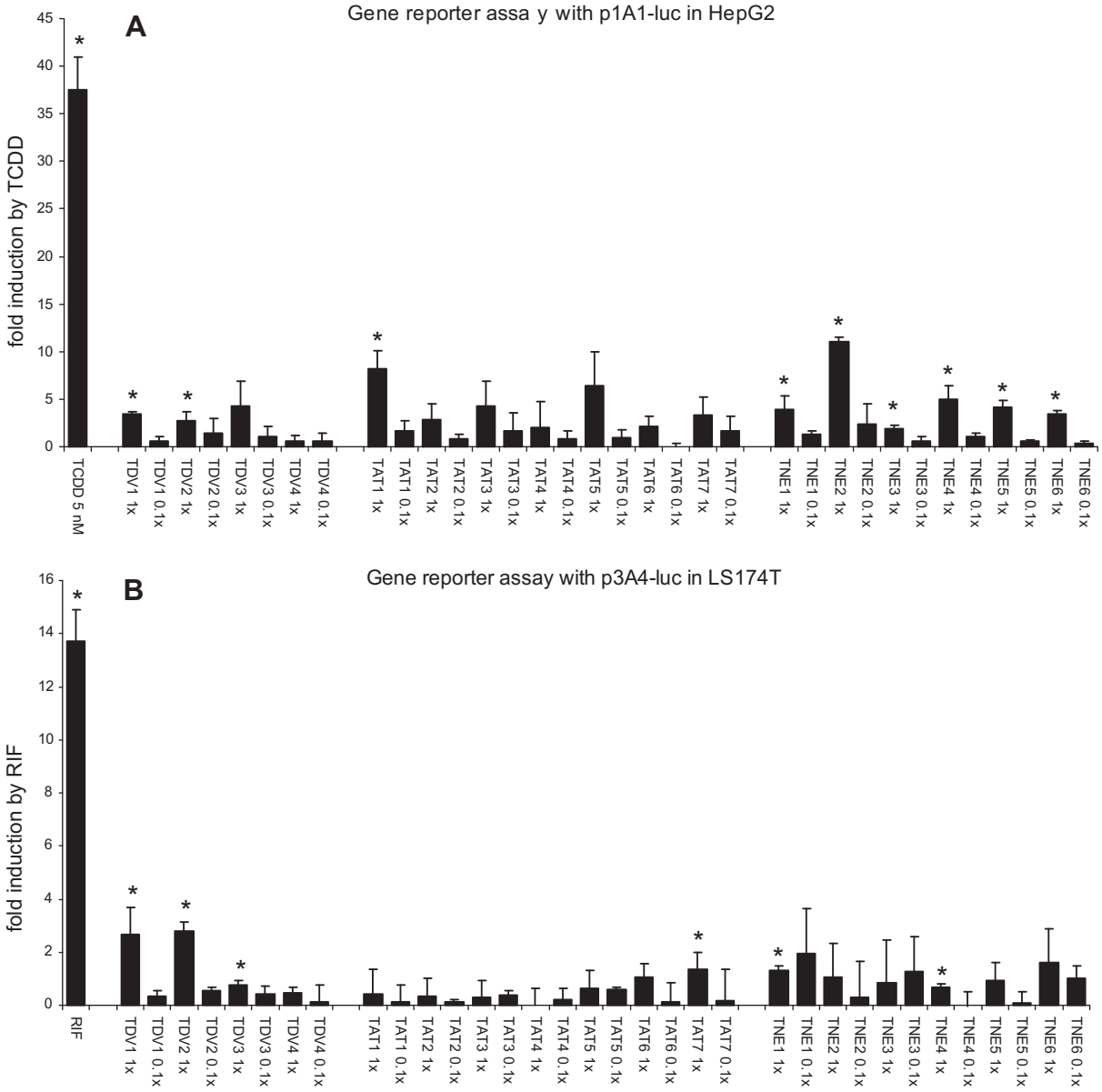

Fig. 3. Effects of RDTs on PXR and AhR transcriptional activity in HepG2 and LS174T cells. HepG2 or LS174T cells were transiently transfected by lipofection (FuGENE 6) either with 300 ng/per well of *p1A1-luc* reporter plasmid (Panel A), or with 300 ng/well of *p3A4-luc* reporter plasmid (Panel B), respectively. Following 16 h of stabilization, the cells were treated for 24 h with ethanolic solutions obtained from 17 different RDTs (dilution 1000× and 10,000×), rifampicin (RIF; 10 μM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (EtOH = UT; 0.1% V/V). After the treatments, cells were lysed and luciferase activity was measured and standardised per milligram of protein. The data are mean ± SD from triplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). * – value is significantly different from EtOH-treated cells ($p < 0.05$).

4. Discussion

In the present paper, we have examined the effects of 17 ready to drink teas (RDTs), marketed in the Czech Republic, on AhR- and PXR- transcriptional activities and on the expression of CYP1A1 and CYP3A4 genes. We used three experimental approaches: (i) gene reporter assays in transiently transfected cancer cell lines – this allows examining net effects of xenobiotics (RDTs) on AhR and PXR transcriptional activities (with implications in drug metabolism and intermediary metabolism); (ii) measurement of CYP1A1 and CYP3A4 mRNAs and protein levels in cancer cell lines – this allows to assess the involvement of other regulators beside xenoreceptors (e.g. VDR, GR); (iii) measurement of CYP1A1 and CYP3A4 mRNAs and protein levels in primary cultures of human hepatocytes – this allows to evaluate the effects of RDTs and their metabolites, i.e. metabolic activation/inactivation. This implies that the data obtained from each of the above models may differ and that the combination of these three approaches allows objective evaluation of the RTDs' effects.

We observed rather general effects of RDTs on AhR–CYP1A signalling pathway, in terms of moderate activation of AhR in gene reporter assays (probably due to the presence of polyphenols). Consistent data were obtained for samples TAT1 (Green tea + lemon) and TAT5 (Red tea + pear), both from brand Aquila Team (Table 1). These RDTs induced CYP1A1 mRNA in HepG2 cells and human hepatocytes, and activated AhR in HepG2 cells transfected with *p1A1-luc* plasmid. On the other hand, the levels of CYP1A1 protein were not significantly altered.

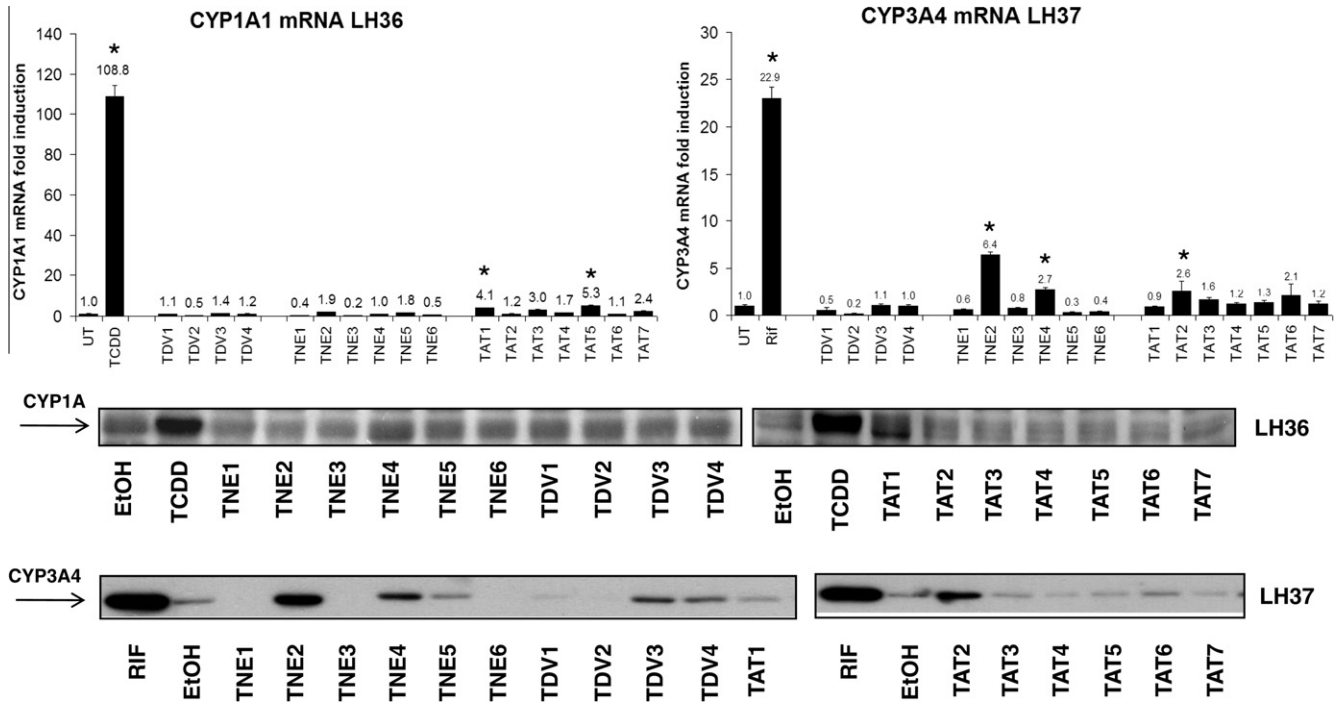

Fig. 4. Effects of RDTs on CYP1A1 and CYP3A4 expression in primary cultures of human hepatocytes. Cells were treated with ethanolic solutions obtained from 17 different RDTs (dilution 1000×), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and vehicle (ethanol = UT; 0.1% V/V). Bar graphs show representative RT-PCR analyses of CYP1A1 and CYP3A4 mRNAs from two human hepatocytes cultures. Treatment duration was 24 h. The data are mean ± SD from duplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). The data were normalised per GAPDH mRNA levels. * – value is significantly different from EtOH-treated cells ($p < 0.05$). Western blots show representative analysis of CYP1A1/2 and CYP3A4 proteins from two human hepatocytes cultures. Treatment duration was 48 h.

We found significant CYP3A4 mRNA induction in LS174T cells for TNE1, TNE5 and TNE6 samples. In addition, we found that RDTs TNE2 (Nestea Vitao; red tea + pear), TNE4 (Nestea Vitao; peach) and TAT2 (Aquila Team; black tea + lemon) strongly induced CYP3A4 mRNA and protein in primary human hepatocytes. The strongest induction was achieved by TNE2 (about 30% of induction in RIF-treated hepatocytes). Since the data from human hepatocytes for these three RDTs were not consistent with data from cells lines, it is likely that CYP3A4 induction was caused by metabolites from RDTs.

This paper is the first report describing the effects of ready to drink teas on AhR- and PXR-dependent expression of CYP1A1 and CYP3A4 in human models. The data obtained demonstrate that commonly marketed RDTs are capable to induce important enzymes involved in drug metabolism and chemically-induced carcinogenesis. Since many drugs metabolized by CYP3A4 are used chronically (hypolipidemics, antihypertensives, antipsychotics, anticonvulsants, etc.), attention should be paid on possible food–drug interactions originating from occasional or regular consumption of several RDTs.

Xenoreceptors PXR and AhR play many physiological function (Puga, Ma, & Marlowe, 2009) and they are important regulators of carbohydrade and lipid metabolism (Gouedard, Barouki, & Morel, 2004; Moreau, Vilarem, Maurel, & Pascussi, 2008). Therefore, another aspect of RDTs role in human health is possible perturbation of intermediary metabolism via PXR- and AhR-mediated transcription.

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APPENDIX II

Kamenickova A., Dvorak Z. (2012) Effects of flavored mineral waters on AhR–CYP1A1 signaling pathway in primary human hepatocytes and in human hepatic and intestinal cancer cells. *Food Chem Toxicol*. 50(6):1933-9

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Effects of flavored mineral waters on AhR–CYP1A1 signaling pathway in primary human hepatocytes and in human hepatic and intestinal cancer cells

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ABSTRACT

Food–drug interaction is an emerging phenomenon, comprising pharmacokinetic or toxicokinetic interactions between food constituents and drugs. The mechanisms include inhibition of enzymes and transporters, and induction of drug metabolizing enzymes. A prominent regulator of drug-metabolizing enzymes is an aryl hydrocarbon receptor (AhR) that transcriptionally regulates CYP1 enzymes, phase II enzymes and many other genes. In the current paper, we have examined the effects of 28 different flavored mineral waters on AhR–CYP1A1 signaling pathway in primary cultures of human hepatocytes and in human cancer cell lines HepG2 (hepatic) and LS174T (intestinal). The techniques of Western blot, RT-PCR and gene reporter assays were employed to determine the expression of CYP1A1 mRNA, protein and activation of AhR, respectively. We have identified four flavored mineral waters which activated AhR and/or induced CYP1A1. These data imply a potential of some flavored mineral waters to cause food–drug interactions. In addition, activation of AhR–CYP1A1 signaling may result in chemically-induced carcinogenesis and alteration of intermediary metabolism.

1. Introduction

In the recent years, a phenomenon of food–drug (or drug–food) interactions emerged. It became of great importance, because of increasing number of herbal preparations (e.g. teas, drops), food supplements and parapharmaceuticals are sold over-the-counter. Common foods and beverages contain natural compounds (e.g. terpenes, polyphenols, alkaloids), but also artificial constituents such as dyes, flavors, stabilizers, sweeteners, antioxidants, and aromas. Similarly, as in case of drug–drug interactions, food–drug interactions are caused by mutual interferences between a drug and a food constituent, resulting in altered pharmacokinetic of the drug, and undesired or even dangerous clinical situation. There are numerous reports on food–drug interactions, occurring by several mechanisms, including enzyme inhibition (e.g. furocoumarins from grapefruit juice) or enzyme induction (e.g. St. John's Worth preparations). The latter is caused by induction of drug-metabolizing enzymes such as cytochromes P450 or phase II enzymes. Transcriptional regulation of drug-metabolizing enzymes is controlled by xenoreceptors, i.e. pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR). In addition, several nuclear and steroid receptors are key regulators of P450s. Regarding food–drug interactions, of particular interest seems to be AhR, since it transcriptionally regulates CYP1A1 expression in human liver, but also in extrahepatic tissues, such as an intestine, placenta, skin and lungs (Pavek and Dvorak, 2008). CYP1A1 is the main extrahepatic form of CYP1A subfamily of genes, while CYP1A2 is hepatospecific enzyme. Both, CYP1A1 and CYP1A2 are involved in drug metabolism, but also in chemically-induced carcinogenesis (Vondracek et al., 2011) and metabolism of endogenous compounds such as eicosanoids or retinoids (Benisek et al., 2011). Flavored mineral waters are very popular in Czech Republic. They contain various fruit extracts (lemon, grapefruit, orange, pear, mango, etc.) and herb extracts (salvia, mellisa, chestnut, linden, wild-thyme, dog-rose, etc.). Therefore, we have examined the effects of 28 different flavored mineral waters marketed in the Czech Republic, on transcriptional activity of AhR and the expression of CYP1A1 and CYP1A2 mRNAs and proteins. We used human hepatic (HepG2) and intestinal (LS174T) cancer cell lines, and primary cultures of human hepatocytes. The techniques of gene reporter assays, RT-PCR and immunoblotting were employed. We have identified four flavored mineral waters that caused either activation of AhR and/or induction of CYP1A enzymes. The data obtained are the first report on the effects of flavored mineral waters on AhR–CYP1A signaling pathway, which has implications in pharmacokinetic of drugs, intermediary metabolism and chemically-induced carcinogenesis.

Abbreviations: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; MW, mineral water; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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Table 1
List of tested flavored mineral waters (MWs).

Abbreviations	Brand	Type	Others
MWDV1	DOBRA VODA	Raspberry	Vitamin C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV2	DOBRA VODA	Mango	Vitamin C 92 mg/L, sugar, citric acid, aroma
MWDV3	DOBRA VODA	Elderberry	Vitamin C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV4	DOBRA VODA	Lemon	Vitamin C 92 mg/L, sugar, citric acid, aroma, acidity regulator
MWDV5	DOBRA VODA	–	–
MWDV6	DOBRA VODA	Orange	Vitamin C 92 mg/L, sugar, citric acid, aroma
MWDV7	DOBRA VODA	Grapefruit	Vitamin C 92 mg/L, sugar, citric acid, aroma
MWDV8	DOBRA VODA	White table grapes	Vitamin C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV9	DOBRA VODA	Pear	Vitamin C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV10	DOBRA VODA	Lemon, green tea, passion-flower	Sugar, aroma, herb extract 2.5 g, sodium benzoate, aspartame, acesulfame K, citric acid, ascorbic acid
MWDV11	DOBRA VODA	Orange, violet, hawthorn, blueberry	Sugar, aroma, herb extract 4 g, sodium benzoate, aspartame, acesulfame K, citric acid, ascorbic acid
MWDV12	DOBRA VODA	Grapefruit, ginseng, guarana	Sugar, aroma, herb extract 1 g, sodium benzoate, aspartame, acesulfame K, citric acid, ascorbic acid
MWPO1	PODEBRADKA	Lemon	Citric acid, aroma, acesulfame A, aspartame
MWPO2	PODEBRADKA	Grapefruit	Citric acid, aroma, acesulfame A, aspartame
MWPO3	PODEBRADKA	Plum and elderberry	Citric acid, sodium citrate, L-carnitine 100 mg, acesulfame A, aspartame, fiber 1 g
MWPO4	PODEBRADKA	Sour cherry	Citric acid, aroma, acesulfame A, aspartame
MWPO5	PODEBRADKA	Pomelo and cranberry	Citric acid, sodium citrate, L-carnitine 100 mg, acesulfame A, aspartame, fiber 1 g
MWPO6	PODEBRADKA	Lime	Citric acid, aroma, acesulfame A, aspartame
MWPO7	PODEBRADKA	Orange	Citric acid, aroma, acesulfame A, aspartame
MWPO8	PODEBRADKA	Plum	Citric acid, sodium benzoate, aroma, glucose-fructose syrup
MWPO9	PODEBRADKA	Passion-flower	Citric acid, sodium benzoate, aroma, glucose-fructose syrup
MWRA1	RAJEC	Dandelion	Glucose syrup, citric acid, herb extract from dandelion 0.1 g, apple juice, aroma, potassium sorbate, sodium benzoate
MWRA2	RAJEC	Dog-rose	Glucose syrup, citric acid, herb extract from dog-rose 0.1 g, apple juice, aroma, potassium sorbate, sodium benzoate
MWRA3	RAJEC	Wild-thyme	Glucose syrup, citric acid, herb extract from wild-thyme 0.1 g, apple juice, aroma, potassium sorbate, sodium benzoate
MWRA4	RAJEC	Salvia	Glucose syrup, citric acid, herb extract from salvia 0.1 g, apple juice, aroma, potassium sorbate, sodium benzoate
MWRA5	RAJEC	Mellisa	Glucose syrup, citric acid, herb extract from mellisa 0.1 g, apple juice, aroma, potassium sorbate, sodium benzoate
MWRA6	RAJEC	Chestnut	Fructose, citric acid, herb extract from chestnut 0.1 g, aroma, sodium benzoate
MWRA7	RAJEC	Linden	Fructose, citric acid, herb extract from linden 0.1 g, aroma, sodium benzoate, potassium sorbate

2. Materials and methods

2.1. Materials

Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCyclerFastStart DNA Master^{PLUS} SYBR Green I was from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). All other chemicals were of the highest quality commercially available.

2.2. Preparation of samples from flavored mineral waters

Flavored mineral waters were purchased in various supermarkets in Olomouc City, Czech Republic. The extracts from 28 different MWs were prepared as follows (for details of MWs see Table 1). Total sample volume of 1000 mL was obtained by pooling the aliquots of 250 mL taken from four different bottles containing MWs. This sample (1000 mL) was concentrated using vacuum evaporation down-to volume of 200 mL. Concentrated (acidic) sample was extracted twice by liquid–liquid extraction, using 2×200 mL of diethyl ether. Thereafter, sample was alkalized using concentrated sodium hydroxide to pH approximately 9–10. The extraction with 2×200 mL of diethyl ether was repeated. Both extracts, acidic and alkaline, were pooled and evaporated to dryness. Dried extracts were dissolved in 1 mL of ethanol. The resulting stock solutions were 1000× concentrated MWs. In experiments, stock solutions were diluted 1000 times in the culture medium; hence the concentrations of MWs' constituents in the cell cultures were identical to those in MWs.

2.3. Human cancer cell lines

Human Caucasian colon adenocarcinoma cells LS174T (ECACC No. 87060401) and human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430) were purchased from ECACC and were cultured in as recommended by manufacturer.

2.4. Human hepatocytes

Human liver tissue used in this study was obtained from two sources: (i) from multiorgan donor LH38 (male; 58 years); 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 HEP220633 (male, 73 years) (Biopredic International, Rennes, France). Cells were cultured in a serum-free medium. Hepatocytes were treated for 24 or 48 h with tested extracts, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (EtOH; 0.1% v/v). Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.5. Gene reporter assays

Experiments were performed in stably transfected gene reporter cell line AZ-AHR, which was derived from HepG2 cells transfected with a construct containing several AhR binding sites upstream of luciferase reporter gene (Novotna et al., 2011). Following the plating, cells were stabilized for 16 h and then treated for 6 and 24 h with tested extracts, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (EtOH; 0.1% v/v). After the treatments, cells were lysed and luciferase activity was measured.

2.6. Protein detection and Western blotting

Total protein extracts were prepared as described elsewhere (Pavek et al., 2007). Following SDS–PAGE separation and Western-blot transfer, blots were probed with antibody against CYP1A1 (goat polyclonal; sc-1616), purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit.

2.7. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent®. cDNA was synthesized according to common protocol, using M-MLV Reverse Transcriptase F-572 (Finnzymes) and random hexamers 3801 (Takara). qRT-PCR was carried out on Light Cycler apparatus 480 II (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP1A1, CYP1A2 and GAPDH mRNAs were determined as described elsewhere (Dvorak et al., 2008). The measurements were performed in triplicates. Gene expression was normalized *per* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by delta–delta method.

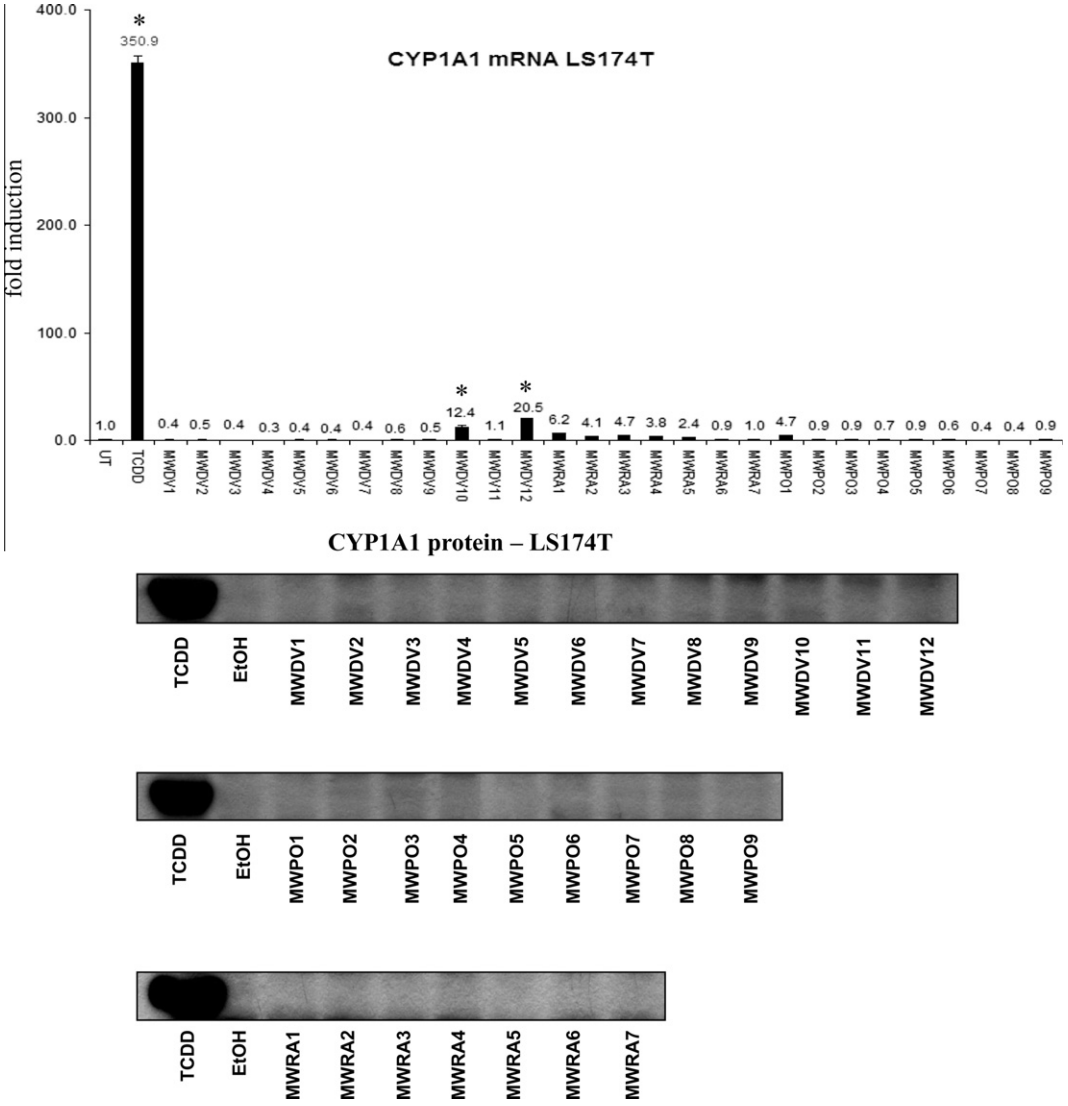

Fig. 1. Effects of mineral waters on CYP1A1 expression in LS174T intestinal cells. Cells were treated with ethanolic solutions obtained from 28 different MWs (dilution 1000×), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% v/v). Bar graph shows representative RT-PCR analyses (three independent experiments) of CYP1A1 mRNA after 24 h treatment. The data are mean ± SD from triplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). The data were normalized *per* GAPDH mRNA levels. ∗ Value is significantly different from EtOH-treated cells ($p < 0.05$). Western blots show representative analysis of CYP1A1 protein after 48 h treatment. Similar profiles were observed in three independent experiments (cell passages).

2.8. Statistical analyses

Results were expressed as mean ± standard deviation. Pair Student's pair test was applied to all analyses.

3. Results

3.1. Effects of mineral waters on CYP1A1 expression in LS174T human intestinal cells

Since intestinal cells are primary target of food constituents, we examined the effects of MWs on CYP1A1 expression in intestinal cell line LS174T. We treated cells for 24 h (mRNA expression) and 48 h (protein expression) with ethanolic solutions of extracts from 28 different MWs (dilution 1.000×), TCDD (5 nM) and vehicle (EtOH; 0.1% v/v). Dioxin (TCDD), a model activator of AhR a inducer of CYP1A1, increased the expression of CYP1A1 mRNA approximately 350-fold, as compared to vehicle-treated cells. Of the mineral waters tested, significant induction of CYP1A1 mRNA was achieved with samples MWDV10 (12-fold) and MWDV12 (20-fold) (Fig. 1). The level of CYP1A1 protein in LS174T cells treated for 48 h with TCDD was drastically increased. However, none of the MWs tested caused significant elevation of CYP1A1 protein level (Fig. 1).

3.2. Effects of mineral waters on CYP1A1 expression in HepG2 human hepatoma cells

The main organ of drug metabolism in human body is liver. Therefore, we examined the effects of MWs on CYP1A1 expression in human hepatoma cell line HepG2. The expression of CYP1A1 mRNA in HepG2 cells treated for 24 h with TCDD was induced approximately 800-fold, as compared to vehicle-treated cells (Fig. 2). Similarly, the level of CYP1A1 protein was strongly increased in HepG2 cells treated with TCDD for 48 h (Fig. 2). Unlike in LS174T cells, none of the tested MWs induced CYP1A1 mRNA or protein.

3.3. Effects of mineral waters on transcriptional activity of AhR in AZ-AHR reporter cell line

The expression of CYP1A1 is transcriptionally regulated by AhR. Therefore, inducers of CYP1A1 are mostly ligands of AhR. For this

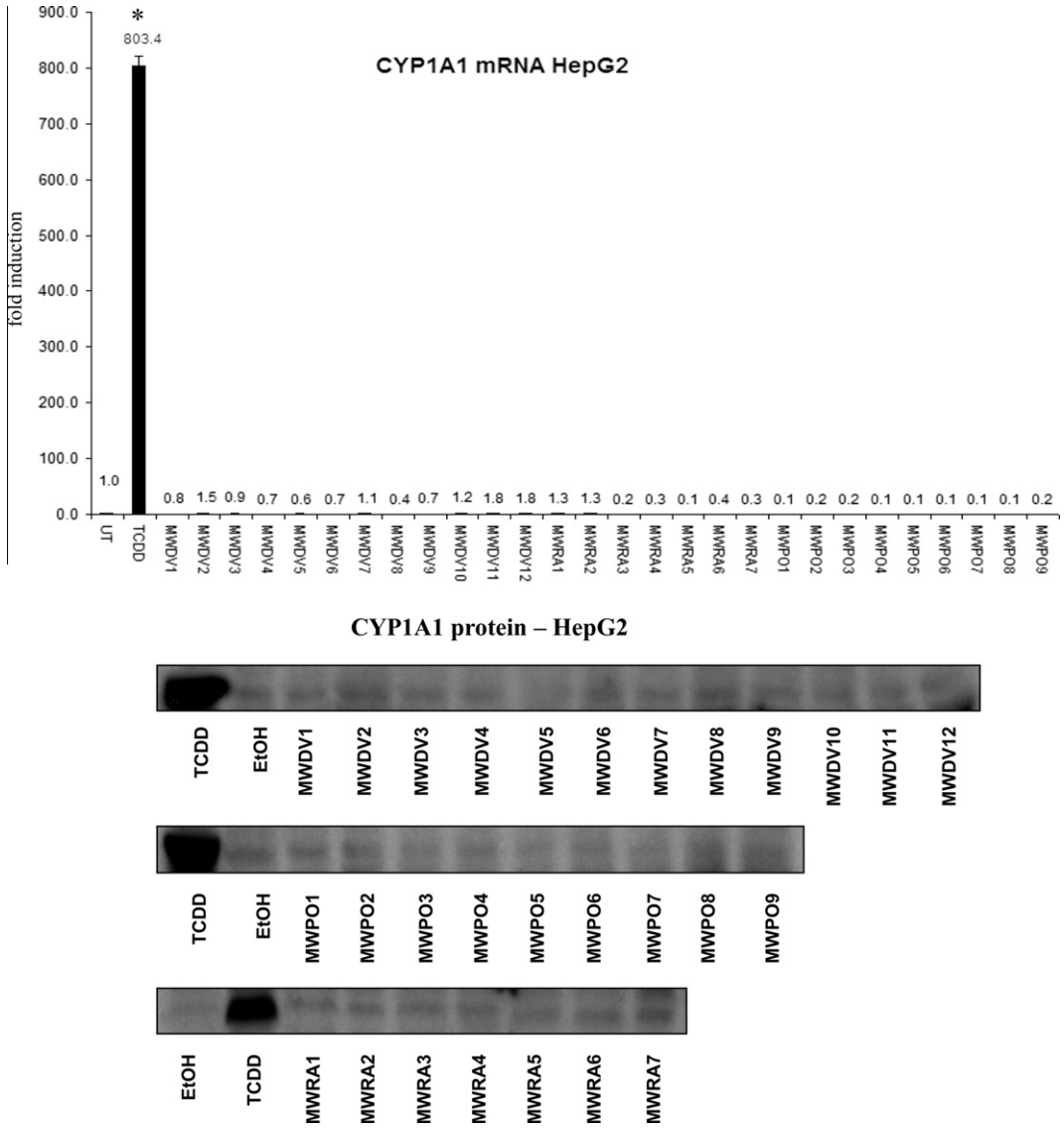

Fig. 2. Effects of mineral waters on CYP1A1 expression in HepG2 hepatoma cells. Cells were treated with ethanolic solutions obtained from 28 different MWs (dilution 1000×), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (ethanol = UT; 0.1% v/v). Bar graph shows representative RT-PCR analyses (three independent experiments) of CYP1A1 mRNA after 24 h treatment. The data are mean ± SD from triplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). The data were normalized *per* GAPDH mRNA levels. ∗ Value is significantly different from EtOH-treated cells ($p < 0.05$). Western blots show representative analysis of CYP1A1 protein after 48 h treatment. Similar profiles were observed in three independent experiments (cell passages).

reason, we have examined the effects of tested MWs in AZ-AHR stable reporter cell line derived from HepG2 cells, containing several dioxin-response elements up-stream of luciferase gene. We have constructed and characterized this cell line recently, and it allows screening for AhR activators in high-throughput format (Novotna et al., 2011). Treatment of AZ-AHR cells with TCDD for 24 h resulted in approximately 157-fold increase in luciferase activity, as compared to vehicle-treated cells. Of 28 MWs tested, the samples MWDV10 (21-fold) and MWDV12 (23-fold) significantly increased luciferase activity (Fig. 3), which is consistent with the data obtained for CYP1A1 mRNA expression in LS174T cells (Fig. 1). We have also examined the effects of MWs after 6 h of the treatment. The luciferase activity was induced by TCDD approximately 10-fold. Tested MWs caused luciferase induction ranging from 0.9- to 5.8-fold. The results for samples MWDV10 (5.8-fold) and MWDV12 (5.0-fold) were consistent with 24 h treatments, but we detected transient increase in luciferase activity with several other samples (Fig. 3).

3.4. Effects of mineral waters on CYP1A1 and CYP1A2 expression in primary cultures of human hepatocytes

In final series of experiments, we examined the effects of MWs in two different primary cultures of human hepatocytes. In addition to cell lines, primary human hepatocytes allow to assess the effects of parent compounds and their metabolites. Dioxin induced CYP1A1/CYP1A2 mRNAs 320/84-fold and 121/121-fold, in cultures Hep220633 and LH38, respectively. The level of CYP1A1 mRNA, but not CYP1A2 mRNA was increased by samples MWPO1 (22-fold) and MWPO9 (11-fold) in culture Hep220633 (Fig. 4A). None of the samples tested induced CYP1A proteins in Hep220633 culture, while TCDD strongly up-regulated CYP1A proteins. The expression

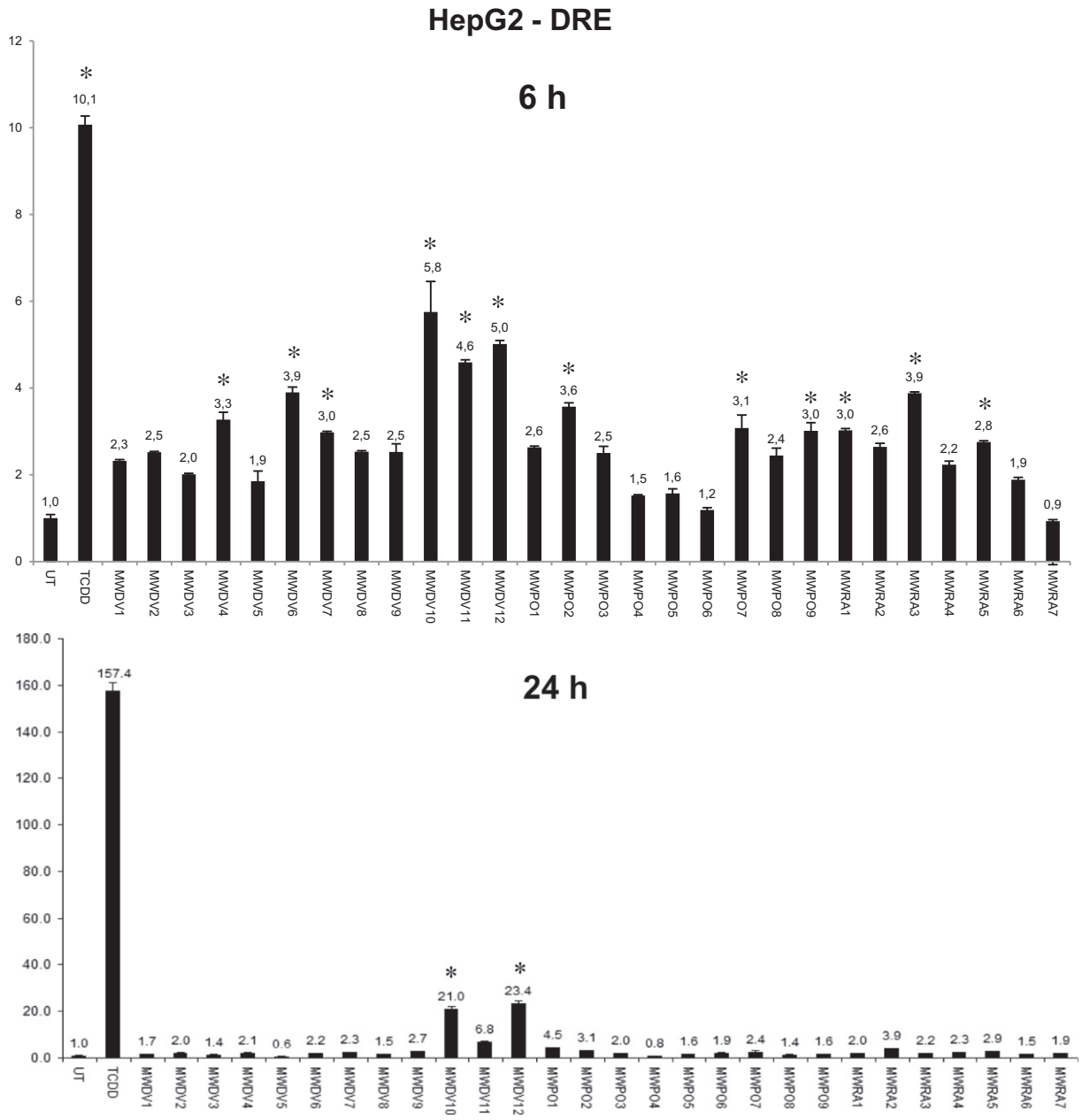

Fig. 3. Effects of mineral waters on transcriptional activity of AhR in AZ-AHR reporter cell line. AZ-AHR cells were seeded on 96-well plates, and allowed to attach and stabilize for 16 h. Thereafter, the cells were treated for 6 and 24 h with ethanolic solutions obtained from 28 different MWs (dilution 1000×), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (EtOH = UT; 0.1% v/v). After the treatments, cells were lysed and luciferase activity was measured. The data are mean ± SD from triplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). ∗ Value is significantly different from EtOH-treated cells ($p < 0.05$). Experiments were performed in three different clones of AZ-AHR cells.

of CYP1A1 and CYP1A2 mRNAs was significantly increased by MWDV10 (7- and 9-fold, respectively) in culture LH38 (Fig. 4B). Besides of TCDD, the level of CYP1A proteins was elevated in culture LH38 treated with MWPO1.

4. Discussion

In the present paper, we have examined the effects of 28 flavored mineral waters, marketed in the Czech Republic, on AhR–CYP1A signaling pathway in human hepatocytes and human cancer cell lines. We found that four samples activated AhR and/or induced CYP1A1: MWDV10 (DOBRA VODA; lemon, green tea, passion-flower), MWDV12 (DOBRA VODA; grapefruit, ginseng, guarana), MWPO1 (PODEBRADKA; lemon) and MWPO9 (PODEBRADKA; passion-flower). We observed some discrepancies between the data from cancer cell lines and human hepatocytes, probably due to the extensive metabolism of mineral waters constituents in hepatocytes. This study is the first report on the effects of flavored MWs on the expression of drug-metabolizing enzymes. Activation of AhR and induction of CYP1A1 and CYP1A2 by constituents of flavored MWs may have several physiological and/or clinical consequences: (i) food–drug interaction, i.e. induction of CYP1A enzymes alter pharmacokinetic of the drug(s) taken by a patient. This may decrease/increase the efficacy of the drug as well as its side effects (toxicity). Hence, undesired or even dangerous clinical situation may occur (Sergent et al., 2009). (ii) Chemically-induced carcinogenesis, i.e. active AhR and elevated activities of

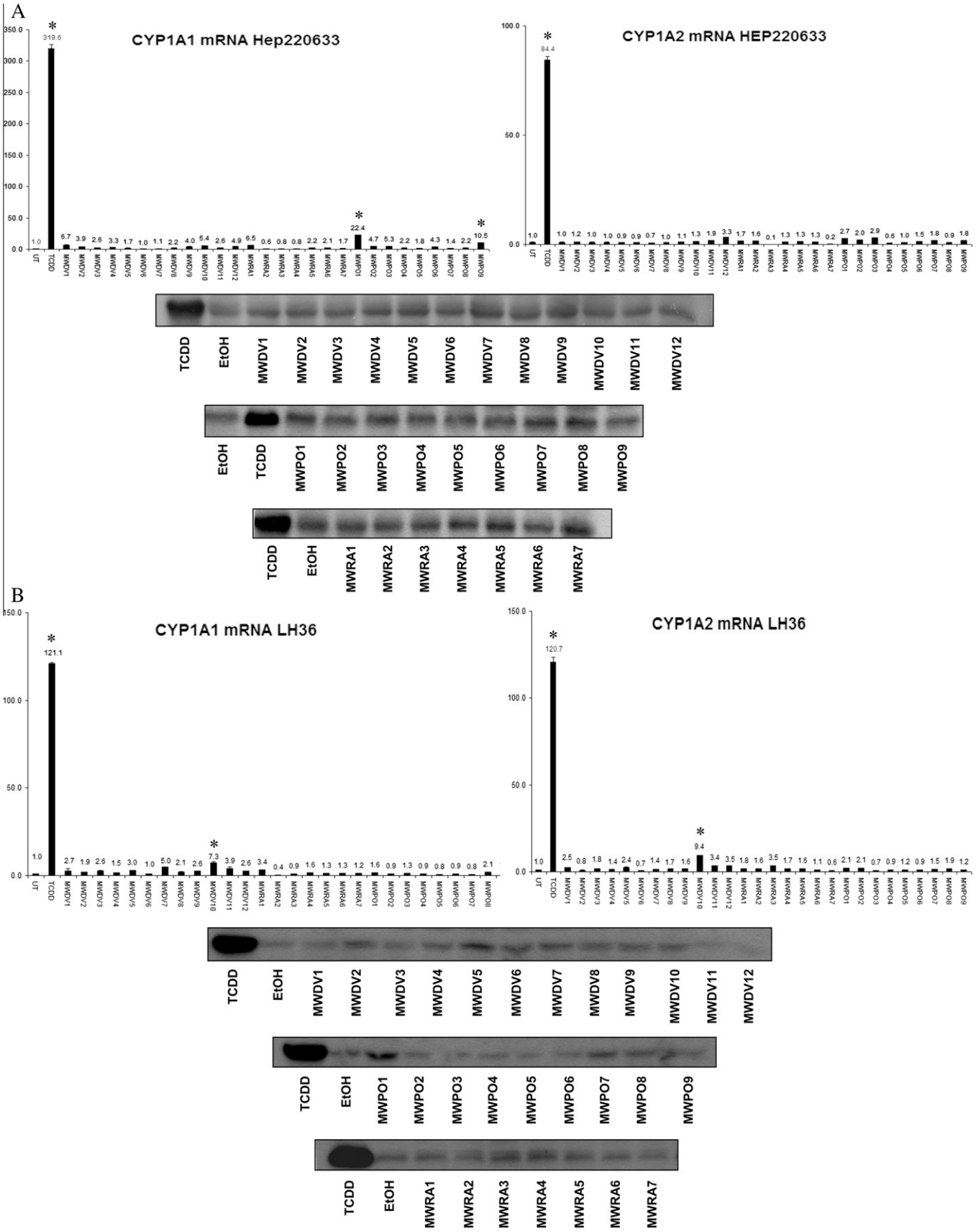

Fig. 4. Effects of mineral waters on CYP1A1 and CYP1A2 expression in primary cultures of human hepatocytes. Cells were treated with ethanolic solutions obtained from 28 different MWs (dilution 1000×), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM), and vehicle (ethanol = UT; 0.1% v/v). Experiments were performed in two different primary human hepatocytes cultures: (Panel A) HEP220633; (Panel B) LH36. Bar graphs show representative RT-PCR analyses of CYP1A1 and CYP1A2 mRNAs. Treatment duration was 24 h. The data are mean ± SD from triplicate measurements and are expressed as fold induction over EtOH-treated cells (UT). The data were normalized *per* GAPDH mRNA levels. ∗ Value is significantly different from EtOH-treated cells ($p < 0.05$). Western blots show the analyses of CYP1A1/2 proteins. Treatment duration was 48 h.

CYP1A1 and CYP1A2 increase the risk of chemically-induced cancers (Vondracek et al., 2011). (iii) Perturbation of intermediary metabolism; i.e. drug-metabolizing enzymes CYP1A1 and CYP1A2 are also involved in intermediary metabolism, in particular in metabolism of eicosanoids and retinoids (Benisek et al., 2011). (iv) Others; i.e. besides the regulation of CYP1A1 and CYP1A2, AhR controls the expression of phase II enzymes and transporters. It is involved in variety of cell functions, including immune response, differentiation, apoptosis, etc. Hence, the activation of AhR may have the effects on all the cell functions controlled by AhR (Abel and Haarmann-Stemmann, 2010).

On the other hand, the majority of drug–drug interactions, and probably also food–drug interactions is caused by an inhibition of the enzyme activity by a drug or food constituent, respectively. Therefore, AhR–CYP1A signaling pathway may be influenced by constituents from mineral waters at the level of drug-metabolizing enzymes activities, as well. Overall, with increasing consumption of prescribed drugs, and with increasing use of OTC products containing natural and synthetic xenobiotics, the phenomenon of food–drug interactions is of growing importance. Therefore, drinking flavored mineral waters in excessive amounts or on daily basis should be considered with prudence in people on regular medications.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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APPENDIX III

Kamenickova A., Anzenbacherova E., Pavek P., Soshilov A.A., Denison M.S., Anzenbacher P., Dvorak Z. (2013) Pelargonidin activates the AhR and induces CYP1A1 in primary human hepatocytes and human cancer cell lines HepG2 and LS174T. *Toxicol Lett.* 218(3):253-9

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Pelargonidin activates the AhR and induces CYP1A1 in primary human hepatocytes and human cancer cell lines HepG2 and LS174T

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HIGHLIGHTS

- Dietary xenobiotics may interact with drug metabolism in humans.
- Food–drug interactions occur through induction of P450 enzymes via xenoreceptors.
- We examined effects of 6 anthocyanidins on AhR-CYP1A1 pathway.
- Human hepatocytes and cell lines HepG2 and LS174T were used as in vitro models.
- Pelargonidin induced CYP1A1 and activated AhR by ligand dependent mechanism.

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Food–drug interactions

ABSTRACT

We examined the effects of anthocyanidins (cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin) on the aryl hydrocarbon receptor (AhR)-CYP1A1 signaling pathway in human hepatocytes, hepatic HepG2 and intestinal LS174T cancer cells. AhR-dependent reporter gene expression in transfected HepG2 cells was increased by pelargonidin in a concentration-dependent manner at 24 h. Similarly, pelargonidin induced the expression of CYP1A1 mRNA up to 5-fold in HepG2 and LS174T cells relative to the induction by 5 nM 2,3,7,8-tetrachlorodibenzodioxin (TCDD), the most potent activator of AhR. CYP1A1 and CYP1A2 mRNAs were also increased by pelargonidin in three primary human hepatocytes cultures (approximately 5% of TCDD potency) and the increase in CYP1A1 protein in HepG2 and LS174T cells was comparable to the increase in catalytic activity of CYP1A1 enzyme. Ligand binding analysis demonstrated that pelargonidin was a weak ligand of AhR.

Enzyme kinetic analyses using human liver microsomes revealed inhibition of CYP1A1 activity by delphinidin (IC_{50} 78 μM) and pelargonidin (IC_{50} 33 μM).

Overall, although most anthocyanidins had no effects on AhR-CYP1A1 signaling, pelargonidin can bind to and activate the AhR and AhR-dependent gene expression, and pelargonidin and delphinidin inhibit the CYP1A1 catalytic activity.

1. Introduction

Anthocyanidins and anthocyanins are naturally occurring flavonoid compounds that are responsible for typical color (bluish-red, orange-red, orange) and biological effects of many fruits, berries and vegetables. Anthocyanidins are aglycon (sugar-free) backbones of anthocyanins, which are conjugated with various sugars, such as xylose, arabinose, glucose, galactose and rhamnose in the latter compounds (Welch et al., 2008). The most commonly occurring anthocyanidins in higher plants are pelargonidin, peonidin, cyanidin, malvidin, petunidin and delphinidin (Kong et al., 2003). Anthocyanidins are liberated from anthocyanins in the gastrointestinal tract mainly due to action of the intestinal microflora (Keppler and Humpf, 2005). Although the bioavailability of anthocyanins and anthocyanidins is generally low, they can be absorbed in the gastrointestinal tract and have been detected in human plasma (McGhie and Walton, 2007). Anthocyanins and anthocyanidins possess antioxidant, antitumor and antimutagenic

Abbreviations: AhR, aryl hydrocarbon receptor; EROD, ethoxyresorufin-*O*-deethylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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properties in vivo (Kong et al., 2003). The aglycones generated from the most abundant anthocyanins have been shown to inhibit the growth of human stomach, colon, lung, breast and CNS cancer cells (Zhang et al., 2005). Both the human intestine and liver are organs rich in drug-metabolizing enzymes, which interact with drugs and food constituents. Among the drug-metabolizing enzymes, cytochromes P450 (CYPs) are the most important and most generally distributed enzymes responsible for more than two thirds of metabolic processes with known mechanisms (Pavek and Dvorak, 2008). Cytochromes P450 1A, namely, CYP1A2 (present mainly in the liver) and CYP1A1 (mostly extrahepatic, but present in the liver after induction) are the evolutionary oldest and best-studied forms of this enzyme and they are known for their roles in activation of carcinogens (e.g. polycyclic aromatic hydrocarbons and heterocyclic amines), and in the metabolism of drugs (e.g. tricyclic antidepressants and theophylline) (Anzenbacher and Anzenbacherova, 2001; Monostory et al., 2009). Both CYP1A1 and CYP1A2 are transcriptionally regulated by the aryl hydrocarbon receptor (AhR), and they are inducible by a variety of xenobiotic AhR ligands, including drugs (e.g. omeprazole), natural compounds (e.g. berberine), synthetic chemicals (e.g. specific inhibitor of c-jun-N-terminal kinase SP600125) and environmental pollutants (e.g. polyhalogenated biphenyls, polycyclic aromatic hydrocarbons, dioxins) (Denison and Nagy, 2003; Stejskalova et al., 2011).

Besides its role in CYP1A genes induction, the AhR plays many physiological functions and it is involved in chemically-induced carcinogenesis (Abel and Haarmann-Stemmann, 2010). Therefore, it is of topical interest to identify chemicals that affect the AhR-CYP1A signaling pathway and resulting enzymatic activities, with regard to putative food–drug interactions and effects on human health. Anthocyanins are contained in common food, beverages and dietary supplements. Structurally, they are considered as polyphenolic compounds together with flavonoids, flavones and isoflavones. While the effects of flavonoids, flavones and isoflavones on AhR-CYP1A have been broadly studied (Amakura et al., 2008; Hodek et al., 2002), there are no reports of interactions between anthocyanins and the AhR-CYP1A signaling pathway. In the present paper, we have examined the effects of the anthocyanidins cyanidin, delphinidin, malvidin, peonidin, petunidin and pelargonidin, on the aryl hydrocarbon receptor (AhR)-CYP1A1 signaling pathway in primary human hepatocytes and, in human hepatic HepG2 and intestinal LS174T cancer cell lines. We found that pelargonidin activates the AhR and induces CYP1A genes by a ligand-dependent mechanism, and that pelargonidin and delphinidin can inhibit CYP1A1 catalytic activity. The other anthocyanidins did not affect AhR-CYP1A1 signaling.

2. Materials and methods

2.1. Compounds and reagents

Dimethylsulfoxide (DMSO), resveratrol and hygromycin B were purchased from Sigma–Aldrich (Prague, Czech Republic). The anthocyanidins, cyanidin chloride (ref.#0909S; purity ≥ 96%), delphinidin chloride (ref.#0904S; purity ≥ 97%), malvidin chloride (ref.#0913S; purity ≥ 97%), peonidin chloride (ref.#0906S; purity ≥ 97%), petunidin chloride (ref.#0942S; purity ≥ 95%) and pelargonidin chloride (ref.#0912S; purity ≥ 97%) were purchased from Extrasynthese (Lyon, France). Luciferase lysis buffer and P450-Glo CYP1A1 assay were from Promega (www.promega.com; Hercules, CA). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCycler FastStart DNA Master^{PLUS} SYBR Green I was from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). All other chemicals were of the highest quality commercially available.

2.2. Human hepatocytes

Human hepatocytes were obtained from two sources: (i) human liver obtained from multiorgan donors LH44 (F, 57 years) and LH45 (M, 46 years); 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). Hepatocytes were treated in a serum-free medium for 24 h or 48 h with the tested compounds, TCDD (5 nM) and/or vehicle (DMSO; 0.1%, v/v). Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.3. Cancer cell lines

Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC no. 85011430) and human Caucasian colon adenocarcinoma cells LS174T (ECACC no. 87060401) were purchased from ECACC and were cultured as recommended by manufacturer. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Gene reporter assay

Experiments were performed in a stably transfected gene reporter cell line AZ-AHR, which was derived from HepG2 cells transfected with a construct containing several AhR binding sites upstream of a luciferase reporter gene (Novotna et al., 2011). Following plating, cells were stabilized for 16 h and then incubated for 24 h with tested compounds, TCDD (5 nM), resveratrol and/or vehicle (DMSO; 0.1%, v/v). After the treatments, cells were lysed using Reporter Lysis Buffer (Promega, Madison, WI, USA) according to manufacturers' instructions, and luciferase activity was measured.

2.5. 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) and random hexamers 3801 (Takara). qRT-PCR was carried out on Light Cycler apparatus 480 II (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP1A1, CYP1A2 and GAPDH mRNAs were determined as described elsewhere (Dvorak et al., 2008). The measurements were performed in triplicates. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. Data were processed by delta-delta method.

2.6. Protein detection and Western blotting

Total protein extracts were prepared as described elsewhere (Pavek et al., 2007). Following SDS-PAGE separation and Western-blot transfer, blots were probed with antibody against CYP1A1 (goat polyclonal; sc-1616), purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescent detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit.

2.7. 7-Ethoxyresorufin-O-deethylase (EROD) activity of CYP1A1/2 in cell cultures

HepG2 cells and/or LS174T cells were seeded in 96-well plates at a density of 2.4×10^4 cells/cm^2 in DMEM supplemented with 10% FCS and stabilized for 24 h. Cells were then incubated for 48 h with test compounds, TCDD (5 nM) and/or vehicle (DMSO; 0.1%, v/v). The 7-ethoxyresorufin-O-deethylase (EROD) activity in cell cultures was measured as described elsewhere (Donato et al., 1993). Briefly, monolayers were washed with PBS and the serum-free medium containing 8 μM 7-ethoxyresorufin and 10 μM dicumarol was applied to cells. Following 30 min of incubation at 37 °C, an aliquot of 75 μL of the medium was mixed with 125 μL of methanol and fluorescence was measured in a 96-well plate with 530 nm excitation and 590 nm emission filters. The data were expressed as the ratio of treated over control values (DMSO-treated cells).

2.8. Enzyme activity of CYP1A1 and CYP1A2 in human liver microsomes

Pooled human liver microsomes were obtained from Biopredic (Rennes, France) in accordance with ethical regulations of the country of origin (France). They were from twenty-six donors (twenty males and six females) with a protein content of 25 mg/ml; the CYP1A1/2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4/5 enzyme activities were verified before the experiment.

Activity of CYP1A1/2 was measured either as EROD (see above) with 7-ethoxyresorufin as substrate according to established method (Leclercq et al., 1996) or by a luciferase based assay with CYP1A1 specific substrate Luciferin-CEE (www.promega.com, Promega, Hercules, CA). Amount of product of the reaction was determined by HPLC (Shimadzu Class VP, Tokyo, Japan) with fluorescence detection or by measuring the luminescence using Infinite M200 spectrophotometer/spectrofluorometer/luminometer (TECAN Austria, Vienna). Anthocyanidins were dissolved in aqueous solution of pH 3.5 to concentration of 1 mM. Incubation mixture contained 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (0.8 mM $NADP^+$, 5.8 mM isocitrate, 0.3 unit/ml of isocitrate dehydrogenase and 8 mM $MgCl_2$), 35 pmol human liver microsomes and 2.6 μM 7-ethoxyresorufin. Final incubation mixture volume was 100 μl. Inhibition experiments were performed with five concentrations of anthocyanidins (10, 20, 40, 80, 100 μM). Experimental conditions were the same as above. Reaction mixtures were pre-incubated with inhibitors at 37 °C for 30 min. With luciferin-CEE luminogenic substrate, microsomes with 22 pmol total CYP were preincubated with 60 μM

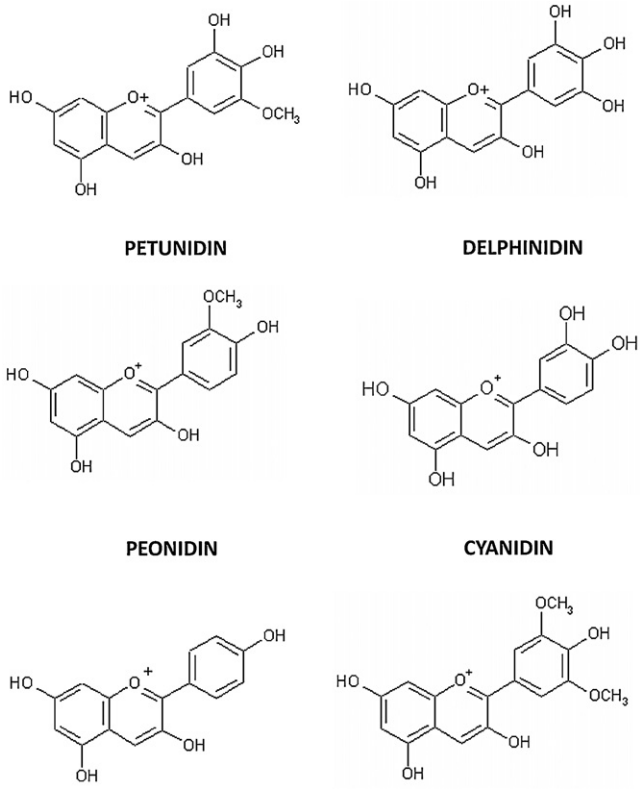

Fig. 1. Chemical structures of anthocyanidins.

luciferin-CEE and pelargonidin or delphinidin (same final concentrations as above) for 30 min at 37 °C; then, NADPH-generating system was added (same as above) and the system was incubated for 10 min. Detection reagent was then added and the reaction mixture was incubated for 20 min according to the recommended protocol (www.promega.com). Two independent experiments were done with duplicates, which did not differ by more than 10%. Inhibition of CYP1A activities was in all cases evaluated by plotting the respective remaining activity versus the inhibitor concentration.

2.9. AhR ligand binding assay

[^{3}H]TCDD was kindly provided by Dr. Steven Safe (Texas A&M university) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) was from Accustandard (New Haven, CT, USA). The competitive displacement of [^{3}H]TCDD from guinea pig hepatic cytosol was as previously described (Korashy et al., 2011). Briefly, hepatic guinea pig cytosol diluted to 8 mg/ml protein in MEDG (25 mM MOPS-NaOH, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) was incubated with different concentrations of pelargonidin chloride or 200 nM TCDF for 30 min at room temperature or on ice, and further incubated for 1 h at room temperature in the presence of 2 nM [^{3}H]TCDD. The amount of [^{3}H]TCDD specific binding was determined by hydroxyapatite protocol, and specific binding was determined as the difference between the 'no competitor' and TCDF reaction (Denison et al., 2002).

3. Results

3.1. Effects of anthocyanidins on transcriptional activity of AhR in AZ-AHR reporter cell line

In the first series of experiments, we examined the effects of the various anthocyanidins (chemical structures shown in Fig. 1) on transcriptional activity of AhR. Experiments were performed in recombinant AZ-AHR cells, which are HepG2 cells that had been stably transfected with an AhR-responsive luciferase reporter plasmid (Novotna et al., 2011). In agonist experiments, the cells were incubated for 24 h with increasing concentrations (10 nM–50 μM) of anthocyanidins (cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin), TCDD (5 nM) or vehicle (0.1%, v/v DMSO). Luciferase activity was increased 1253-fold by TCDD, the most

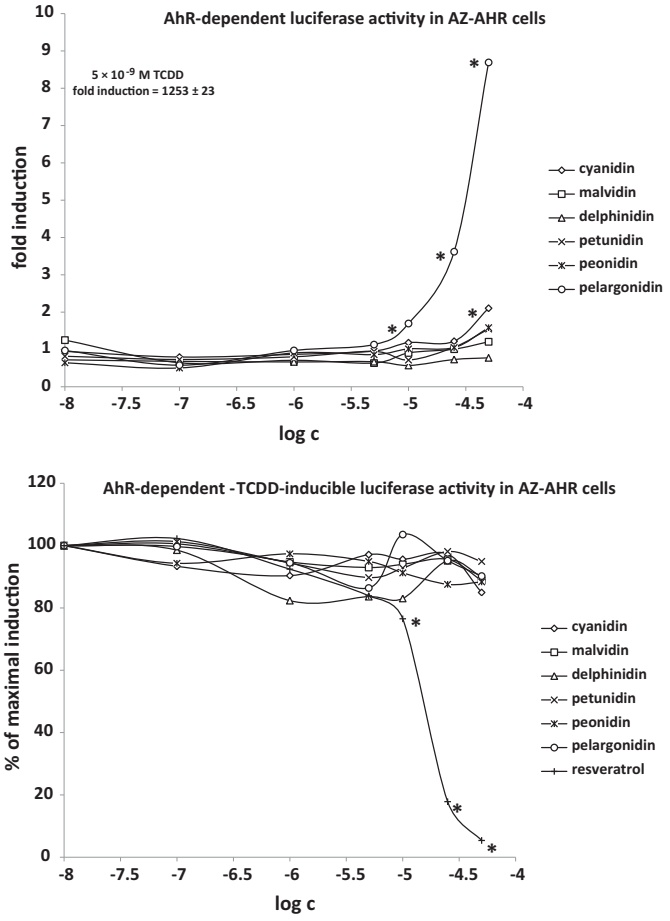

Fig. 2. Effects of anthocyanidins on transcriptional activity of AhR in AZ-AHR reporter cell line. AZ-AHR cells were seeded in 96-well plates and stabilized for 16 h. Thereafter, the cells were treated for 24 h with anthocyanidins (10 nM–50 μM; i.e. cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1%, v/v). Upper panel: Agonist mode, i.e. incubation with individual compounds; lower panel: antagonist mode, i.e. combined incubation of 5 nM TCDD plus tested compounds and/or resveratrol in increasing concentrations. After the treatments, cells were lysed and luciferase activity was measured. The data are the mean from triplicate measurements and are expressed either as fold induction over DMSO-treated cells (agonist mode) or as percentage of the induction by TCDD (antagonist mode). Experiments were performed in three different passages of AZ-AHR cells. The differences between individual measurements were lower that 5%. * – values significantly different from the vehicle value ($p < 0.05$) as determined by the Student's *t*-test.

potent activator of AhR. Pelargonidin produced a concentration-dependent induction of luciferase activity, which was significantly different from vehicle at concentrations of 10 μM and higher (Fig. 2, upper panel). Although the magnitude of induction by 50 μM pelargonidin was 9-fold greater than that of the vehicle (DMSO) control, the magnitude of the induction response was very low when compared to TCDD (i.e. less than 1% of the maximal level of induction by TCDD). While very low, but significant level of induction of luciferase activity was observed for 50 μM cyanidin (Fig. 2, upper panel), no significant induction of luciferase activity was observed with delphinidin, malvidin, peonidin or petunidin. In antagonist experiments, the cells were incubated with increasing concentrations of anthocyanidins and/or resveratrol in the presence of TCDD. While the induction of luciferase activity was not significantly altered by any of the anthocyanidins tested, resveratrol, a known antagonist of AhR, produced a concentration-dependent decrease of TCDD-induced luciferase activity to a maximum of 95% inhibition at 50 μM resveratrol (Fig. 2,

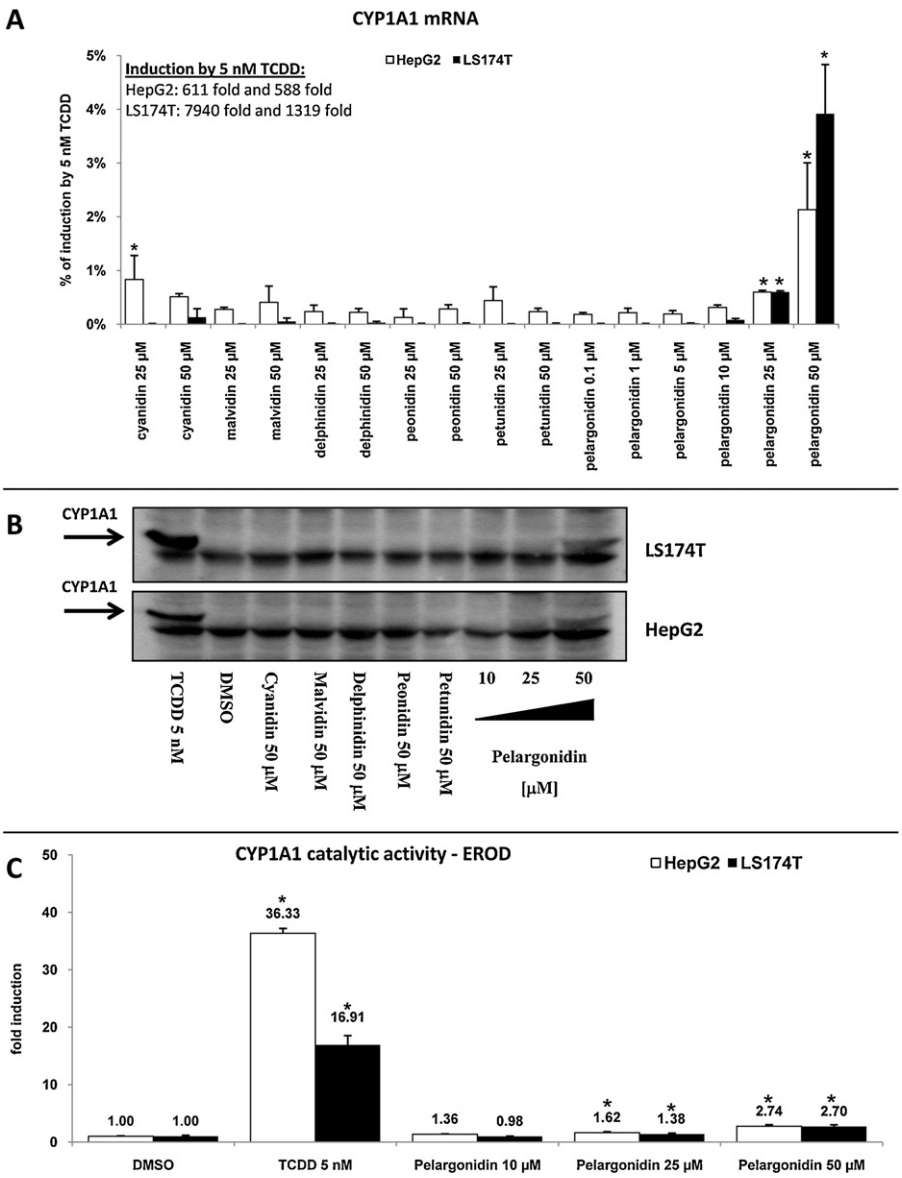

Fig. 3. Effects of anthocyanidins on CYP1A1 expression in HepG2 and LS174T cells. Panel A: cells were treated with cyanidin (25 μM and 50 μM), delphinidin (25 μM and 50 μM), malvidin (25 μM and 50 μM), peonidin (25 μM and 50 μM), petunidin (25 μM and 50 μM), pelargonidin (0.1 μM, 1 μM, 5 μM, 10 μM, 25 μM and 50 μM), TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v). Bar graph shows representative RT-PCR analyses (3 independent experiments) of CYP1A1 mRNA after 24 h treatment. The data are the mean from triplicate measurements and are expressed as percentage of maximal induction attained in TCDD-treated cells. The data were normalized *per* GAPDH mRNA levels. * – value is significantly different from DMSO-treated cells ($p < 0.05$) as determined by the Student's *t*-test. Panel B: cells were treated with cyanidin (50 μM), delphinidin (50 μM), malvidin (50 μM), peonidin (50 μM), petunidin (50 μM), pelargonidin (10 μM, 25 μM and 50 μM), TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v) for 48 h. Western blots show a representative analysis of CYP1A1 protein. Similar profiles were observed in three independent experiments. As a loading control, the blots were probed to actin (data not shown). Panel C: cells were treated with pelargonidin (10 μM, 25 μM and 50 μM), TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v) for 48 h. Catalytic activity of CYP1A1 (EROD) was measured as described in detail in Section 2. The data are mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. * – value is significantly different from DMSO-treated cells ($p < 0.05$) as determined by the Student's *t*-test.

lower panel). Taken together, these results indicate that pelargonidin is a weak AhR agonist with no apparent antagonist activity.

3.2. Effects of anthocyanidins on CYP1A1 expression in cancer cells HepG2 and LS174T

A typical target gene of ligand- and AhR-dependent signal transduction is CYP1A1. Therefore, we studied the effects of anthocyanidins on the expression of CYP1A1 mRNA, protein and enzyme catalytic activity in hepatic HepG2 and intestinal LS174T human cancer cells. Cells were incubated with anthocyanidins, TCDD (5 nM) and DMSO (0.1%, v/v) for 24 h and 48 h. TCDD strongly induced CYP1A1 mRNA after 24 h of incubation in HepG2 cells (588-fold and 611-fold) and in LS174T cells (1319-fold and 7940-fold). While cyanidin, delphinidin, malvidin, peonidin and petunidin did not influence CYP1A1 mRNA, protein and enzyme activity levels, pelargonidin increased CYP1A1 mRNA expression in concentration-dependent manner in both cell lines. However, pelargonidin was a relatively weak inducer of CYP1A1 mRNA in HepG2 and LS174T cells increasing CYP1A1 mRNA levels by only about 2% and 4% of that observed with a maximal inducing concentration of TCDD, respectively (Fig. 3A). Pelargonidin (50 μM) incubation did increase CYP1A1 protein levels in HepG2 and LS174T cell lines after 48 h incubation (Fig. 3B). EROD activity in HepG2 and LS174T cells was induced 36-fold and 17-fold by TCDD after 24 h of incubation, respectively. Similarly to its effect on CYP1A1

Table 1
Effects of anthocyanidins on the expression of CYP1A1 and CYP1A2 mRNAs in primary human hepatocytes treated for 24 h with tested compounds. Results are expressed as fold induction over the vehicle-treated cells. Data are mean ± S.D. from triplicate measurements. nd = not determined.

Compound	HH 44		HH 45		HEP220670	
	CYP1A1	CYP1A2	CYP1A1	CYP1A2	CYP1A1	CYP1A2
DMSO	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.36	1.00 ± 0.36	1.00 ± 0.14	1.00 ± 0.13
TCDD	159.79 ± 10.5	63.34 ± 0.93	330.84 ± 57.4	51.62 ± 9.13	229.13 ± 6.74	143.51 ± 0.70
Cyanidin 10 μM	2.29 ± 0.07	4.71 ± 0.61	0.95 ± 0.26	1.42 ± 0.40	nd	nd
Cyanidin 50 μM	2.11 ± 0.23	4.96 ± 1.35	0.96 ± 0.10	2.02 ± 0.26	1.80 ± 0.20	1.90 ± 0.21
Malvidin 10 μM	0.90 ± 0.05	1.89 ± 0.01	1.51 ± 0.03	1.51 ± 0.06	nd	nd
Malvidin 50 μM	1.35 ± 0.03	2.29 ± 0.08	1.83 ± 0.15	2.96 ± 0.38	1.11 ± 0.03	1.40 ± 0.05
Delphinidin 10 μM	1.20 ± 0.20	2.47 ± 0.14	2.19 ± 0.27	1.99 ± 0.25	nd	nd
Delphinidin 50 μM	0.96 ± 0.20	013 ± 0.02	1.16 ± 0.19	1.26 ± 0.38	1.13 ± 0.03	1.33 ± 0.02
Peonidin 10 μM	1.64 ± 0.01	1.05 ± 0.05	0.84 ± 0.12	1.07 ± 0.13	nd	nd
Peonidin 50 μM	3.52 ± 0.80	1.61 ± 0.19	1.11 ± 0.10	1.53 ± 0.13	1.57 ± 0.08	1.82 ± 0.08
Petunidin 10 μM	1.40 ± 0.32	0.69 ± 0.01	2.58 ± 0.15	2.12 ± 0.14	nd	nd
Petunidin 50 μM	2.35 ± 0.13	0.67 ± 0.02	1.48 ± 0.18	2.57 ± 0.29	1.13 ± 0.07	0.85 ± 0.03
Pelargonidin 10 μM	14.62 ± 1.64	0.53 ± 0.02	3.43 ± 0.39	2.13 ± 0.25	1.59 ± 0.03	1.89 ± 0.04
Pelargonidin 50 μM	22.24 ± 3.06	0.73 ± 0.07	15.3 ± 0.34	2.61 ± 0.11	5.90 ± 0.09	5.41 ± 0.06

mRNA and protein levels, pelargonidin increased EROD activity ~3-fold in concentration-dependent manner in both cell lines (Fig. 3C). Collectively, these results further support the conclusion that pelargonidin is an AhR agonist, albeit relatively weak compared to TCDD.

3.3. Effects of anthocyanidins on CYP1A1 and CYP1A2 expression in primary human hepatocytes

In the next series of experiments, we tested the effects of anthocyanidins on the expression of CYP1A genes in primary human hepatocytes. In contrast to the cell lines, human hepatocytes express a full panel of drug-metabolizing enzymes, hence, the mixed effects of the parent compounds and their metabolites are examined. Three different primary human hepatocytes cultures were used (HH44, HH45 and HEP220670). Dioxin strongly induced the expression of CYP1A1 and CYP1A2 mRNAs in all human hepatocytes cultures at 24 h, and the magnitude of induction ranged between 160 and 331 fold and 52 and 144 fold, respectively. Pelargonidin induced CYP1A1 and CY1A2 mRNAs in concentration-dependent manner in all of the primary hepatocytes cultures (Table 1). While slight induction of CYP1A1 and CYP1A2 mRNAs was observed for other tested anthocyanidins, these increases occurred in a culture dependent manner, i.e. they were not systematic. The levels of CYP1A proteins in two human hepatocytes cultures were moderately increased only by incubation with 50 μM pelargonidin for 48 h, as compared to the vehicle-treated cells (Fig. 4).

3.4. Ligand binding assay

Activation of AhR may occur by ligand-dependent or ligand-independent mechanisms. Therefore, we tested whether the effects of pelargonidin on AhR-CYP1A1 signaling pathway involved binding of pelargonidin to the AhR. For this purpose, we performed AhR ligand binding assays using guinea pig hepatic cytosol. Pelargonidin competitively inhibited [^{3}H]-TCDD binding to the AhR when present in the binding incubation at 50 μM (13–16% inhibition) or 100 μM (25% inhibition), when cytosol was pre-incubated with pelargonidin on ice or at room temperature; there were no consistent differences between pre-incubation conditions (Fig. 5). These data are consistent with the previous results which suggest that pelargonidin is a weak ligand/agonist of the AhR, and that the effects of pelargonidin on AhR-CYP1A1 signaling pathway likely occur via a ligand-dependent mechanism.

3.5. Effects of anthocyanidins on CYP1A1 and CYP1A2 catalytic activity in human liver microsomes

Given the ability of many AhR ligands to also bind to and be metabolized by the coordinately induced CYP1A1/2, we directly examined the effects of anthocyanidins on the activities of two drug metabolizing enzymes by determining their effects on CYP1A1/2 catalytic (EROD) and to CYP1A1-specific luciferin activating activity using human microsomes. Results of these competitive kinetic analyses revealed a weak concentration-dependent inhibition of

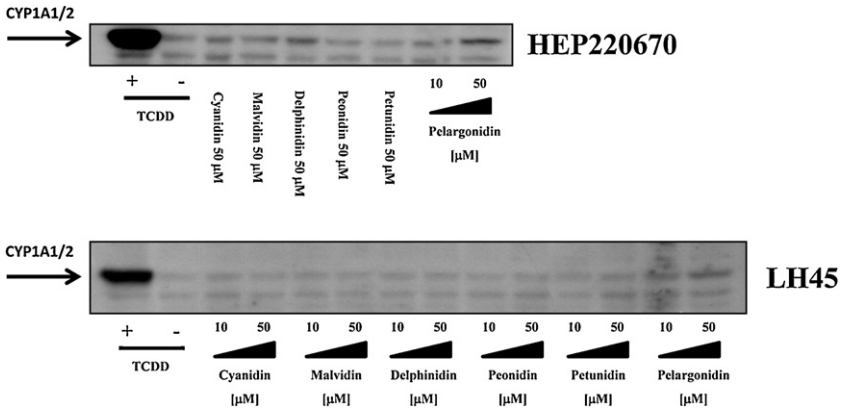

Fig. 4. Effects of anthocyanidins on CYP1A protein expression in primary human hepatocytes. Cells were treated with cyanidin (10 μM and 50 μM), delphinidin (10 μM and 50 μM), malvidin (10 μM and 50 μM), peonidin (10 μM and 50 μM), petunidin (10 μM and 50 μM), pelargonidin (10 μM and 50 μM), TCDD (5 nM) and vehicle (DMSO; 0.1%, V/V) for 48 h. Western blots show analyses of CYP1A proteins from two different primary human hepatocytes cultures. As a loading control, the blots were probed to actin (data not shown).

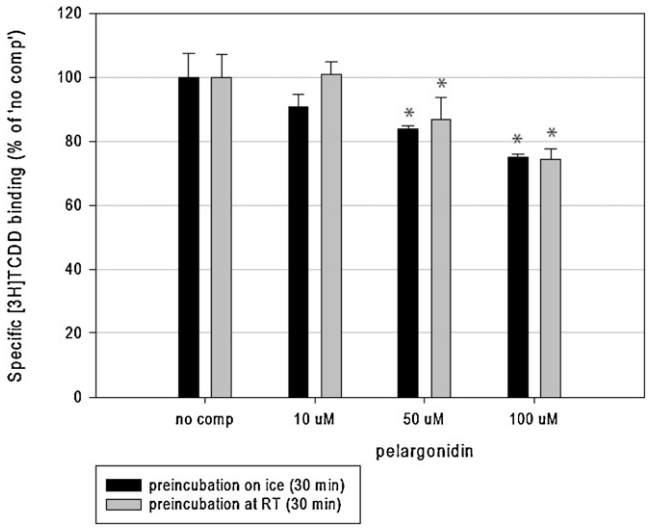

Fig. 5. Ligand binding assay. Pelargonidin chloride is a weak AhR ligand. Guinea pig hepatic cytosol was pre-incubated with indicated concentrations of pelargonidin chloride or TCDF for 30 min at room temperature or on ice, followed by addition of 2 nM [^{3}H]TCDD and further incubation for 1 h at room temperature. Ligand binding to the cytosolic proteins was determined by the hydroxyapatite binding protocol and scintillation counting. Specific binding was determined as a difference between total and non-specific (TCDF) reactions. The values are presented as mean ± SD of three independent reactions. * – values significantly different from the 'no competitor' reaction at $p < 0.05$ as determined by the Student's *t*-test. The results are representative of two independent experiments.

CYP1A1/2 EROD activity (the EROD deethylating activity is not specific either to CYP1A1 or CYP1A2 as both enzymes share this activity with CYP1A1 being more active) following the addition of petunidin, cyanidin, peonidin and malvidin, albeit to a relatively low extent (to 80–95% of the original activity at the highest concentration (i.e. 100 μM) of the respective anthocyanidin). In contrast, delphinidin inhibited CYP1A1/2 EROD activity down to 28% and pelargonidin to 75% of the initial activity (data not shown). This was why with these two anthocyanidins the second, luciferin-based activity specific for CYP1A1 has been followed. CYP1A1 has been chosen for detailed study on the basis of results with AhR activation and CYP1A1 expression in cancer cells (see the respective paragraphs). The data (Fig. 6) clearly show the inhibition of CYP1A1 activity down to 22% and 40% of the initial activity (i.e. without anthocyanidin added) corresponding to IC_{50} values of 33 μM and 77 μM for pelargonidin and delphinidin, respectively. These results thus document the ability of at least two anthocyanidins to interact with drug metabolizing system of CYP1A1/2.

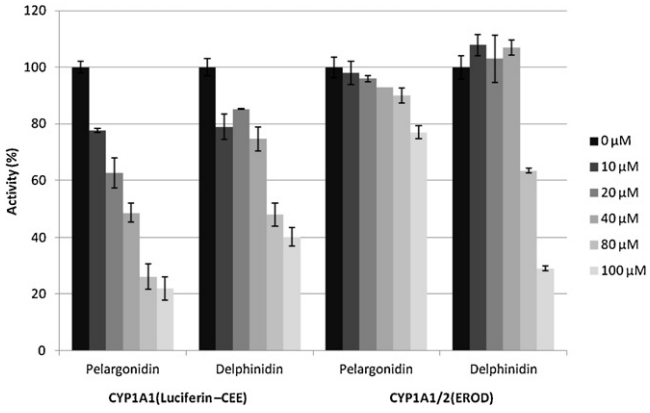

Fig. 6. Effects of anthocyanidins on CYP1A1 catalytic activity in human liver microsomes. Inhibition of human microsomal CYP1A1 catalytic activity by pelargonidin and delphinidin expressed as the amount of activity remaining relative to control (without anthocyanidin) in percent. Concentration of respective anthocyanidins in the reaction mixture was 0, 10, 20, 40, 80 and 100 μM. For experimental details, see the Section 2.

4. Discussion

The phenomenon of *food–drug interactions* has emerged in the last few years. It comprises pharmacokinetic and toxicokinetic interactions between food constituents and drugs that modify their relative potency and efficacy. While drug–drug interactions may be effectively managed by a physician who prescribes the drugs, the food–drug interactions are not subject to such control. Therefore, it is of value to search for food constituents that may cause or contribute to food–drug interactions. These compounds may be natural food constituents (e.g. alkaloids, polyphenolics, terpenes), artificial food additives (e.g. sweeteners, preservatives, taste and flavor enhancers, colorings, stabilizers etc.) and even contaminants (e.g. persistent organic pollutants). Food–drug interactions may occur in two main ways, i.e. by inhibition or induction of drug metabolizing enzymes. Recently, we described the effects of extracts from ready-to-drink teas (Kamenickova et al., 2012) and flavored mineral waters (Kamenickova and Dvorak, 2012) on the AhR-CYP1A1 signaling pathway in human hepatocytes and human cancer cell lines. We found that extracts from some of non-alcoholic beverages activated AhR and induced CYP1A1/2 and CYP3A4 genes. In the present paper, we studied the effects of six major anthocyanidins on AhR-dependent gene expression and on CYP1A1/2 expression, production and enzymatic activity. Analysis of inhibition of the CYP1A1/2 activity revealed that the effects of the majority of these compounds on metabolic pathways mediated by CYP1A1/2 enzymes are not likely. However, pelargonidin and delphinidin were shown to interfere with CYP1A1/2 drug metabolizing system (Fig. 6), although this effect occurred at concentrations higher than those corresponding to the average values reached in human plasma after fruit or juice consumption, i.e. 274 nmol/L and 1220 ng/L for pelargonidin (Mullen et al., 2008) and delphinidin glycosides (Frank et al., 2005), respectively. Since CYP1A enzymes are responsible for activation of many pro-carcinogens (e.g. polycyclic aromatic hydrocarbons) and detoxication of many others (Anzenbacher and Anzenbacherova, 2001; Monostory et al., 2009), interference of anthocyanidins present in food with these enzymes may be even beneficial.

The major finding of the current paper is that pelargonidin is a weak ligand/agonist of the AhR, as revealed by ligand binding assay in guinea pig cytosols (Fig. 5) and gene reporter assays in recombinant human AZ-AHR cells (Fig. 2), respectively. Pelargonidin also induced CYP1A1 mRNA, protein and catalytic activity in human hepatic HepG2 and human intestinal LS174T cancer cells (Fig. 3) and also induced CYP1A1 mRNA and to a lesser extent that of CYP1A2 mRNA in three different primary human hepatocytes cultures (Table 1). The levels of CYP1A proteins were slightly elevated by 50 μM pelargonidin in human hepatocytes (Fig. 4). While we also observed slight induction of CYP1A1/2 mRNAs in human hepatocytes by 50 μM cyaniding these effects were not consistent, possibly due to the metabolic transformation of cyanidin in human hepatocytes. Other antocyanidins, i.e. delphinidin, peonidin, petunidin and malvidin did not display significant and systematic effects on AhR transcriptional activity and CYP1A1 expression. Regarding structure–activity relationship, pelargonidin is the only anthocyanidin mono-substituted (monohydroxylated) at the phenyl group bound at 2-position of the chromenylium backbone. Speculatively, this feature may be one of the explanations for the unique activity of pelargonidin towards the AhR, because other anthocyanidins are either di-substituted (cyanidin, peonidin) or tri-substituted (malvidin, petunidin, delphinidin) (Fig. 1).

Taken together, pelargonidin activates AhR through a ligand dependent mechanism, and induces CYP1A and AhR-dependent reporter genes expression in human cancer cell lines and human hepatocytes, which may be of toxicological significance, with respect to multiple roles of AhR in human organism.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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APPENDIX IV

Kamenickova A., Anzenbacherova E., Pavek P., Soshilov A.A., Denison M.S., Zapletalova M., Anzenbacher P., Dvorak Z. (2013) Effects of anthocyanins on the AhR-CYP1A1 signaling pathway in human hepatocytes and human cancer cell lines. *Toxicol Lett*. 221(1):1-8

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Effects of anthocyanins on the AhR–CYP1A1 signaling pathway in human hepatocytes and human cancer cell lines

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HIGHLIGHTS

- Food constituents may interact with drug metabolizing pathways.
- AhR–CYP1A1 pathway is involved in drug metabolism and carcinogenesis.
- We examined effects of 21 anthocyanins on AhR–CYP1A1 signaling.
- Human hepatocytes and cell lines HepG2 and LS174T were used as the models.
- Tested anthocyanins possess very low potential for food–drug interactions.

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ABSTRACT

Anthocyanins are plant pigments occurring in flowers and berry fruits. Since a phenomenon of food–drug interactions is increasingly emerging, we examined the effects of 21 major anthocyanins and the extracts from 3 food supplements containing anthocyanins on the aryl hydrocarbon receptor (AhR)–cytochrome P450 CYP1A1 signaling pathway in human hepatocytes and human hepatic HepG2 and intestinal LS174T cancer cells. Pelargonidin-3-*O*-rutinoside (PEL-2) and cyanidin-3,5-*O*-diglucoside (CYA-3) dose-dependently activated AhR, as revealed by gene reporter assay. PEL-2 and CYA-3 induced CYP1A1 mRNA but not protein in HepG2 and LS174T cells. Neither compounds induced CYP1A1 mRNA and protein in four different primary human hepatocytes cultures. The effects of PEL-2 and CYA-3 on AhR occurred by ligand-dependent and ligand-independent mechanisms, respectively, as demonstrated by ligand binding assay. In a direct enzyme inhibition assay, none of the antocyanins tested inhibited the CYP1A1 marker activity to less than 50% even at 100 μM concentration. PEL-2 and CYA-3 at 100 μM inhibited CYP1A1 to 79% and 65%, respectively. In conclusion, with exception of PEL-2 and CYA-3, there were no effects of 19 major anthocyanins and 3 food supplements containing anthocyanins on AhR–CYP1A1 signaling, implying zero potential of these compounds for food–drug interactions with respect to AhR–CYP1A1 pathway.

1. Introduction

Anthocyanins, the sub-group of flavonoids, are the plant pigments responsible for red, blue or purple colors of berries, grapes, apples, purple cabbage and corn. They are water-soluble compounds occurring in plants primarily in the form of glycosidic polyhydroxyl and polymethoxyl derivatives of flavylium salts (Winkel-Shirley, 2001). Anthocyanins are attached with one or more sugars such as glucose, galactose, arabinose, xylose, rhamnose and glucoronid acid (Welch et al., 2008). They differ in the number of hydroxyl and methoxyl groups, in the position of attachment of sugars and in a number of aliphatic or aromatic acids attached to sugars in the molecule (Fig. 1). Epidemiologic studies demonstrated that anthocyanins-containing foodstuffs possess anti-oxidant, anti-obesity and anti-inflammatory effects and due to them they lower the risk of diabetes, cardiovascular disease, arthritis and cancer (Prior and Wu, 2006). Consequently, a vast number of dietary supplements, containing anthocyanins is available at

Abbreviations: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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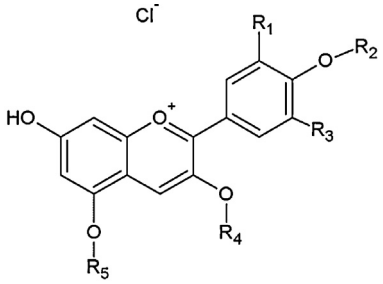

	Anthocyanin	R1	R2	R3	R4	R5
PEL-1	pelargonidin-3,5-di-O-glucoside chloride	H	H	H	glucoside	glucose
PEL-2	pelargonidin-3-O-rutinoside chloride	H	H	H	rutinoside	H
DEL-1	delphinidin-3-O-glucoside chloride	OH	H	OH	glucoside	H
DEL-2	delphinidin-3-O-rutinoside chloride	OH	H	OH	rutinoside	H
DEL-3	delphinidin-3,5-di-O-glucoside 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

Fig. 1. Chemical structures of anthocyanins.

the market worldwide. These supplements are provided in several forms, including dried juice, dried fruits or water extracts from fruits. Major source for the supplements are blueberries, cranberries, raspberries and grape wine fruits and leaves. The concentration (content) of anthocyanins in single dose of dietary supplement can be in some cases unexpectedly high. For instance, food supplement Urinal Akut® (Walmark a.s., Czech Republic), used in the current study, contains in one tablet the amount of extract equivalent to 338,400 mg of fresh cranberries, as declared by manufacturer. Therefore, very high concentrations of anthocyanins are attained in gastrointestinal tract, and perhaps in portal vein and liver of consumers, leading potentially to food–drug interactions.

Signaling pathway aryl hydrocarbon receptor (AhR)–cytochrome P450 CYP1A1 is involved in the metabolism of xenobiotics, as well as in homeostasis of endogenous compounds such as eicosanoids or retinoids. In addition, AhR plays a role in chemically induced carcinogenesis (Haarmann-Stemmann et al., 2012), immune response, cell differentiation, cell cycle, adaptive skin responses (Haarmann-Stemmann et al., 2013) and many other cellular functions (Abel and Haarmann-Stemmann, 2010). It was demonstrated that some flavonoids act as AhR agonists leading to increased transcription of CYP1A1 gene (Hodek et al., 2002). However, the complex study focused on anthocyanins was not performed yet. We have recently examined the effects of six major anthocyanidins, the aglycones of anthocyanins, on AhR–CYP1A1 pathway (Kamenickova et al., 2013).

In the present study, we investigated the effects of 21 major anthocyanins and the extracts from 3 food supplements containing anthocyanins on the AhR–CYP1A1 signaling pathway in human hepatocytes and human hepatic HepG2 and intestinal LS174T cancer cells.

2. Materials and methods

2.1. Compounds and reagents

Dimethylsulfoxide (DMSO) and hygromycin B were purchased from Sigma–Aldrich (Prague, Czech Republic). The anthocyanins, peonidin-3-*O*-glucoside chloride (PEO-1; ref.#0929S; purity ≥95%), peonidin-3-*O*-rutinoside chloride (PEO-2; ref.#0945S; purity ≥95%), pelargonidin-3,5-di-*O*-glucoside chloride (PEL-1; syn. pelargonin; ref.#0903S; purity ≥90%), pelargonidin-3-*O*-rutinoside chloride (PEL-2; ref.#0943S; purity ≥90%), delphinidin-3-*O*-glucoside chloride (DEL-1; syn. myrtillin; ref.#0938S; purity ≥95%), delphinidin-3-*O*-rutinoside chloride (DEL-2; syn. tulipanin; ref.#0901S; purity ≥95%), delphinidin-3,5-di-*O*-glucoside chloride (DEL-3; syn. delphin; ref.#0941S; purity ≥97%), delphinidin-3-*O*-sambubioside chloride (DEL-4; ref.#0948S; purity ≥90%), delphinidin-3-*O*-rhamnoside chloride (DEL-5; ref.#0940S; purity ≥90%), malvidin-3-*O*-glucoside chloride (MAL-1; syn. oenin; ref.#0911S; purity ≥95%), malvidin-3,5-di-*O*-glucoside chloride (MAL-2; syn. malvin; ref.#0930S; purity ≥95%), malvidin-3-*O*-galactoside chloride (MAL-3; syn. primulin; ref.#0931S; purity ≥97.5%), cyanidin-3-*O*-glucoside chloride (CYA-1; syn. kuromanin, asterin, chrysanthemin; ref.#0915S; purity ≥96%), cyanidin-3-*O*-rutinoside chloride (CYA-2; syn. keracyanin; ref.#0914S; purity ≥96%), cyanidin-3,5-di-*O*-glucoside chloride (CYA-3; cyanin; ref.#0932S; purity ≥97%), cyanidin-3-*O*-sophoroside chloride (CYA-4; ref.#0937S; purity ≥95%), cyanidin-3-*O*-arabinoside chloride (CYA-5; ref.#0908S; purity ≥97%), cyanidin-3-*O*-rhamnoside chloride (CYA-6; ref.#0939S; purity ≥90%), cyanidin-3-*O*-galactoside chloride (CYA-7; syn. ideain; ref.#0923S; purity ≥97%), cyanidin-3-*O*-sambubioside chloride (CYA-8; ref.#0949S; purity ≥95%), cyanidin-3-*O*-lathyroside chloride (CYA-9; ref.#0936S; purity ≥97%) and proanthocyanidin A2 (PAC, purity ≥97%) were purchased from extrasynthese (Lyon, France). Luciferase lysis buffer and P450-Glo CYP1A1 assay were from Promega (www.promega.com, Hercules, CA). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCycler FastStart DNA Master^{PLUS} SYBR Green I was from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). Following dietary supplements were purchased in public pharmacy store: SUP-1 (Antistax®, Tablets à 360 mg of *Vitis viniferae folii extractum aquosum siccum*, total amount of anthocyanins 24 mg/100 g with the major components being malvidin-3-glucoside (17.5 mg/100 g), and cyanidin-3-glucoside (4.8 mg/100 g), Boehringer Ingelheim International GmbH, Germany), SUP-2 (Urinal Akut®, Tablets à 36 mg of proanthocyanidins, the amount of anthocyanins 44 mg/100 g with the highest representation of cyanidin-3-arabinoside (32.9 mg/100 g), and cyanidin-3-galactoside (9.8 mg/100 g), Walmark a.s., Czech Republic), SUP-3 (Ostrovid, Tablets à 40 mg of extract from *Vaccinium myrtillus*, the amount of anthocyanins 911 mg/100 g with the highest amount of cyanidin-3-glucoside (714.2 mg/100 g), cyanidin-3-galactoside (66.7 mg/100 g), delphinidin-3-glucoside (58.2 mg/100 mg), and malvidin-3-glucoside (41.6 mg/100 g), Swiss Herbal Remedies Ltd., Canada). Amount of anthocyanins was determined by HPLC by a standard method (Durst, 2001). The extracts were prepared by pulverizing and dissolving 1 tablet of each supplement in 3 mL of sterile distilled water. All other chemicals were of the highest quality commercially available.

2.2. 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); tissue acquisition protocol was in accordance with the requirements issued by local ethical commission in the Czech Republic. Hepatocytes were treated in a serum-free medium for 24 h or 48 h with the tested compounds, TCDD (5 nM) and/or vehicle (DMSO; 0.1%, v/v). Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.3. Human cancer cell lines

Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430) and human Caucasian colon adenocarcinoma cells LS174T (ECACC No. 87060401) were purchased from ECACC and were cultured as recommended by manufacturer. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Gene reporter assay

Stably transfected gene reporter cell line AZ-AHR, which was derived from HepG2 cells transfected with a construct containing several AhR binding sites upstream of a luciferase reporter gene, were used (Novotna et al., 2011). Following plating, cells were stabilized for 16 h and then incubated for 24 h with tested compounds, tested extracts, TCDD (5 nM), and/or vehicle (DMSO; 0.1%, v/v). After the treatments, cells were lysed using Reporter Lysis Buffer (Promega, Madison, WI, USA) according to manufacturers' instructions, and luciferase activity was measured.

2.5. 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) and random hexamers 3801 (Takara). qRT-PCR was carried out on Light Cycler apparatus 480 II (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP1A1 and GAPDH mRNAs were determined as described elsewhere (Dvorak et al., 2008). The measurements were performed in triplicates. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. Data were processed by delta–delta method.

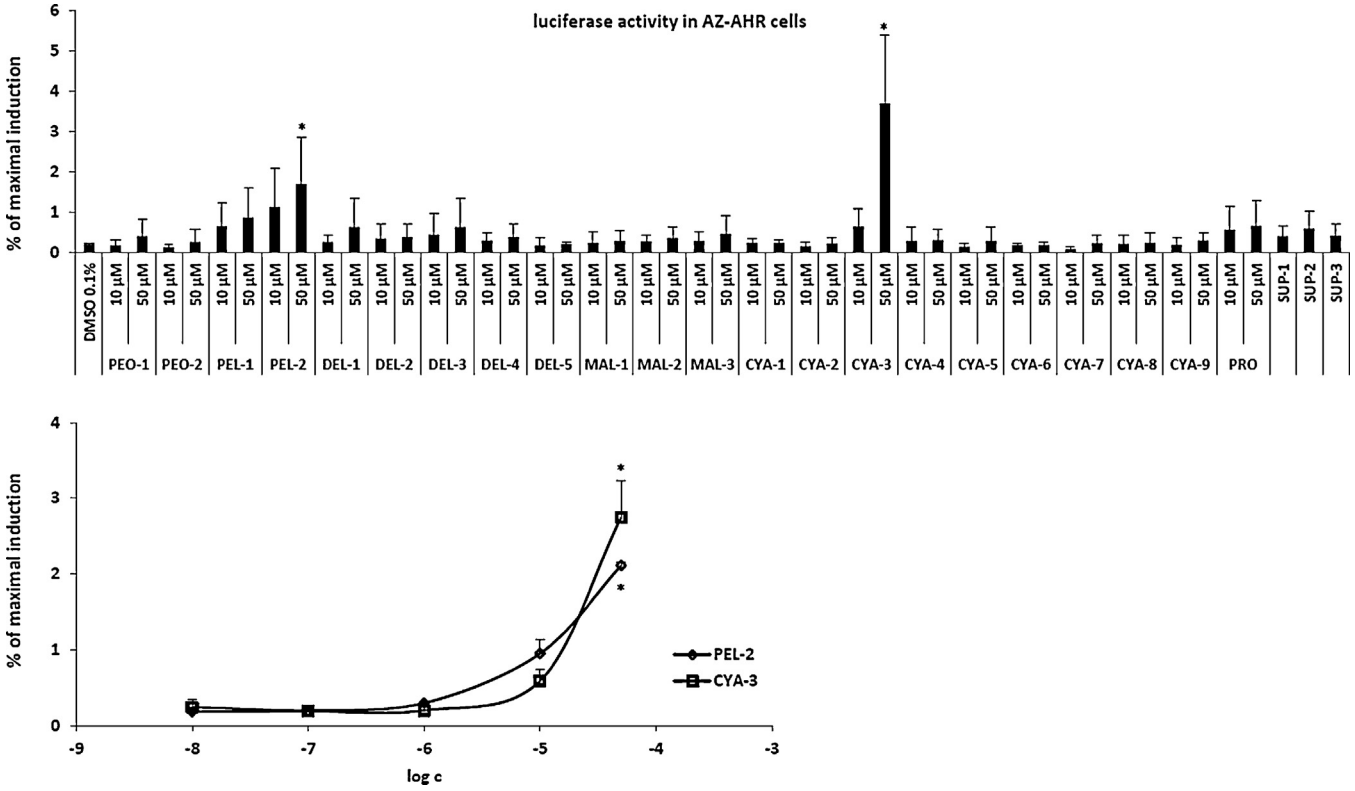

Fig. 2. Effects of anthocyanins on transcriptional activity of AhR in AZ-AHR transgenic cell line. AZ-AHR cells were plated at 96-well plates and stabilized for 16 h. *Bar graph*: Cells were treated for 24 h with 21 anthocyanins (10 μM and 50 μM; i.e. PEO-1, PEO-2, PEL-1, PEL-2, DEL-1, DEL-2, DEL-3, DEL-4, DEL-5, MAL-1, MAL-2, MAL-3, CYA-1, CYA-2, CYA-3, CYA-4, CYA-5, CYA-6, CYA-7, CYA-8, CYA-9), proanthocyanidin A2 (10 μM and 50 μM; PRO), extracts from food supplements (SUP-1, SUP-2, SUP-3), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1%, v/v). *Plot graph*: Cells were treated for 24 h with PEL-2 and CYA-3 in concentrations of 10 nM, 100 nM, 1 μM, 10 μM and 50 μM. After the treatments, cells were lysed and luciferase activity was measured. The data are the mean from triplicate measurements and are expressed as a percentage of the induction attained by TCDD. Experiments were performed in three different passages of AZ-AHR cells. The differences between individual measurements were lower that 5%. *Values significantly different from the vehicle value ($p < 0.05$) as determined by the Student's *t*-test.

2.6. Protein detection and Western blotting

Total protein extracts were prepared as described elsewhere (Pavek et al., 2007). Following SDS-PAGE separation and Western blot transfer, blots were probed with antibody against CYP1A1 (goat polyclonal; sc-1616), purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescent detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit.

2.7. Enzyme activity of CYP1A1 in human liver microsomes

Pooled human liver microsomes were obtained from Biopredic (Rennes, France) in accordance with ethical regulations of the country of origin (France). They were from 26 donors (20 males and 6 females) with a protein content of 25 mg/mL; the CYP1A1/2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4/5 enzyme activities were verified before the experiment.

Activity of CYP1A1 was evaluated using a luciferase based assay with CYP1A1 specific substrate Luciferin-CEE (www.promega.com, Promega, Hercules, CA). Formation of product of the reaction was determined by measuring the luminescence using Infinite M200 spectrophotometer/spectrofluorometer/luminometer (TECAN Austria, Vienna). Incubation mixture contained 100 mM potassium phosphate buffer (pH 7.4). Aqueous stock solution of anthocyanins and proanthocyanidin A2 was 1 mM (pH 6.7). Final reaction mixture volume was 100 μL. Inhibition experiments were performed with five concentrations of anthocyanins (0, 10, 20, 40, 80, 100 μM). With luciferin-CEE luminogenic substrate, microsomes with 22 pmol total CYP were preincubated with 60 μM luciferin-CEE and with the respective anthocyanin (same final concentrations as above) for 30 min at 37 °C; then, NADPH-generating system was added (0.8 mM $NADP^+$, 5.8 mM isocitrate, 0.3 unit/mL of isocitrate dehydrogenase and 8 mM $MgCl_2$), and the system was incubated for 10 min. Detection reagent was then added and the reaction mixture was incubated again for 20 min according to the recommended protocol (www.promega.com). Two independent experiments were done with duplicates which did not differ by more than 10%. Inhibition of CYP1A1 activity was in all cases evaluated by plotting the respective remaining activity versus the inhibitor concentration.

2.8. AhR ligand binding assay

[^{3}H]TCDD was kindly provided by Dr. Steven Safe (Texas A&M University) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) was from Accustandard (New Haven, CT, USA). The competitive displacement of [^{3}H]TCDD from guinea pig hepatic cytosol was as previously described (Korashy et al., 2011). Briefly, hepatic guinea pig cytosol diluted to 8 mg/mL protein in MEDG (25 mM MOPS-NaOH, pH 7.5, 1 mM EDTA, 1 mM DTT, 10%, v/v glycerol) was incubated with different concentrations of PEL-2 (pelargonidin-3-*O*-rutinoside chloride), CYA-3 (cyanidin-3,5-di-*O*-glucoside chloride) or 200 nM TCDF for 1 h at room temperature in the presence of 2 nM [^{3}H]TCDD. The amount of [^{3}H]TCDD specific binding was determined by hydroxyapatite protocol, and specific binding was determined as the difference between the 'no competitor' and TCDF reaction (Denison et al., 2002). [^{3}H]TCDD specific activity was 9.5 Ci/mmol. Non-specific binding was approximately 30% of total binding, i.e. specific binding was approximately 70% of total binding.

3. Results

3.1. Effects of anthocyanins on transcriptional activity of AhR in AZ-AHR reporter cells

In the first series of experiments, we examined the effects of 21 different anthocyanins (chemical structures shown in Fig. 1; abbreviations explained in Section 2.1), proanthocyanidin A2, and extracts from 3 dietary supplements containing anthocyanins, on transcriptional activity of AhR in recombinant AZ-AHR cells, which are HepG2 cells that had been stably transfected with an AhR-responsive luciferase reporter plasmid (Novotna et al., 2011). Cells were incubated for 24 h with 21 different anthocyanins (10 μM and 50 μM), proanthocyanidin A2 (10 μM and 50 μM), extracts from 3 dietary supplements (1000× dilution), TCDD (5 nM) or vehicle (0.1%, v/v DMSO). In nine independent experiments, induction of luciferase activity by 5 nM TCDD varied from 87-fold to

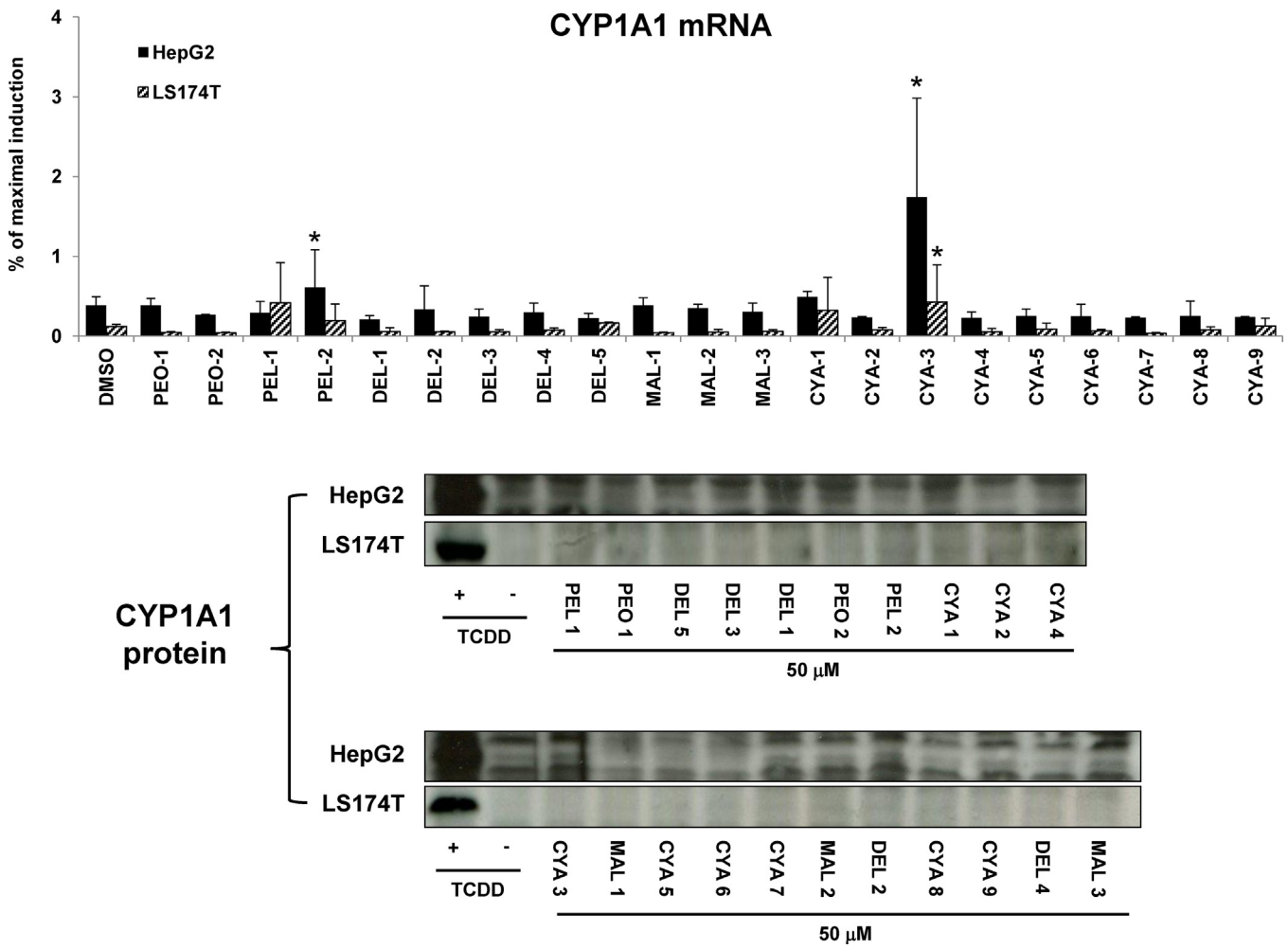

Fig. 3. Effects of anthocyanins on the expression of CYP1A1 in HepG2 and LS174T cells. *Upper panel*: Cells were incubated for 24 h with 21 anthocyanins (10 μM and 50 μM; i.e. PEO-1, PEO-2, PEL-1, PEL-2, DEL-1, DEL-2, DEL-3, DEL-4, DEL-5, MAL-1, MAL-2, MAL-3, CYA-1, CYA-2, CYA-3, CYA-4, CYA-5, CYA-6, CYA-7, CYA-8, CYA-9), TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v). Bar graph shows RT-PCR analyses (2 independent experiments) of CYP1A1 mRNA. The data are the mean from triplicate measurements and are expressed as a percentage of maximal induction attained in TCDD-treated cells. The data were normalized *per* GAPDH mRNA levels. *Value is significantly different from DMSO-treated cells ($p < 0.05$) as determined by the Student's *t*-test. *Lower panel:* Cells were incubated for 48 h with 21 anthocyanins (10 μM and 50 μM; i.e. PEO-1, PEO-2, PEL-1, PEL-2, DEL-1, DEL-2, DEL-3, DEL-4, DEL-5, MAL-1, MAL-2, MAL-3, CYA-1, CYA-2, CYA-3, CYA-4, CYA-5, CYA-6, CYA-7, CYA-8, CYA-9), TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v). Western blots show a representative analysis of CYP1A1 protein. Similar profiles were observed in two independent experiments. As a loading control, the blots were probed to actin (data not shown).

1023-fold. Anthocyanins PEL-2 (pelargonidin-3-*O*-rutinoside) and CYA-3 (cyaniding-3,5-di-*O*-glucoside) produced a concentration-dependent induction of luciferase activity, which was significantly different from vehicle at concentration of 50 μM (Fig. 2). Although the magnitude of induction by 50 μM PEL-2 and CYA-3 was 10-fold and 17-fold greater than that of the vehicle (DMSO) control, respectively, the magnitude of the induction response was very low when compared to TCDD (i.e. between 2% and 3% of the maximal level of induction by TCDD). No significant induction of luciferase activity was observed with other 19 anthocyanins, proanthocyanidin A2 and extracts from food supplements (dilution 1000×; SUP-1, SUP-2, SUP-3). Taken together, these results indicate that PEL-2 and CYA-3 are weak AhR agonists. Based on the data above, we did not further test extracts from food supplements and proanthocyanidin A2 for their effects on AhR–CYP1A1 pathway in cell lines.

3.2. *Effects of anthocyanins on CYP1A1 expression in HepG2 and LS174T cancer cells*

We studied the effects of anthocyanins on the expression of CYP1A1, a typical target gene of ligand- and AhR-dependent signal transduction. We determined the levels of CYP1A1 mRNA and protein in hepatic HepG2 and intestinal LS174T human cancer cells, which were incubated with 21 different anthocyanins (50 μM), TCDD (5 nM) and DMSO (0.1%, v/v) for 24 h and 48 h. In two independent experiments, TCDD strongly induced CYP1A1 mRNA after 24 h of incubation in HepG2 cells (322-fold and 216-fold) and in LS174T cells (1003-fold and 724-fold). The level of CYP1A1 mRNA was significantly increased by PEL-2 and CYA-3 in HepG2 cells, and by CYA-3 in LS174T cells, while other 19 anthocyanins had no significant effects on CYP1A1 mRNA in either cell line. However, PEL-2 and CYA-3 were relatively weak inducers of CYP1A1 mRNA in both cell lines, increasing CYP1A1 mRNA levels by only about 0.5–2% of that observed with a maximal inducing concentration of TCDD (Fig. 3). These results are consistent with those obtained from AZ-AHR cells, and further support the conclusion that PEL-2 and CYA-3 are AhR agonists, albeit relatively weak compared to TCDD. In contrast, none of the 21 anthocyanins tested increased the levels of CYP1A1 protein in HepG2 and LS174T cells after 48 h incubation, whereas TCDD strongly induced CYP1A1 protein in both cell lines (Fig. 3). This discrepancy is probably due to weak induction of mRNA and hence not detectable change in protein level.

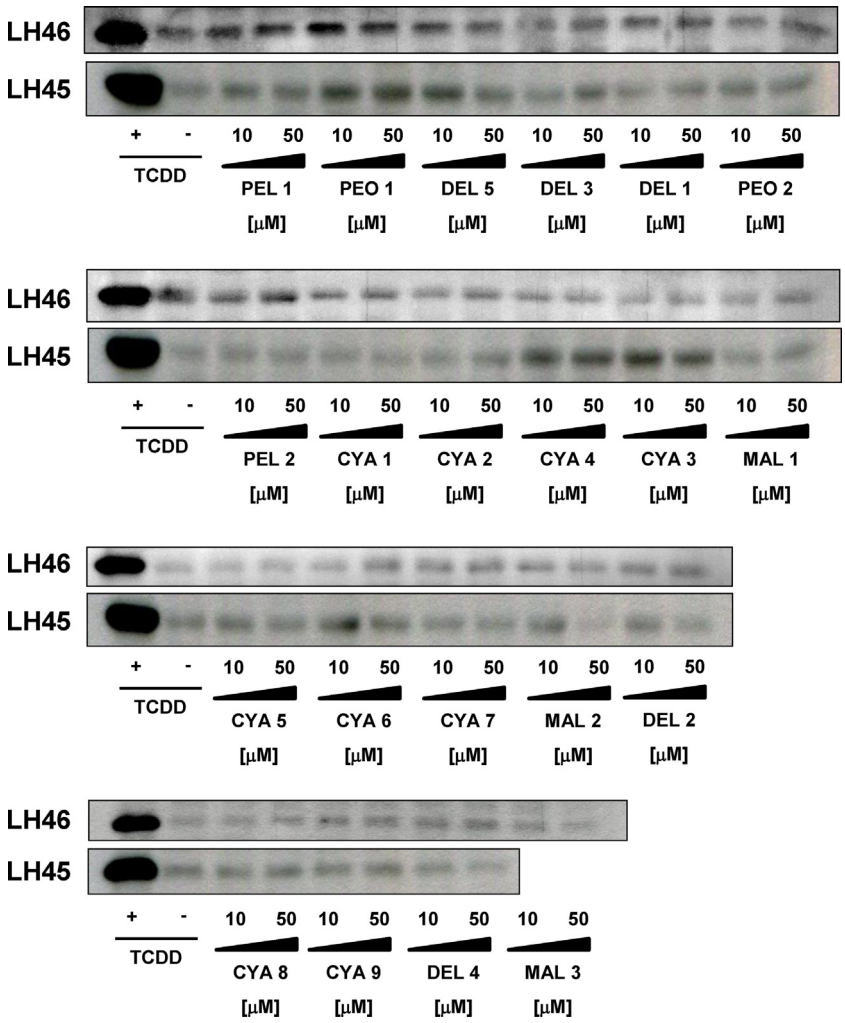

Fig. 4. Effects of anthocyanins on CYP1A protein expression in primary human hepatocytes. Cells were incubated for 48 h with 21 anthocyanins (10 μM and 50 μM; i.e. PEO-1, PEO-2, PEL-1, PEL-2, DEL-1, DEL-2, DEL-3, DEL-4, DEL-5, MAL-1, MAL-2, MAL-3, CYA-1, CYA-2, CYA-3, CYA-4, CYA-5, CYA-6, CYA-7, CYA-8, CYA-9), TCDD (5 nM) and vehicle (DMSO; 0.1%, v/v). Western blots show analyses of CYP1A proteins from two different primary human hepatocytes cultures (LH45 and LH46). As a loading control, the blots were probed to actin (data not shown).

3.3. Effects of anthocyanins on CYP1A1 expression in primary human hepatocytes

In the next series of experiments, we tested the effects of anthocyanins on the expression of CYP1A1 mRNA and protein in primary human hepatocytes. In contrast to the cell lines, human hepatocytes express a full panel of drug-metabolizing enzymes, hence, the mixed effects of the parent compounds and their metabolites are examined. Therefore, we also tested the effects of extracts from food supplements. Four different primary human hepatocytes cultures were used (LH45, LH46, LH47, LH49). Dioxin strongly induced the expression of CYP1A1 mRNAs in all human hepatocytes cultures at 24 h, and the magnitude of induction in cultures LH45, LH46, LH47 and LH49 was 125-fold, 344-fold, 105-fold and 71-fold, respectively. The levels of CYP1A1 protein were strongly induced after 48 h of incubation with 5 nM TCDD in four human hepatocytes cultures; data shown for two cultures (Fig. 4). While slight induction of CYP1A1 mRNA and protein was observed for some of the tested anthocyanins, these increases occurred in a culture dependent manner, i.e. they were not systematic (Table 1 and Fig. 4).

3.4. Ligand binding assay

Since the activation of AhR may occur by ligand-dependent or ligand-independent mechanisms, we tested whether the effects of PEL-2 and CYA-3 on AhR–CYP1A1 signaling pathway involved binding of PEL-2 and CYA-3 to the AhR. We performed AhR ligand binding assays using guinea pig hepatic cytosol. PEL-2 competitively inhibited [^{3}H]-TCDD binding to the AhR when present in the binding incubation at 50 μM (61–71% inhibition) (Fig. 5). These data are consistent with the previous results which suggest that PEL-2 is a weak ligand/agonist of the AhR, and that the effects of PEL-2 on AhR–CYP1A1 signaling pathway likely occur via a ligand-dependent mechanism. On the other hand, CYA-3 did not displace radiolabeled TCDD from AhR in any tested concentration (Fig. 5). Therefore, effects of CYA-3 on AhR–CYP1A1 signaling pathway are probably ligand-independent.

3.5. Effects of anthocyanins on CYP1A1 catalytic activity in human liver microsomes

Given the ability of many AhR ligands to also bind to and be metabolized by the CYP1A enzymes, we directly examined the effects of anthocyanins on the activity of the CYP1A1 by following a specific luciferin activating activity using human microsomes. Tested compounds were selected based on the composition of food supplements tested in the current study. Results of competitive kinetic analyses revealed a weak concentration-dependent inhibition of CYP1A1 after the addition of anthocyanins CYA-1, CYA-5,

Table 1
Effects of anthocyanins on the expression of CYP1A1 mRNA in primary human hepatocytes treated for 24 h with tested compounds. Results are expressed as a fold induction over the vehicle-treated cells. Data are mean S.D. from triplicate measurements. n.d., not determined.

Abbr.	Compound	Conc.	LH45	LH46	LH47	LH49
	DMSO	0.1%	1.00 ± 0.38	1.00 ± 0.25	1.00 ± 0.10	1.00 ± 0.11
	TCDD	5 nM	125.4 ± 17.7	343.7 ± 45.6	104.7 ± 13.4	70.5 ± 1.8
PEO-1	Peonidin-3-*O*-glucoside	10 μM	0.21 ± 0.03	2.14 ± 0.18	n.d.	n.d.
		50 μM	0.80 ± 0.22	2.48 ± 0.57	0.51 ± 0.07	0.08 ± 0.03
PEO-2	Peonidin-3-*O*-rutinoside	10 μM	0.20 ± 0.03	1.04 ± 0.02	n.d.	n.d.
		50 μM	0.67 ± 0.07	1.15 ± 0.09	0.54 ± 0.03	0.08 ± 0.00
PEL-1	Pelargonidin-3,5-di-*O*-glucoside	10 μM	0.70 ± 0.17	1.49 ± 0.10	n.d.	n.d.
		50 μM	1.17 ± 0.14	2.21 ± 0.66	0.59 ± 0.52	0.98 ± 0.27
PEL-2	Pelargonidin-3-*O*-rutinoside	10 μM	0.87 ± 0.04	2.66 ± 0.20	n.d.	n.d.
		50 μM	0.24 ± 0.01	2.39 ± 0.22	0.61 ± 0.01	0.60 ± 0.17
DEL-1	Delphinidin-3-*O*-glucoside	10 μM	1.80 ± 0.11	1.99 ± 0.12	n.d.	n.d.
		50 μM	1.50 ± 0.21	0.80 ± 0.07	1.01 ± 0.12	0.01 ± 0.00
DEL-2	Delphinidin-3-*O*-rutinoside	10 μM	1.53 ± 0.36	1.34 ± 0.10	n.d.	n.d.
		50 μM	0.53 ± 0.11	0.68 ± 0.01	0.23 ± 0.05	0.02 ± 0.01
DEL-3	Delphinidin-3,5-di-*O*-glucoside	10 μM	0.82 ± 0.02	0.85 ± 0.06	n.d.	n.d.
		50 μM	1.70 ± 3.26	1.14 ± 0.21	0.39 ± 0.06	0.02 ± 0.01
DEL-4	Delphinidin-3-*O*-sambubioside	10 μM	0.26 ± 0.21	1.11 ± 0.20	n.d.	n.d.
		50 μM	0.34 ± 0.09	1.31 ± 0.05	0.21 ± 0.26	0.07 ± 0.01
DEL-5	Delphinidin-3-*O*-rhamnoside	10 μM	0.48 ± 0.01	0.98 ± 0.15	n.d.	n.d.
		50 μM	0.40 ± 0.09	1.19 ± 0.18	0.26 ± 0.01	0.01 ± 0.01
MAL-1	Malvinidin-3-*O*-glucoside	10 μM	1.13 ± 0.35	2.34 ± 0.25	n.d.	n.d.
		50 μM	0.93 ± 0.11	1.56 ± 0.23	0.14 ± 0.13	0.29 ± 0.03
MAL-2	Malvinidin-3,5-di-*O*-glucoside	10 μM	1.14 ± 0.28	1.72 ± 0.39	n.d.	n.d.
		50 μM	0.44 ± 0.01	1.64 ± 0.38	0.56 ± 0.07	0.93 ± 0.09
MAL-3	Malvinidin-3-*O*-galactoside	10 μM	0.12 ± 0.00	0.87 ± 0.02	n.d.	n.d.
		50 μM	0.28 ± 0.04	1.32 ± 0.05	0.54 ± 0.02	0.33 ± 0.02
CYA-1	Cyanidin-3-*O*-glucoside	10 μM	1.22 ± 0.04	1.14 ± 0.20	n.d.	n.d.
		50 μM	0.73 ± 0.22	1.04 ± 0.12	0.28 ± 0.01	0.03 ± 0.00
CYA-2	Cyanidin-3-*O*-rutinoside	10 μM	2.34 ± 0.28	0.55 ± 0.03	n.d.	n.d.
		50 μM	0.69 ± 0.25	0.91 ± 0.04	0.57 ± 0.09	0.07 ± 0.02
CYA-3	Cyanidin-3,5-di-*O*-glucoside	10 μM	0.68 ± 0.20	2.21 ± 0.16	n.d.	n.d.
		50 μM	0.48 ± 0.07	2.59 ± 0.09	0.45 ± 0.03	0.13 ± 0.03
CYA-4	Cyanidin-3-*O*-sophoroside	10 μM	1.31 ± 0.25	2.97 ± 0.39	n.d.	n.d.
		50 μM	0.46 ± 0.03	0.28 ± 0.05	0.51 ± 0.16	0.05 ± 0.02
CYA-5	Cyanidin-3-*O*-arabinoside	10 μM	0.69 ± 0.06	0.67 ± 0.07	n.d.	n.d.
		50 μM	1.16 ± 0.17	0.61 ± 0.08	0.34 ± 0.11	0.07 ± 0.01
CYA-6	Cyanidin-3-*O*-rhamnoside	10 μM	0.77 ± 0.12	1.19 ± 0.14	n.d.	n.d.
		50 μM	0.73 ± 0.04	1.36 ± 0.30	0.31 ± 0.03	0.07 ± 0.00
CYA-7	Cyanidin-3-*O*-galactoside	10 μM	0.65 ± 0.11	2.62 ± 0.41	n.d.	n.d.
		50 μM	0.34 ± 0.06	1.85 ± 0.09	0.25 ± 0.02	0.05 ± 0.02
CYA-8	Cyanidin-3-*O*-sambubioside	10 μM	0.26 ± 0.01	1.48 ± 1.14	n.d.	n.d.
		50 μM	0.20 ± 0.04	1.72 ± 0.72	2.44 ± n.d.	0.02 ± 0.00
CYA-9	Cyanidin-3-*O*-lathyroside	10 μM	0.32 ± 0.12	0.91 ± 0.11	n.d.	n.d.
		50 μM	0.61 ± 0.05	1.77 ± 0.49	0.11 ± 0.17	0.09 ± 0.05
SUP-1	Ostrovid		n.d.	n.d.	0.57 ± 0.72	n.d.
SUP-2	Antistax		n.d.	n.d.	0.91 ± 1.16	n.d.
SUP-3	Urinal		n.d.	n.d.	0.77 ± 1.00	n.d.

CYA-7, MAL-1, PEO-1, PEO-2, DEL-1, CYA-3, PAC, albeit to a relatively low extent (to 80–65% of the initial activity, i.e. without anthocyanidin added) at the highest concentration of the respective anthocyanin (i.e. 100 μM). The data (Fig. 6) clearly document the course of the inhibition of CYP1A1 activity by various anthocyanins with maximum extent down to 62% with 100 μM of malvidine 3-glucoside (MAL-1). These results thus document the relatively weak ability of anthocyanins to interact with drug metabolizing system of CYP1A1.

4. Discussion

A phenomenon of food–drug (or drug–food) interactions may principally occur in two ways: (i) inhibition of drug-metabolizing enzymes, or by competition for this enzyme; (ii) induction of drug-metabolizing enzymes via activation of nuclear receptors (e.g. vitamin D receptor – VDR, farnesoid X receptor – FXR), receptors for steroid hormones (e.g. glucocorticoid receptor – GR, estrogen receptor – ER) or xenoreceptors (e.g. aryl hydrocarbon

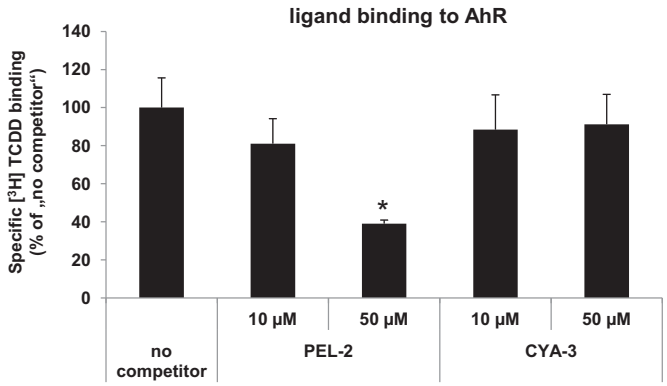

Fig. 5. Ligand binding assay. Guinea pig hepatic cytosol was incubated with indicated concentrations of PEL-2 (pelargonidin-3-*O*-rutinoside chloride), CYA-3 (cyanidin-3,5-di-*O*-glucoside chloride) or 200 nM TCDF for 1 h at room temperature in the presence of 2 nM [^{3}H]TCDD. Ligand binding to the cytosolic proteins was determined by the hydroxyapatite binding protocol and scintillation counting. Specific binding was determined as a difference between total and non-specific (TCDF) reactions. The values are presented as mean ± SD of three independent reactions. *Values significantly different from the 'no competitor' reaction at $p < 0.05$ as determined by the Student's *t*-test. The results are representative of two independent experiments.

receptor – AhR, pregnane X receptor – PXR). Activation of nuclear, steroid and xeno-receptors by xenobiotics, such as drugs or food constituents may have plenty of physiological and pathophysiological consequences, other than induction of drug-metabolizing enzymes. For instance, AhR is a key transcriptional regulator of phase I enzymes (CYP1A1, CYP1A2, CYP1B1) and phase II enzymes (GSTA1, UGT1A2). On the other hand, AhR plays various physiological roles (e.g. in immune response, in cellular proliferation and differentiation) and pathophysiological roles (e.g. in chemically induced carcinogenesis). The behavior of AhR resembles Dr. Jekyll and Mr. Hyde from R.L. Stevenson's novel, when activation of AhR by environmental pollutants leads to toxic responses to human organism, while activation by endogenous ligands is necessary for human health. Given the complex role of AhR in toxicological response and in human physiology and pathophysiology, this is of topical interest to examine the effects of xenobiotics, including food constituents, on the activity of AhR.

In the present study, we examined the effects of 21 major anthocyanins and 3 extracts from food supplements containing anthocyanins, on AhR–CYP1A1 signaling pathway in primary human hepatocytes and in human hepatic HepG2 and intestinal LS174T cancer cells. Anthocyanins are plant pigments occurring in grape wines and berry fruits such as blueberries, cranberries and raspberries. In addition, anthocyanins are the active constituents of food supplements based on the extracts from berry fruits. The amounts of anthocyanins and other phenolics in food supplements are sometimes so high that their consumption results in intestinal or plasma concentrations in order of magnitude higher as compared to that attained by consumption of fresh fruits. Indeed, a food supplement SUP-2 (Urinal Akut®, Tablets à 36 mg of proanthocyanidins Walmark a.s., Czech Republic), used in the current study, contains in one tablet the amount of extract equivalent to 338,400 mg of fresh cranberries, as declared by manufacturer. The data presented in the current paper show that only two anthocyanins of 21 tested displayed an activity toward AhR–CYP1A1 signaling pathway. It was the case of pelargonidin-3-*O*-rutinoside (PEL-2) and cyanidin-3,5-*O*-diglucoside (CYA-3), which dose-dependently activated AhR, as revealed by gene reporter assay. In addition, these two compounds induced CYP1A1 mRNA but not protein in HepG2 and LS174T cells. The effects of PEL-2 and CYA-3 on AhR occurred by ligand-dependent and ligand-independent mechanisms, respectively, as demonstrated by ligand binding assay. The effects of PEL-2 are analogical to those observed for pelargonidin, an aglycone for PEL-2, as we described recently (Kamenickova et al., 2013). Neither compound nor extract from food supplements induced CYP1A1 mRNA and protein in four different primary human hepatocytes cultures. This is an important finding with regard to the fact that examination in primary human hepatocytes comprises both maternal compounds and metabolites (Vanzo et al., 2011). In line with these results, also the CYP1A1 enzyme activity was not prominently influenced by anthocyanins tested. Inhibition of CYP1A1 was concentration-dependent, however, it did not reach 50% even at the highest anthocyanin concentration, i.e. 100 µM. On the contrary, the aglycon parts of the anthocyanins tested, i.e. the anthocyanidins exhibited in at least two cases, of pelargonidin (PEL) and delphinidin (DEL), an inhibition of CYP1A1 with IC_{50} values of 33 and 77 µM, respectively, documenting the ability of aglycons (anthocyanidins) to interact with drug metabolism by CYP1A enzyme (Kamenickova et al., 2013). Conjugation with sugar part, however, lowered the inhibition of enzyme activity as shown in this paper and led to less prominent activation of the AhR.

Collectively, tested anthocyanins and the extracts from food supplements that contain anthocyanins possess very low, if any, potential for food–drug interactions with respect to AhR–CYP1A1 pathway. This result is – taken from the consumer's point of view – important for the safety of their use.

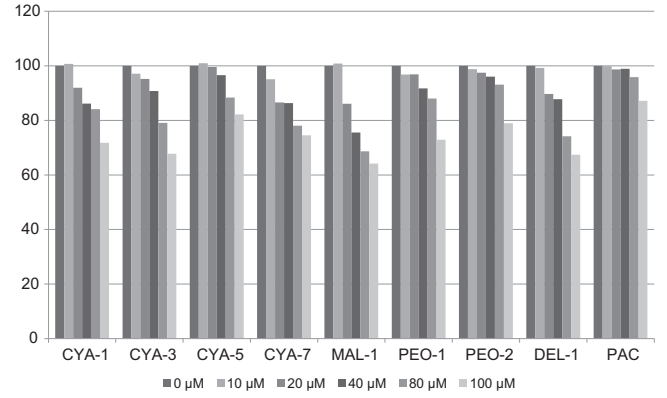

Fig. 6. Effects of anthocyanins on CYP1A1 catalytic activity in human liver microsomes. Inhibition of human microsomal CYP1A1 catalytic activity by eight anthocyanins and proanthocyanidin A2 (PAC) expressed as the amount of activity remaining relative to control (without anthocyanin) in percent. Concentration of respective anthocyanins in the reaction mixture was 0, 10, 20, 40, 80 and 100 µM. For experimental details, see Section 2.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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APPENDIX V

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Effects of artificial sweeteners on the AhR- and GR-dependent CYP1A1 expression in primary human hepatocytes and human cancer cells

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ABSTRACT

Food constituents may cause a phenomenon of food–drug interactions. In the current study, we examined the effects of artificial sweeteners (aspartame, acesulfame, cyclamate, saccharin) on the aryl hydrocarbon receptor (AhR) and glucocorticoid receptor (GR)–dependent expression of CYP1A1 in human hepatocytes, hepatic HepG2 and intestinal LS174T cancer cell lines. Sweeteners were tested in concentrations up to those occurring in non-alcoholic beverages. Basal and ligand-inducible AhR- and GR-dependent reporter gene activation in stably transfected HepG2 and HeLa cells, respectively, were not affected by either of the sweeteners tested after 24 h of incubation. The expression of CYP1A1 mRNA and protein in primary cultures of human hepatocytes and in LS174T and HepG2 cells was not induced by any of the tested sweeteners. Overall, aspartame, acesulfame, saccharin and cyclamate had no effects on CYP1A1 expression and transcriptional activities of AhR and GR. These data imply the safety of artificial sweeteners in terms of interference with AhR, GR and CYP1A1.

1. Introduction

The roles of receptors for steroid hormones (e.g. GR = glucocorticoid receptor; ER = estrogen receptor), nuclear receptors (e.g. RXR = retinoic X receptor; LXR = liver X receptor) and xenoreceptors (e.g. PXR = pregnane X receptor, CAR = constitutive androstane receptor; AhR = aryl hydrocarbon receptor) in the regulation of drug metabolizing enzymes, were elucidated in late 1990s (Pavek and Dvorak, 2008). Regarding the latter, the transcriptional regulation of CYP1A1 in various tissues and CYP1A2 in liver has long been considered as the primary function of AhR. CYP1A1 is inducible by a variety of xenobiotic AhR ligands (but also by endogenous ligands), including drugs, natural compounds, synthetic chemicals and environmental pollutants (Denison and Nagy, 2003; Stejskalova et al., 2011). Cytochromes P450 1A are known for their roles in activation of carcinogens and in the metabolism of drugs (Anzenbacher and Anzenbacherova, 2001; Monostory et al., 2009). Besides its role in CYP1A genes induction, the AhR plays many physiological functions and it is involved in chemically-induced carcinogenesis (Abel and Haarmann-Stemmann, 2010). The role of glucocorticoid receptor (GR) in the regulation of drug metabolizing enzymes was demonstrated (Dvorak and Pavek, 2010), and multiple cross-talks between AhR–GR were described (Monostory and Dvorak, 2011). In the recent years, a phenomenon of *food–drug* interactions emerged, since pharmacokinetic and pharmacodynamics interactions between food constituents and drugs were described. *Food–drug* interactions by food constituents occur mainly in two ways; i.e. by inhibition of drug-metabolizing enzymes or by induction of drug-metabolizing enzymes that comprises transcriptional activation of xenoreceptors (e.g. AhR) or receptors for steroid hormones (e.g. GR). Therefore, it is of value to identify chemicals that influence CYP1A1 expression and transcriptional activities of AhR and GR, with regard to putative *food–drug* interactions and effects on human health. Indeed, we have recently identified several ready-to-drink teas (Kamenickova et al., 2012) and flavored mineral waters (Kamenickova and Dvorak, 2012), which activated AhR and induced CYP1A1.

In the present paper, we have examined the effects of the most common artificial sweeteners, (aspartame, acesulfame, cyclamate, saccharin) on the transcriptional activities of aryl hydrocarbon receptor (AhR) and glucocorticoid receptor (GR), and on expression of CYP1A1 in primary human hepatocytes and, in human intestinal LS174T cancer cell line. We found that aspartame, acesulfame,

Abbreviation: AhR, Aryl hydrocarbon Receptor; CAR, Constitutive androstane receptor; ER, estrogen receptor; GR, glucocorticoid receptor; LXR, liver X receptor; PXR, pregnane X receptor; RXR, retinoic X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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saccharin and cyclamate had no effects on CYP1A1 induction and activation of AhR and GR receptors.

2. Materials and methods

2.1. Compounds and reagents

DMSO, aspartame, acesulfame, cyclamate, saccharin, resveratrol, dexamethasone and hygromycin B were purchased from Sigma–Aldrich (Prague, Czech Republic). Luciferase lysis buffer and P450-Glo CYP1A1 assay were from Promega (www.promega.com; Hercules, CA). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCycler FastStart DNA Master^{PLUS} SYBR Green I was from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). All other chemicals were of the highest quality commercially available.

2.2. Human hepatocytes

Human hepatocytes were obtained from two sources: (i) from multiorgan donors: LH47 (M, 47 years), LH49 (M, 38 years), LH50 (F, 55 years); 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 HEP220708 (F, 64 years) (Biopredic International, Rennes, France). Hepatocytes were treated in a serum-free medium for 24 h (for mRNA analyses) or 48 h (for protein analyses) with the tested compounds, TCDD (5 nM) and/or vehicle (DMSO; 0.1% v/v). Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.3. Human cancer cell lines

Human Caucasian colon adenocarcinoma cells LS174T (ECACC No. 87060401) and human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430) were purchased from ECACC and were cultured as recommended by manufacturer. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Gene reporter assay

A stably transfected gene reporter cell line AZ-AHR, derived from HepG2 cells transfected with a construct containing several AhR binding sites upstream of a luciferase reporter gene, was used for assessment of AhR transcriptional activity (Novotna et al., 2011). A stably transfected gene reporter cell line AZ-GR, derived from HeLa cells transfected with a construct containing several GR response elements upstream of a luciferase reporter gene, was used for assessment of GR transcriptional activity (Novotna et al., 2012). Cells were incubated for 24 h with tested compounds and/or vehicle (DMSO; 0.1% v/v), in the presence or absence of TCDD (5 nM) or dexamethasone (DEX; 100 nM). After the treatments, cells were lysed and luciferase activity was measured.

2.5. 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) and random hexamers 3801 (Takara). qRT-PCR was carried out on Light Cycler apparatus 480 II (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were determined as described elsewhere (Dvorak et al., 2008). The measurements were performed in triplicates. Gene expression levels were normalized to GAPDH, a housekeeping gene. Data were processed by delta–delta method.

2.6. Protein detection and Western blotting

Total protein extracts were separated by SDS–PAGE, and after Western-blot transfer, blots were probed with antibody against CYP1A1 (goat polyclonal; sc-1616), purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescent detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit.

3. Results

3.1. Effects of sweeteners on transcriptional activities of AhR and GR in gene reporter cell lines

The effects of artificial sweeteners on transcriptional activity of the AhR and GR were examined in recombinant AZ-AHR and AZ-GR cells, which are HepG2 and HeLa cells that had been stably transfected with an AhR- and GR-responsive luciferase reporter plasmid, respectively (Novotna et al., 2011, 2012). The cells were incubated for 24 h with sweeteners (aspartame, acesulfame, cyclamate, saccharin) or vehicle (0.1% v/v DMSO), in the presence (antagonist mode) or absence (agonist mode) of ligand (5 nM TCDD and 100 nM DEX). Sweeteners were tested in concentrations up to those occurring in non-alcoholic beverages (Bergamo et al., 2011). In three independent experiments, luciferase activity in AZ-AHR cells was increased 1011-fold, 181-fold and 153-fold by TCDD (5 nM), the most potent activator of AhR. No significant induction of luciferase activity was observed with saccharin, aspartame, acesulfame or cyclamate. In antagonist mode, TCDD-induced luciferase activity was drastically inhibited by AhR antagonist resveratrol, but not by any of the sweeteners tested (Fig. 1). Inductions in luciferase activity in three different passages of AZ-GR cells by 100 nM DEX were 74-fold, 37-fold and 41-fold. Transcriptional activity of GR was not affected by tested sweeteners, regardless the absence or presence of DEX (Fig. 1).

3.2. Effects of sweeteners on CYP1A1 expression in LS174T and HepG2 cancer cells

Cytochrome P450 isoform CYP1A1 is a typical target gene of AhR-dependent signal transduction, which is also modulated by GR. Therefore, we tested the effects of sweeteners on the expression of CYP1A1 mRNA and protein in intestinal LS174T and hepatic HepG2 human cancer cell lines. Cells were incubated with sweeteners, TCDD (5 nM) and DMSO (0.1% v/v) for 24 h and 48 h. TCDD strongly induced CYP1A1 mRNA and protein after 24 h and 48 h of incubation in both types of cells, respectively. None of the tested artificial sweeteners did significantly induce the expression of CYP1A1 mRNA or protein in LS174T and HepG2 cells (Fig. 2).

3.3. Effects of sweeteners on CYP1A1 expression in primary human hepatocytes

In the last series of experiments, we tested the effects of sweeteners on the expression of CYP1A1 mRNA and protein in primary cultures of human hepatocytes. In contrast to the cell lines, human hepatocytes express functional drug-metabolizing enzymes, hence, the mixed effects of the parent compounds and their metabolites are examined. Four different primary human hepatocytes cultures were used (LH47, LH49, LH50 and HEP220708). Dioxin strongly induced the expression of CYP1A1 mRNA in all human hepatocytes cultures after 24 h of incubation (105-fold, 152-fold, 221-fold and

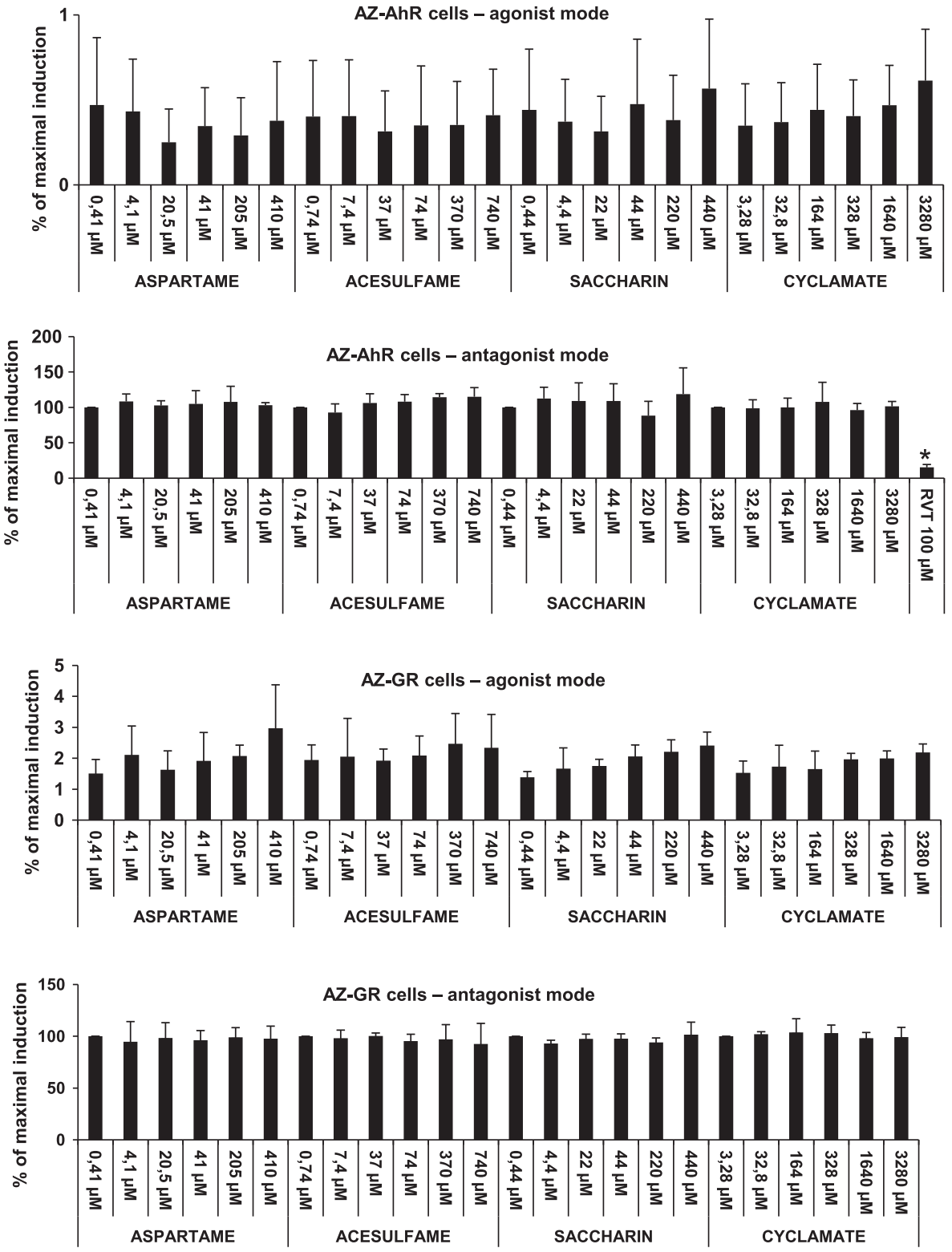


Fig. 1. Effects of sweeteners on transcriptional activities of AhR and GR in transgenic reporter cell lines. AZ-AHR and AZ-GR cells were seeded in 96-well plates, stabilized for 16 h and then incubated for 24 h with aspartame (0.41 μM, 4.1 μM, 20.5 μM, 41 μM, 205 μM, 410 μM), acesulfame (0.74 μM, 7.4 μM, 37 μM, 74 μM, 370 μM, 740 μM), cyclamate (3.28 μM, 32.8 μM, 164 μM, 328 μM, 1640 μM, 3280 μM), saccharin (0.44 μM, 4.4 μM, 22 μM, 44 μM, 220 μM, 440 μM), resveratrol (RVT; 100 μM) and vehicle (DMSO; 0.1% v/v) in the absence (*agonist mode*) or in the presence (*antagonist mode*) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and dexamethasone (DEX; 100 nM). After the treatments, cells were lysed and luciferase activity was measured. Treatments were performed in triplicates. The data are the mean from experiments performed in three different passages of AZ-AhR and AZ-GR cells, and are expressed as a percentage of maximal induction attained by TCDD (i.e. value for TCDD is 100%) or by DEX (i.e. value for DEX is 100%). Inductions in luciferase activity in three different passages of AZ-AhR cells by 5 nM TCDD were 1011-fold, 153-fold and 181-fold. Inductions in luciferase activity in three different passages of AZ-GR cells by 100 nM DEX were 74-fold, 37-fold and 41-fold. * – value is significantly different from TCDD value ($p < 0.05$), as determined by the Student's *t*-test.

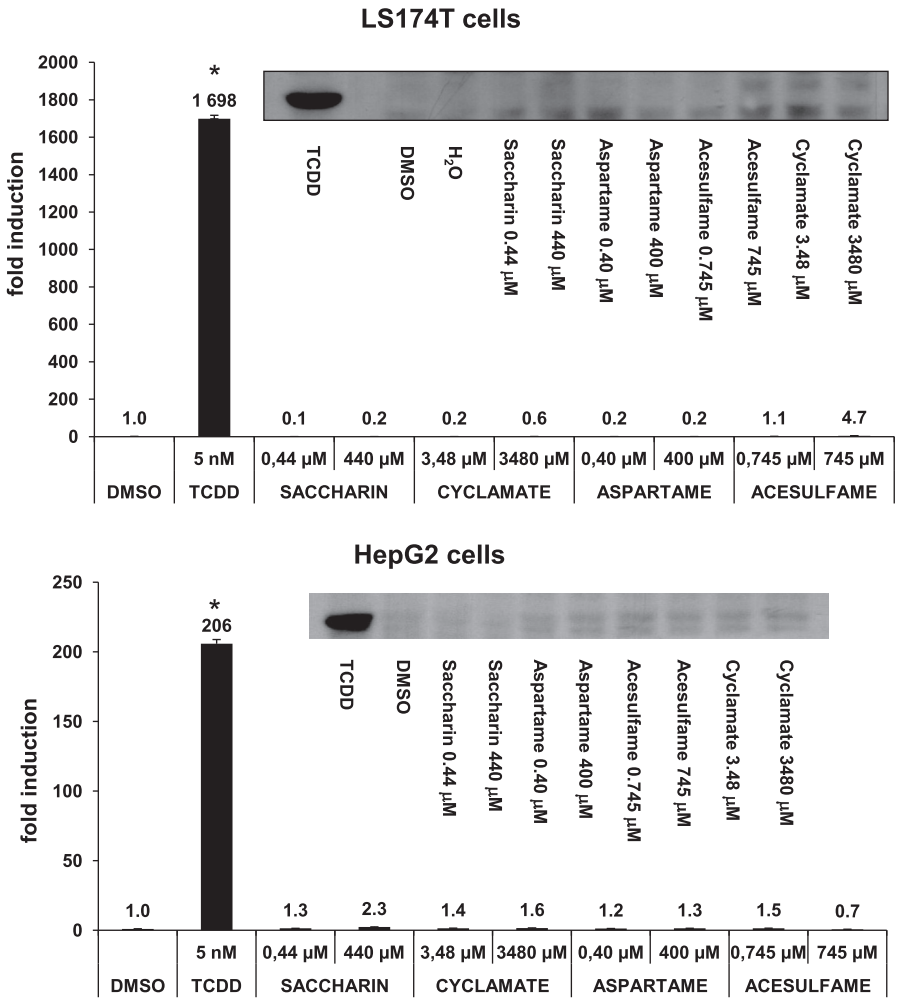


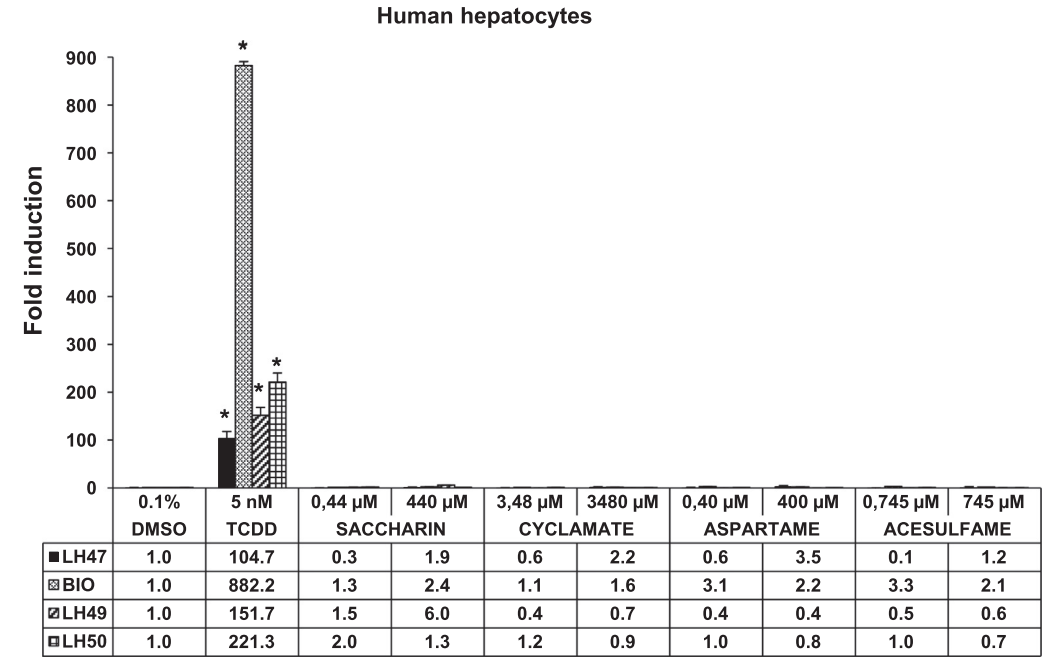
Fig. 2. Effects of sweeteners on CYP1A1 expression in LS174T intestinal and HepG2 hepatic cancer cells. LS174T cells and HepG2 cells were treated for 24 h and 48 h with aspartame (0.4 µM and 400 µM), acesulfame (0.745 µM and 745 µM), cyclamate (3.48 µM and 3480 µM), saccharin (0.44 µM and 440 µM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1% v/v). Bar graphs show representative RT-PCR analyses of CYP1A1 mRNA after 24 h of incubation (3 independent experiments were performed). The data are the mean ± SD from triplicate measurements and are expressed as a fold induction over DMSO-treated cells. The data were normalized *per* GAPDH mRNA levels. * – value is significantly different from DMSO-treated cells ($p < 0.05$) as determined by the Student's *t*-test. Western blot shows a representative analysis of CYP1A1 protein after 48 h of incubation. Similar profiles were observed in two independent experiments. As a loading control, the blots were probed to actin (data not shown).

882-fold). Accordingly, TCDD caused drastic induction of CYP1A1 protein after 48 h of incubation in all human hepatocytes cultures. The levels of CYP1A1 mRNA and protein in four human hepatocytes cultures were not significantly altered by tested sweeteners, implying also inactivity of metabolites originating from sweeteners (Fig. 3).

4. Discussion

The understanding of pharmacokinetic drug-drug interactions that involves induction of drug-metabolizing enzymes has advanced with discovery of roles for xenoreceptors PXR, CAR and AhR in the regulation of drug metabolism. The phenomenon of *food–drug interactions* has emerged in the last few years. It is now well established that food constituents can modify relative potency and efficacy of drugs *via* xenoreceptors. Therefore, it is of value to search for food constituents that may cause *food–drug* interactions. Indeed, the number of publications dealing with *food–drug* interactions dramatically increases (Won et al., 2012). We have focused on AhR, GR and CYP1A1 for three reasons: (i) AhR and GR are involved in the regulation of drug-metabolizing enzymes such as CYP1A1, CYP1A2, phase II enzymes and several drug transporters; (ii) AhR and CYP1A1 play prominent role in chemically-induced carcinogenesis; (iii) AhR and GR have multiple physiological roles, e.g. in immune system, inflammation, steroid hormone signaling, cell cycle control etc.

Food constituents may be of natural origin (e.g. alkaloids, polyphenolics, terpenes), artificial food additives (e.g. sweeteners, preservatives, taste and flavor enhancers, colorings, stabilizers etc.) or contaminants (e.g. heavy metals, pesticides, persistent organic pollutants). Recently, we found that extracts from some of non-alcoholic beverages activated AhR and induced CYP1A1/2 and CYP3A4 genes (Kamenickova and Dvorak, 2012; Kamenickova et al., 2012). In the current paper, we have studied the effects of the most common artificial sweeteners, i.e. aspartame, acesulfame, cyclamate and saccharin on the expression of CYP1A1 and



	0.1% DMSO	5 nM TCDD	0,44 µM SACCHARIN	440 µM SACCHARIN	3,48 µM CYCLAMATE	3480 µM CYCLAMATE	0,40 µM ASPARTAME	400 µM ASPARTAME	0,745 µM ACESULFAME	745 µM ACESULFAME
■LH47	1.0	104.7	0.3	1.9	0.6	2.2	0.6	3.5	0.1	1.2
⊠BIO	1.0	882.2	1.3	2.4	1.1	1.6	3.1	2.2	3.3	2.1
▨LH49	1.0	151.7	1.5	6.0	0.4	0.7	0.4	0.4	0.5	0.6
⊡LH50	1.0	221.3	2.0	1.3	1.2	0.9	1.0	0.8	1.0	0.7

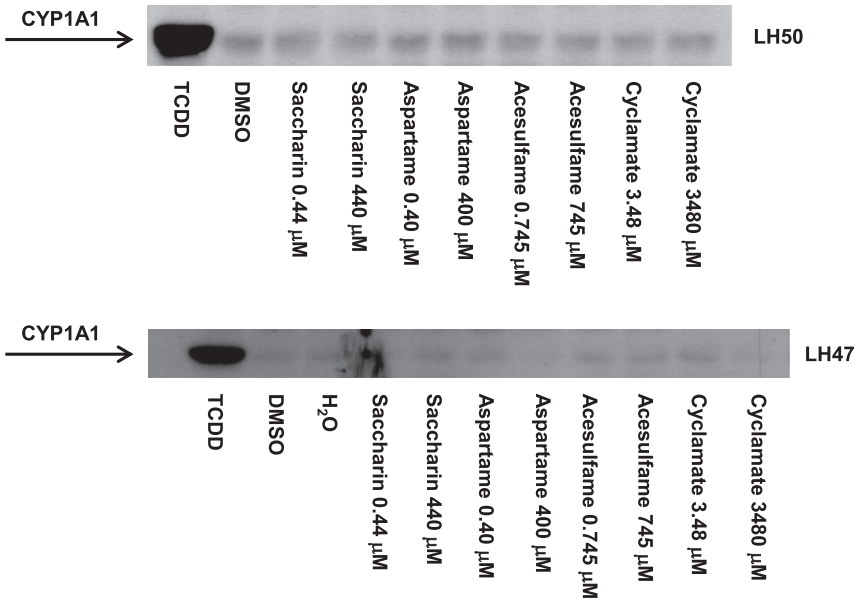


Fig. 3. Effects of sweeteners on CYP1A1 expression in primary human hepatocytes. Hepatocytes were treated for 24 h and 48 h with aspartame (0.4 µM and 400 µM), acesulfame (0.745 µM and 745 µM), cyclamate (3.48 µM and 3480 µM), saccharin (0.44 µM and 440 µM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and/or vehicle (DMSO; 0.1% v/v). Bar graph shows RT-PCR analyses of CYP1A1 mRNA after 24 h of incubation (four independent human hepatocytes cultures were used). The data are the mean ± SD from triplicate measurements and are expressed as a fold induction over DMSO-treated cells. The data were normalized *per* GAPDH mRNA levels.* – value is significantly different from DMSO-treated cells ($p < 0.05$) as determined by the Student's *t*-test. Western blot shows the representative analyses of CYP1A1 protein after 48 h of incubation in human hepatocytes cultures LH47 and LH50. Similar profile was observed in four independent cultures. As a loading control, the blots were probed to actin (data not shown).

transcriptional activities of AhR and GR. We found that tested sweeteners did not influence basal and ligand-inducible transcriptional activity of AhR and GR, as revealed by gene reporter assays. The expression of CYP1A1 mRNA and protein was not induced by any of the sweeteners tested in primary human hepatocytes and in human intestinal LS174T and hepatic HepG2 cancer cells. Taken together, the use of aspartame, acesulfame, cyclamate and saccharin in foods may be considered safe, with regard to no effects on CYP1A1 induction and activation of AhR and GR receptors.

5. Conflict of Interest

The authors declare that they have no conflict of interest.

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PALACKY UNIVERSITY OLOMOUC

Faculty of Science

Department of Cell Biology and Genetics

IN VITRO EFFECTS OF FOOD CONSTITUENTS ON THE ARYL HYDROCARBON RECEPTOR-CYP1A1 SIGNALING PATHWAY

Ph.D. Thesis Summary

P1416 Biology

Mgr. Alžběta Srovnalová

OLOMOUC 2014

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SUMMARY

A number of foreign substances that have possible harmful effects on human health, come from food intake. Compounds involved in the diet are either natural or synthetic origin, having the ability to activate the xenoreceptors, resulting in the induction of drug- and xenobiotic-metabolizing enzymes. Induction of various enzymes results in alteration of the drug metabolism (food-drug interactions), the distortion of the intermediary metabolism and activation of pro-carcinogens to carcinogens. This work is focused on the study food constituents' influence on the activity of arylhydrocarbon receptor (AhR) and subsequent cytochrome P-450 1A1 induction. Constituents of soft drinks, artificial sweeteners, anthocyanins and anthocyanidins were studied. The theoretical background summarizes findings about food-drug interactions, AhR receptor, and cytochrome P-450 1A and also describes the studied compounds. In the methodological section, the techniques of RT-PCR, immunodetection and gene reporter assay were used for determination of expression CYP1A1 mRNA, protein, and activation of AhR. The ligand binding assay and enzyme activity measurement were also done. Primary cultures of human hepatocytes, cancer cell lines HepG2 (liver) and LS174T (intestinal) were used as in vitro models. Our findings points to food constituents, that have not been studied till now, and their influence on the activity of the AhR receptor, leading to induction of CYP1A1 enzyme involved in drug metabolism as well as in the process of chemically induced carcinogenesis.

SOUHRN

Řada cizorodých látek, se kterými člověk přijde do kontaktu, je přijímána potravou a může mít neblahé účinky na lidské zdraví. Sloučeniny obsažené ve stravě jsou buď přírodního, nebo syntetického charakteru se schopností aktivovat tzv. xenoreceptory, což vede k indukci enzymů metabolizujících cizorodé látky a léčiva. V důsledku indukce jednotlivých enzymů dochází ke změně v metabolismu léčiv (tzv. lékovým interakcím), k narušení intermediárního metabolismu a aktivaci prokarcinogenů na karcinogeny. Předkládaná práce je zaměřena na studium vlivu látek obsažených v potravě na aktivitu arylhydrokarbonového receptoru (AhR) a následnou indukci cytochromu P-450 1A1. Ve studii byly testovány obsahové složky nealkoholických nápojů, umělá sladidla, antokyaniny a antokyanidiny. Teoretická část práce shrnuje poznatky o lékových interakcích, AhR receptoru, cytochromech P-450 1A a popisuje studované sloučeniny. V praktické části byly využity techniky RT-PCR, imunodetekce a reporter gene assay pro stanovení exprese CYP1A1 mRNA, proteinů a aktivaci AhR. Byly také použity metody ligand binding assay a měření enzymové aktivity. Jako in vitro modely byly použity primární kultury lidských hepatocytů a nádorových buněčných linií HepG2 (jaterní) a LS174T (střevní). Nově získané poznatky objasňují vliv dosud nestudovaných složek potravy na aktivitu AhR receptoru, vedoucí k indukci enzymu CYP1A1, který se kromě metabolizace léčiv podílí také na procesu chemicky indukované karcinogeneze.

CONTENT

1. AIMS

The main goal of the present thesis was to evaluate the effects of food constituents on the AhR-CYP1A1 signaling pathway. Ready to drink teas, mineral waters, anthocyanidins, anthocyanins and artificial sweeteners were selected for this study. The particular goals were evaluation of *in vitro* effects of food constituents on

1. AhR transcriptional activity by the means of gene reporter assays.

2. The expression of CYP1A1 and CYP1A2 mRNAs and proteins by the means of RT-PCR and western blotting, respectively, in primary cultures of human hepatocytes and in human cancer cell lines.

3. Catalytic activity of CYP1A1 and CYP1A2 by measurement of EROD assay.

4. Ligand binding to AhR.

2. INTRODUCTION

Human populations are daily exposed to a plethora of xenobiotics such as drugs, environmental pollutants, tobacco smoke, plant alkaloids, agrochemicals, dyes, etc. A large number of xenobiotics are absorbed in food substances (tea, spices, fruit, and vegetables). In addition to naturally occurring constituents, additives such as stabilizers, flavors, aromas, antioxidants, colorants, sweeteners, etc. are found in common foods. Both natural and artificial food ingredients can interfere with the regulatory pathways of drug metabolizing enzymes in humans. This can result in so-called food-drug interactions which is undesirable clinically as the drug pharmacokinetics is affected by another compound, i.e. xenobiotics (Mandlekar et al., 2006). Examples of serious food-drug interactions are acceleration of the metabolism of steroid hormones by hypericin (St. John's wort tea), and due to enzyme induction drugs, which finally leads to failure of oral contraceptives (Mai et al., 2004).

Another aspect of undesirable food ingredients and other substances on the human body is the activation of pro-carcinogens to ultimate carcinogens. Examples of chemically induced carcinogenesis is auto-induction of cytochrome P450 by polycyclic aromatic hydrocarbons (PAHs) contained in cigarette smoke, grilled and smoked meat, which ultimately result in the conversion of PAHs into powerful carcinogens and in colorectal tumors. The molecular mechanisms involved in heavy metal-mediated drug interactions or chemically-induced carcinogenesis include induction (increased de novo synthesis) of drug

metabolizing enzymes, especially cytochrome P450 (Gonzalez and Gelboin, 1994). The most prominent and important inducible cytochrome P450 is CYP1A1, CYP1A2, CYP2B6, CYP2C9 and CYP3A4-the enzyme responsible for the metabolism of about 60% of drugs.

Xenobiotic-mediated induction of cytochrome P450 comprises activation of xenoreceptors, which are ligand-activated transcription factors that are responsible for initiation of the synthesis of cytochrome P450. The receptor for polyaromatic hydrocarbons (AhR) is involved in many cellular processes and it mediates response to endogenous signals or xenobiotics (Abel and Haarmann-Stemmann, 2010). When AhR was discovered, it was believed that it regulated discrete cell response to foreign chemicals, i.e. (i) the induction of cytochrome P450 CYP1A1 and CYP1A2, and (ii) the toxicity of dioxin-like compounds (Denison et al., 2002). In the last two decades, however, it has become increasingly clear that in addition to its role as a receptor for xenobiotics, AhR plays a role in such vital cellular storylines as development, cell differentiation and the immune response (Haarmann-Stemmann et al., 2012).

Currently, much is known about the effects of chemicals on the AhR, in particular environmental pollutants (polychlorinated biphenyls, polyaromatic hydrocarbons, etc.), chemicals and pharmaceuticals (omeprazole, lansoprazole) (Quattrochi and Tukey, 1993). However, the effects of food additives on the AhR have not been systematically studied and the data in the literature are diverse. While the massive development and progress in research on the AhR have led to improvements in the safety/testing of new drugs, research on food safety and food quality has been neglected. Despite many indications that food additives affect the AhR receptor and the consequent metabolism of prescription drugs or result in carcinogenesis, systematic examination of these substances in food on the activity of AhR is result lacking.

3. MATERIAL AND METHODS

3.1 Biological material

3.1.1 Human cancer cell lines

Human Caucasian colon adenocarcinoma cells LS174T (ECAC No. 87060401) and human Caucasian hepatocellular carcinoma cells HepG2 (ECAC No. 85011430) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 µg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acid, and 1 mM sodium pyruvate. Cells were maintained at 37^{o}C and 5% CO_2 in a humidified incubator.

3.1.2 Human hepatocytes

Human hepatocytes were obtained from two sources: (i) Hepatocytes were prepared from liver tissue, resected from multiorgan donors. The tissue acquisition protocol was in accordance with requirements issued by the local ethics commission of the Czech Republic. Human liver tissue used in the study was obtained from nine multiorgan donors: LH36 (male, 74 years), LH37 (male, 28 years), LH38 (male, 58 years), LH44 (female, 57 years), LH45 (male, 46 years), LH46 (male, 37 years), LH47 (male, 47 years), LH49 (male, 38 years), LH50 (female, 55 years). Culture medium was enriched for plating with 2% fetal calf serum (v/v). The medium was exchanged for serum-free medium the day after the culture was allowed to stabilize for an additional 24 h. (ii) Long-term human hepatocytes in monolayer batch were purchased from Biopredic International, France: HEP220633 (male, 73 years), HEP220670 (female, 64 years) and HEP220708 (female, 64 years). Hepatocytes were treated for 24 h or 48 h with tested compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5 nM) and vehicle (DMSO, ethanol; 0,1% V/V). Cultures were maintained at 37^{o}C and 5% CO_2 in a humidified incubator.

3.2 Compounds

Dimethylsulfoxide (DMSO), resveratrol, hygromycin B, aspartame, acesulfane, cyclamate, saccharin, Dulbecco´s modified Eagle´s medium, fetal bovine serum, streptomycin, penicillin, L-glutamine, sodium pyruvate, non-essential amino acids were purchased from Sigma-Aldrich (Czech Republic); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (USA). The anthocyanidins and anthocyanins (Table 1, Figure 1) were purchased from Extrasynthese (France), where the purity was ≥ 90-97% by all of the compounds. Saccharin, aspartame, acesulfame K and cyclamate were purchased from Sigma – Aldrich.

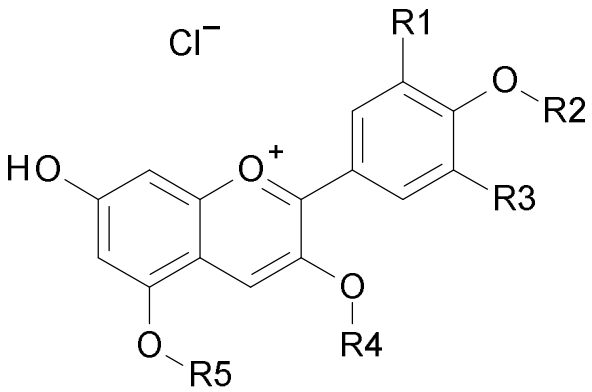

Figure 1. Structure of anthocyanins and anthocyanidins.

Table 1. Anthocyanins and anthocyanidins used in the study

	Anthocyanin	R1	R2	R3	R4	R5
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
	Anthocyanidin	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

3.3 Methods

3.3.1 Preparation of samples from ready to drink teas and mineral waters

Ready to drink teas (RDTs) and flavored mineral waters (MWs) were purchased in various supermarkets in Olomouc City, Czech Republic (Table 2 and Table 3, Figure 2). The extracts from 17 different RDTs and 28 different MW were prepared as follows: Aliquots of 250 ml were taken from four different bottles containing RDTs and MWs. The aliquots were mixed together, to a total sample volume of 1000 ml. The entire sample (1000 ml) was concentrated using vacuum evaporation down-to volume of 200 ml. Concentrated (acidic) sample was extracted twice with 2 x 200 ml of diethyl ether. The sample was then alkalized using concentrated sodium hydroxide to pH approximately 9-10. The extraction with 2 x 200 ml of diethyl ether was performed again. All the extracts, both acidic and alkaline, were pooled and evaporated to dryness. Dried extracts were dissolved in 1 ml of ethanol. The resulting ethanolic solutions were 1000x concentrated RDTs and MWs. In cell experiments, ethanolic samples were diluted 1000 times in the culture medium; hence the concentrations of RDTs' and MWs' constituents in the cell cultures were identical to those in RDTs and MWs.

Figure 2. Photo of tested flavored mineral waters and ready to drink teas.

Table 2. List of ready to drink teas

Code	Brand	Type	Added substances
TDV1	DOBRA VODA	Green Tea + Lemon	Sugar, tea extract 5 g, natural aroma, citric acid, sodium benzoate, potasium sorbate, dimethyldicarbonate
TDV2	DOBRA VODA	White Tea + Apricot	Sugar, tea extract 4 g, natural aroma, citric acid, sodium benzoate, potasium sorbate, dimethyldicarbonate
TDV3	DOBRA VODA	Tea Citron	Sugar, tea extract 2.1 g, natural aroma, citric acid, sodium citrate, sodium benzoate, potasium sorbate, dimethyldicarbonate
TDV4	DOBRA VODA	Tea Peach	Sugar, tea extract 2.1 g, natural aroma, citric acid, sodium citrate, sodium benzoate, potasium sorbate, dimethyldicarbonate
TAT1	AQUILA TEAM	Green Tea + Lemon	Sugar, green tea extract, natural aroma green tea + lemon, natural lemon juice, sodium benzoate
TAT2	AQUILA TEAM	Black Tea + Lemon	Sugar, citric acid, caramel dye, black tea extract, phosphoric acid, sodium benzoate, natural lemon aroma, vitamin C
TAT3	AQUILA TEAM	Yellow tea + apple	Sugar, yellow tea extract, citric acid, ascorbic acid, natural apple aroma, apple juice, sodium citrate, sodium benzoate
TAT4	AQUILA TEAM	White tea + pomegranate	Sugar, white tea extract, citric acid, ascorbic acid, natural pomegranate aroma, pomegranate juice, sodium citrate, sodium benzoate
TAT5	AQUILA TEAM	Red tea + pear	Sugar, red tea extract, citric acid, ascorbic acid, natural pear aroma, pear juice, sodium citrate, sodium benzoate
TAT6	AQUILA TEAM	Black tea + peach	Sugar, citric acid, caramel dye, black tea extract, phosphoric acid, sodium benzoate, natural peach aroma, vitamin C
TAT7	AQUILA TEAM	Black tea + forest fruits	Sugar, natural forest fruits aroma, citric acid, caramel dye, black tea extract, sodium benzoate, vitamin C
TNE1	NESTEA VITAO	White tea + apricot	Sugar, citric acid, sodium citrate, white tea extract 0.2%, apricot juice 0.1% of concentrate, aroma, ascorbic acid, polyphenols 700 mg
TNE2	NESTEA VITAO	Red tea + pear	Sugar, citric acid, sodium citrate, roibois extract 0.1%, pear juice 0.1% of concentrate, aroma, ascorbic acid, polyphenols 440 mg
TNE3	NESTEA VITAO	Green Tea + aloe vera + strawberry	Sugar, malic acid, sodium citrate, strawberry aroma, aloe vera aroma, green tea extract 0.15%, strawberry juice 0.1% of concentrate, ascorbic acid, polyphenols 570 mg
TNE4	NESTEA	Peach	Sugar, citric acid, sodium citrate, tea extract 0.1%, aroma, peach juice 0.1% of concentrate,
TNE5	NESTEA	Lemon	Sugar, citric acid, sodium citrate, tea extract 0.1%, aroma, lemon juice 0.1% of concentrate, ascorbic acid
TNE6	NESTEA VITAO	Green Tea + lemon	Sugar, citric acid, sodium citrate, green tea extract 0.2%, aroma, orange and lemon juice 0.2% of concentrate, ascorbic acid, polyphenols 800 mg

Table 3. List of flavored mineral waters

Code	Trade name	Type	Added substances
MWDV1	DOBRA VODA	Raspberry	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV2	DOBRA VODA	Mango	Vit C 92 mg/L, sugar, citric acid, aroma
MWDV3	DOBRA VODA	Elderberry	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV4	DOBRA VODA	Lemon	Vit C 92 mg/L, sugar, citric acid, aroma, acidity regulator
MWDV5	DOBRA VODA		-
MWDV6	DOBRA VODA	Orange	Vit C 92 mg/L, sugar, citric acid, aroma
MWDV7	DOBRA VODA	Grapefruit	Vit C 92 mg/L, sugar, citric acid, aroma
MWDV8	DOBRA VODA	White Grapes	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV9	DOBRA VODA	Pear	Vit C 92 mg/L, sodium benzoate, sugar, citric acid, dimethyldicarbonate, aroma, conservant
MWDV10	DOBRA VODA	Lemon, Green Tea, Passion-flower	Sugar, aroma, herb extract 2.5 g, sodium benzoate, aspartam, acesulpham K, citric acid, ascorbic acid
MWDV11	DOBRA VODA	Orange, Violet,Howthorn, Blueberry	Sugar, aroma, herb extract 4 g, sodium benzoate, aspartam, acesulpham K, citric acid, ascorbic acid
MWDV12	DOBRA VODA	Grapefruit, Ginseng, Guarana	Sugar, aroma, herb extract 1 g, sodium benzoate, aspartam, acesulpham K, citric acid, ascorbic acid
MWPO1	PODEBRADKA	Lemon	Citric acid, aroma, acesulphame A, aspartam
MWPO2	PODEBRADKA	Grapefruit	Citric acid, aroma, acesulphame A, aspartam
MWPO3	PODEBRADKA	Plum & Elderberry	Citric acid, sodium citrate, L-carnitin 100 mg, acesulpham A, aspartam, fiber 1 g
MWPO4	PODEBRADKA	Sour cherry	Citric acid, aroma, acesulphame A, aspartam
MWPO5	PODEBRADKA	Pomelo & Cranberry	Citric acid, sodium citrate, L-carnitin 100 mg, acesulphame A, aspartam, fiber 1 g
MWPO6	PODEBRADKA	Lime	Citric acid, aroma, acesulphame A, aspartam
MWPO7	PODEBRADKA	Orange	Citric acid, aroma, acesulphame A, aspartam
MWPO8	PODEBRADKA	Plum	Citric acid, sodium benzoate, aroma, glucose-fructose syrup
MWPO9	PODEBRADKA	Passion-flower	Citric acid, sodium benzoate, aroma, glucose-fructose syrup
MWRA1	RAJEC	Dandelion	Glucose syrup, citric acid, herb extract from damdelion 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA2	RAJEC	Dog-rose	Glucose syrup, citric acid, herb extract from dog-rose 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA3	RAJEC	Wild-thyme	Glucose syrup citric acid, herb extract from wild-thyme 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA4	RAJEC	Salvia	Glucose syrup, citric acid, herb extract from salvia 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA5	RAJEC	Mellisa	Glucose syrup, citric acid, herb extract from mellisa 0.1 g, apple juice, aroma, potasium sorbate, sodium benzoate
MWRA6	RAJEC	Chestnut	Fructose, citric acid, herb extract from chestnut 0.1 g, aroma, sodium benzoate
MWRA7	RAJEC	Linden	Fructose, citric acid, herb extract from linden 0.1 g, aroma, sodium benzoate, potasium sorbate

3.3.2 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

HepG2 and LS174T Cells were seeded on six-well plates in a density of $1x10^6$ cells/well using culture media enriched with fetal bovine serum (10% v/v). After 16 hours of cultivation, cells were treated by tested. After another 24 hours, the total RNA was isolated using TRI Reagent® according to the manufacturer´s instructions. cDNA was synthetized according to a common protocol, using M-MLV Reverse Transcriptase and random hexamers. One tenth of new synthetized cDNA was used for qRT-PCR using a Light Cycler apparatus 480 II (Roche Diagnostic Corporation, Prague, Czech Republic). The following program was used: an activation step at 95°C for 10 min was followed by 40 cycles of PCR (denaturation at 95°C for 10 seconds; annealing for 7 seconds at 65 °C for CYP1A1 and CYP3A4 or 68°C for GAPDH; elongation for 17 seconds at 72°C). The levels of mRNAs were determined, as described elsewhere (Dvorak et al., 2008). The measurements were performed in triplicate. Gene expression was normalized *per* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The data were processed according to the delta-delta method.

3.3.3 Western blotting and protein detection

Total protein extracts were analyzed on SDS-PAGE gels (10%) using a Bio-Rad apparatus according to the standard procedure. Protein transfer onto polyvinylidine difluoride (PVDF) membrane was carried out. After the transfer, the membrane was stained with Ponceau S red dye for control of transfer and then saturated with 5% non-fat dried milk dissolved in a TBS-T buffer for 1 hour at room temperature, followed by incubation with primary antibody overnight at 4°C. The dilution of antibodies against CYP1A1 and CYP3A4 was 1/500 and against actin 1/2000. Subsequent incubation with an appropriate secondary antibody was carried out for 1 hour at room temperature. Chemiluminiscence detection was performed using horseradish peroxidase-conjugated secondary antibody and ECL kit.

3.3.4 Plasmid, transfection and gene reporter assay

HepG2 cells were transiently transfected by lipofection (Fugene 6) in 24-well plates with 300 ng/well of reporter *p1A1-luc* plasmid containing 5´flanking region (-1566-1999) of human CYP1A1 gene subcloned into the *KpnI-HindIII* double digested pGL3-Basic vector upstream of the firefly luciferase reporter gene. Following 16 h stabilization, the cells were treated for 24 h with tested extracts. After treatments, cells were lysed and luciferase activity was measured and standardized per milligram of protein. Experiments were also performed in stably transfected gene reporter cell line AZ-AHR, which was derived from HepG2 cells transfected with a construct containing several binding sites upstream of luciferase reporter gene (Novotna et al., 2011). The cells were seeded on a 96-well plate in a density of 25 000

cells per well. After plating, cells were stabilized for 16 hours and then incubated for 24 hours with tested compounds, extracts, TCDD (5nM) and/or vehicle (DMSO; 0.1% v/v). After the treatments, cells were lysed using Reporter Lysis Buffer according to the manufacturers´ instruction and luciferase activity was measured in 96-well plate format, using Infinite M200 luminometer (Tecan, Schoeller, Prague).

3.3.5 7-Ethoxyresorufin-O-deethylase activity of CYP1A1/2 in cell cultures

Cells were seeded in 96-well plates at a density of 2.4×10^4 cells/cm^2 in DMEM supplemented with 10% FBS and stabilized for 24 h. Cells were then incubated for 48 h with tested compounds. The 7-ethoxyresorufin-O-deethylase (EROD) activity in cell cultures was measured as described elsewhere (Donato et al., 1993).

3.3.6 Enzyme activity of CYP1A1 and CYP1A2 in human liver microsomes

The activity of CYP1A1/2 was measured in pooled human liver microsomes either as EROD (see above) with 7-ethoxyresorufin as substrate according to an established method (Leclercq et al., 1996) or by a luciferase based assay with CYP1A1 specific substrate Luciferin-CEE). The quantity of product of the reaction was determined by HPLC (Shimadzu Class VP, Tokyo, Japan) with fluorescence detection or by measuring the luminescence using Infinite M200 spectrophotometer/spectrofluorometer/luminometer.

3.3.7 AhR ligand binding assay

[^{3}H]TCDD was kindly provided by Dr Steven Safe (Texas A&M university) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) was from Accustandard (New Haven, CT USA). The competitive displacement of [^{3}H]TCDD from guinea pig hepatic cytosol was as previously described (Korashy et al., 2011). Briefly, hepatic guinea pig cytosol diluted to 8 mg/mL protein in MEDG (25 mM MOPS-NaOH, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% v/v glycerol) was incubated with different concentrations of tested compound or 200 nM TCDF for 30 min at room temperature or on ice, and further incubated for 1 h at room temperature in the presence of 2 nM [^{3}H]TCDD. The amount of [^{3}H]TCDD specific binding was determined by hydroxyapatite protocol, and specific binding was determined as the difference between the ‘no competitor’ and TCDF reaction (Denison et al., 2002).

4. RESULTS AND DISCUSSION

In the present study, we examined the effects of 17 ready to drink teas (RDTs), 28 flavored mineral waters (MWs), 6 major anthocyanidins, 21 anthocyanins and 4 artificial sweeteners on the AhR-CYP1A1 signaling pathway. *In vitro* models of primary cultures of human hepatocytes and cancer cell lines (HepG2 and LS174T) were chosen. Three main experimental approaches were used: (i) gene reporter assays in stably or transiently transfected cancer cell lines, allowing the examination of xenobiotic effects on AhR transcriptional activity; (ii) measurement of CYP1A1 mRNA and protein levels in cell lines, allowing assessment of the functional consequences of AhR activation with regard to drug metabolism; (iii) measurement of CYP1A1 mRNA and protein levels in primary cultures of human hepatocytes, allowing evaluation of the xenobiotics and their metabolites - metabolic activation/inactivation. The foregoing implies that while the data from each model may differ, the combination of these approaches increases the probability of fair evaluation of the xenobiotic effects.

4.1 Effects of ready to drink teas (RDTs) on AhR-CYP1A1 signaling pathway

The results reported in *Food Chemistry* in 2012 firstly showed that some ready to drink teas were able to increase CYP1A1 activity. Consistent data were obtained for two samples, TAT1 (Green tea + lemon) and TAT5 (Red tea + pear), both from the brand Aquila. These RDTs induced CYP1A1 mRNA in HepG2 cells and human hepatocytes (Figure 3), and activated AhR in HepG2 cells transfected with *p1A1-luc* plasmid (Figure 4). The protein levels of CYP1A1 were not significantly altered (Figure 5). We found no significant changes in CYP1A1 induction by the remaining 15 samples of RDTs, suggesting that the elevated level of mRNA and increased activity of AhR by TAT1 and TAT5 were due to the specific content of these products. Several *in vitro* studies have shown the effects of food polyphenols (citrus fruits, isoflavones and flavones) on AhR, resulting in its activation (agonist/antagonist) mode (Amakura et al., 2008) and which could be the case for the RTDs.

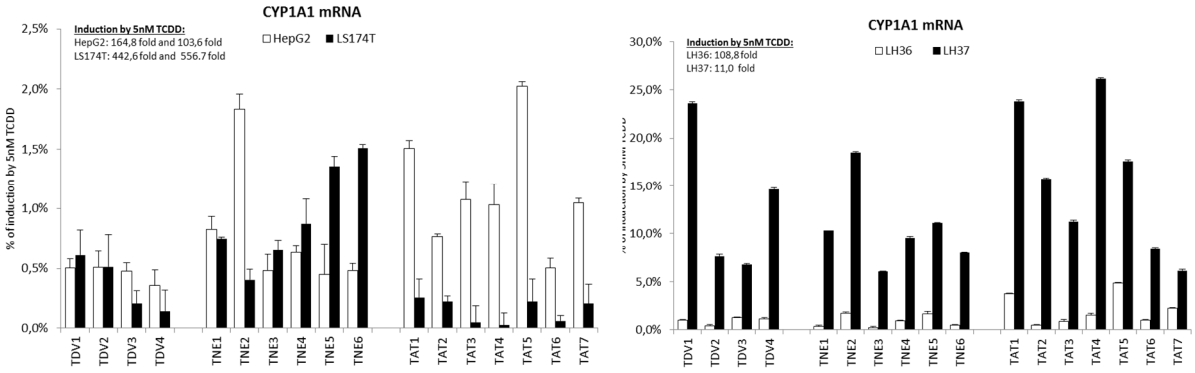

Figure 3. Effects of RDTs on CYP1A1 mRNA expression in cell lines and human hepatocytes.

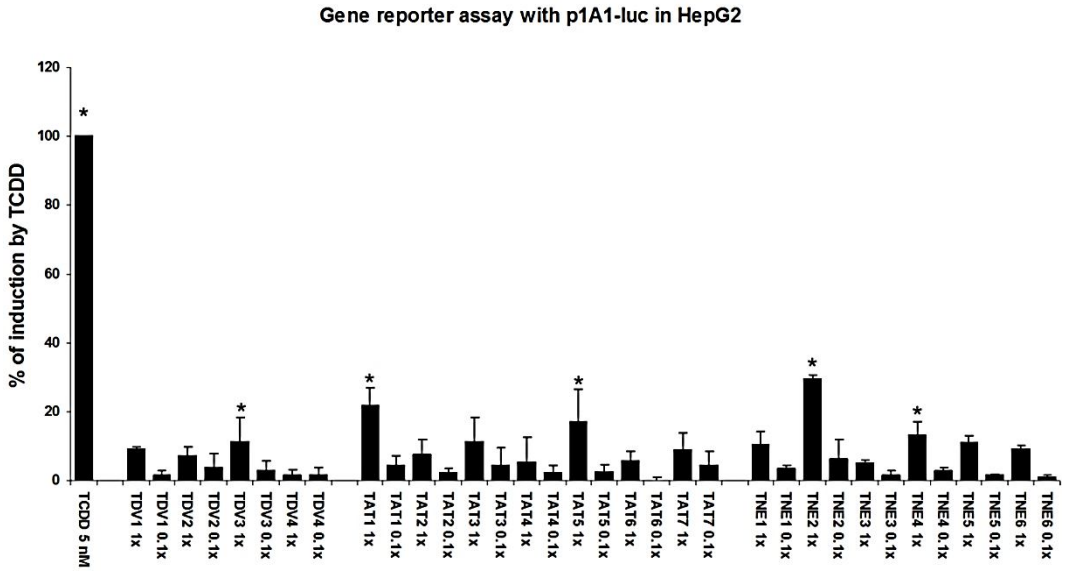

Figure 4. Effects of RDTs on AhR transcriptional activity in HepG2 cells.

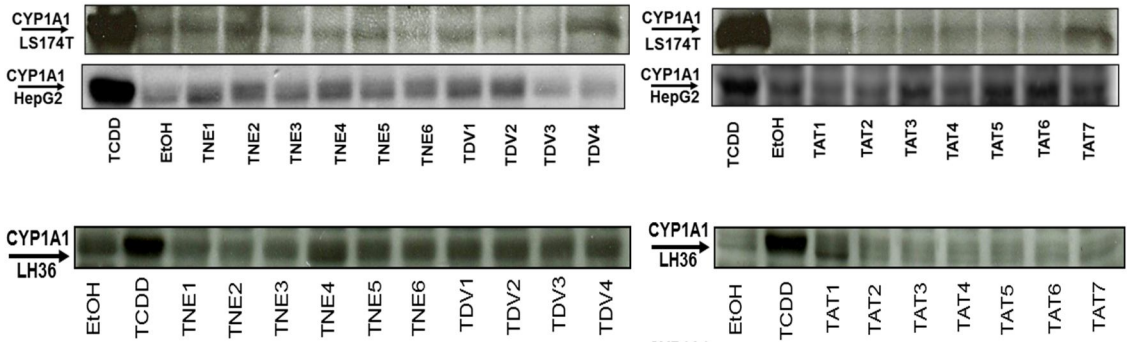

Figure 5. Effects of RDTs on CYP1A1 protein expression in primary cultures in cell lines and human hepatocytes.

4.2 Effects of flavored mineral waters (MWs) on AhR-CYP1A1 signaling pathway

In the study published in *Food and Chemical Toxicology* in 2012 we found that the following four samples of flavored mineral waters (MWs) activated AhR and/or induced CYP1A1: MWDV10 (DOBRA VODA; lemon, green tea, passion-flower), MWDV12 (DOBRA VODA; grapefruit, ginseng, guarana), MWPO1 (PODEBRADKA; lemon) and MWPO9 (PODEBRADKA; passion-flower) (Figure 6, Figure 7). There were some discrepancies between the data from human cancer cell lines and human hepatocytes, probably due to the extensive metabolism of mineral water constituents in hepatocytes. These results suggest, as well as those from RDTs´, that some active constituents of MWs´ have the ability to activate the AhR receptor with subsequent induction of CYP1A1 at the levels of mRNA and protein. We observed no significantly consistent changes caused by the other 24 MWs and their interference with the AhR-CYP1A1 signaling pathway.

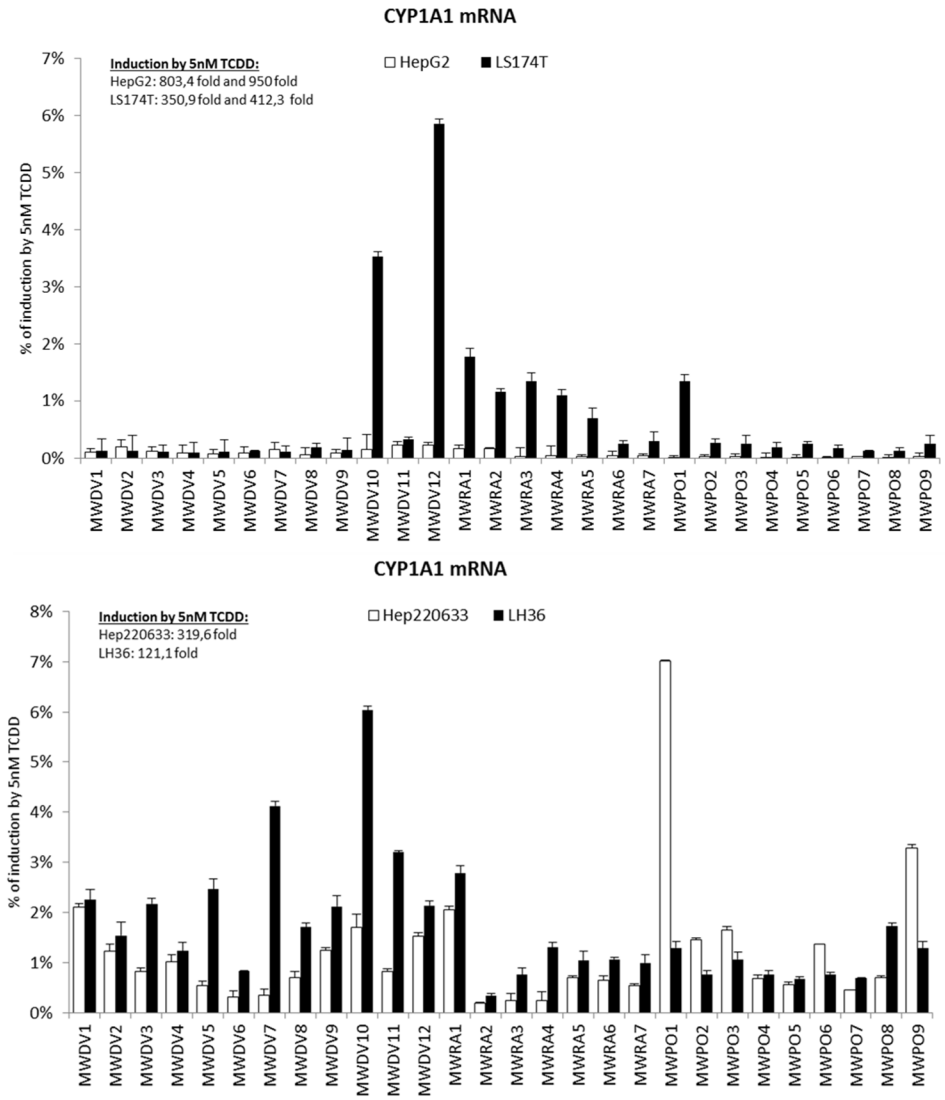

Figure 6. Effects of MWs on CYP1A1 mRNA expression in cell lines and human hepatocytes.

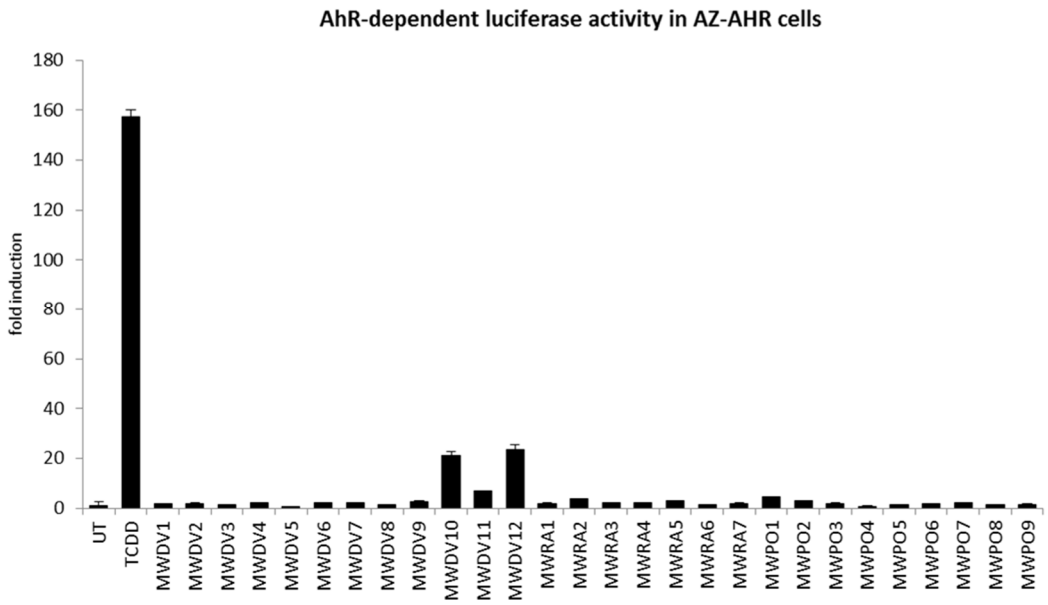

Figure 7. Effects of mineral waters on transcriptional activity of AhR in AZ-AHR reporter cell line.

4.3 Effects of anthocyanidins on AhR-CYP1A1 signaling pathway

The study published in *Toxicology Letters* in 2013 by our research team with Professor Michael Denison from University of California in Davis, USA, was focused on the effects of anthocyanidins on AhR-dependent gene expression and on CYP1A1/2 expression, production and enzymatic activity. Analysis of the inhibition of CYP1A1/2 activity revealed that the effects of the majority of these compounds on the metabolic pathways mediated by CYP1A1/2 enzymes are unlikely. However, pelargonidin and delphinidin were shown to interfere with the CYP1A1/2 drug metabolizing system (Figure 11), although this effect occurred at concentrations higher than those corresponding to the average values reached in human plasma after fruit or juice consumption, i.e. 274 nmol/L and 1220 ng/L for pelargonidin (Mullen et al., 2008) and delphinidin glycosides (Frank et al., 2005), respectively. Since CYP1A enzymes are responsible for activation of many pro-carcinogens (e.g. polycyclic aromatic hydrocarbons) and detoxification of many others (Anzenbacher and Anzenbacherova, 2001; Monostory et al., 2009), interference by anthocyanidins present in food with these enzymes may be even beneficial. The major finding of this study is that pelargonidin is a weak ligand/agonist of the AhR receptor, as revealed by ligand binding assay in guinea pig cytosols (Figure 11) and gene reporter assays in recombinant human AZ-AHR cells (Figure 9), respectively. Pelargonidin also induced CYP1A1 mRNA (Figure 8), protein and catalytic activity in human hepatic HepG2 and human intestinal LS174T cancer cells (Figure 10) and also induced CYP1A1 mRNA in three different primary human hepatocytes cultures (Figure 8). The levels of CYP1A proteins were slightly elevated by 50 μM pelargonidin in human hepatocytes. While we also observed slight induction of

CYP1A1/2 mRNAs in human hepatocytes by 50 µM cyanidin, these effects were not consistent, possibly due to the metabolic transformation of cyanidin in human hepatocytes. Other anthocyanidins did not display significant or systematic effects on AhR transcriptional activity or CYP1A1 expression. Regarding structure–activity relationship, pelargonidin is the only anthocyanidin mono-substituted (monohydroxylated) at the phenyl group bound at 2-position of the chromenylium backbone. Speculatively, this feature may be one of the explanations for the unique activity of pelargonidin towards the AhR because other anthocyanidins are either di-substituted (cyanidin, peonidin) or tri-substituted (malvidin, petunidin, delphinidin) (Figure 1, Table 1). Taken together, pelargonidin activates AhR through a ligand dependent mechanism and induces CYP1A and AhR-dependent reporter gene expression in human cancer cell lines and human hepatocytes, which may be of toxicological significance given the multiple roles of the AhR in the human organism.

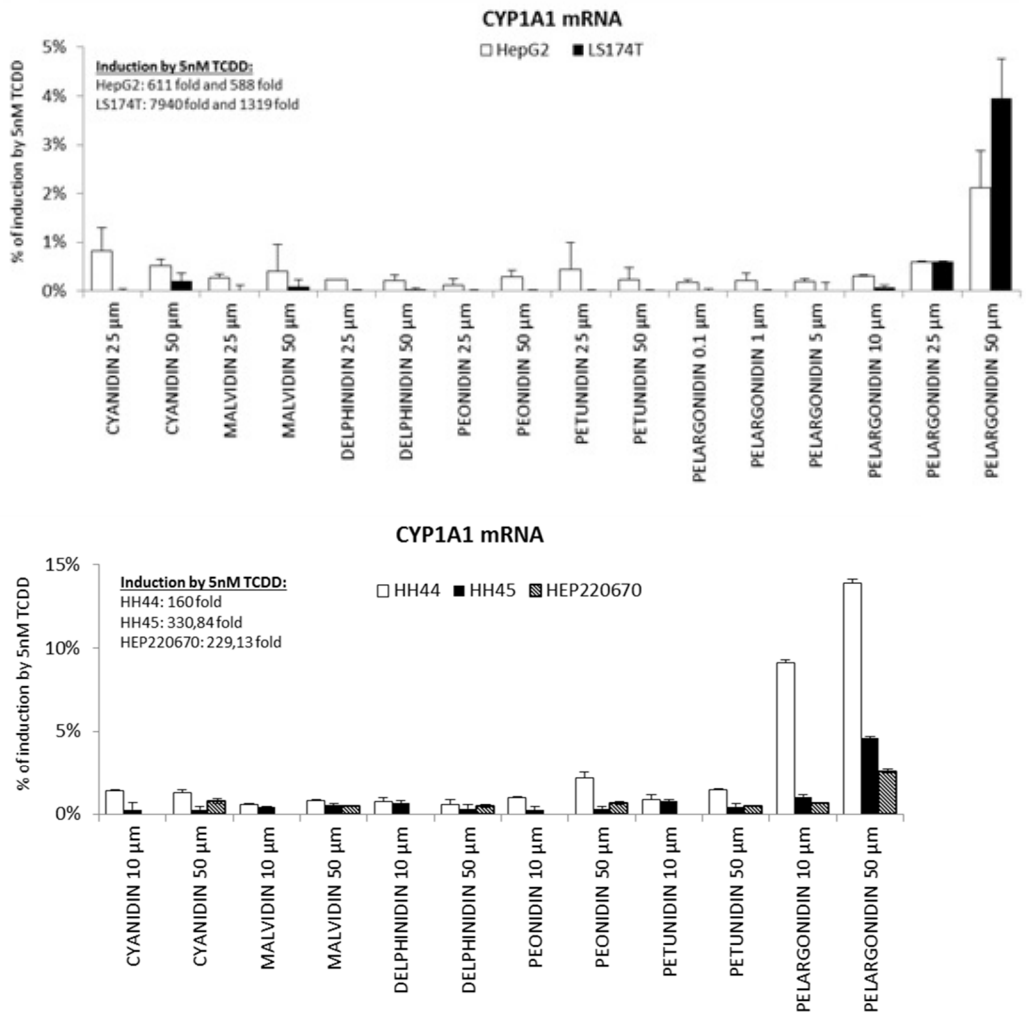

Figure 8. Effects of anthocyanidins on CYP1A1 mRNA expression in cell lines and human hepatocytes.

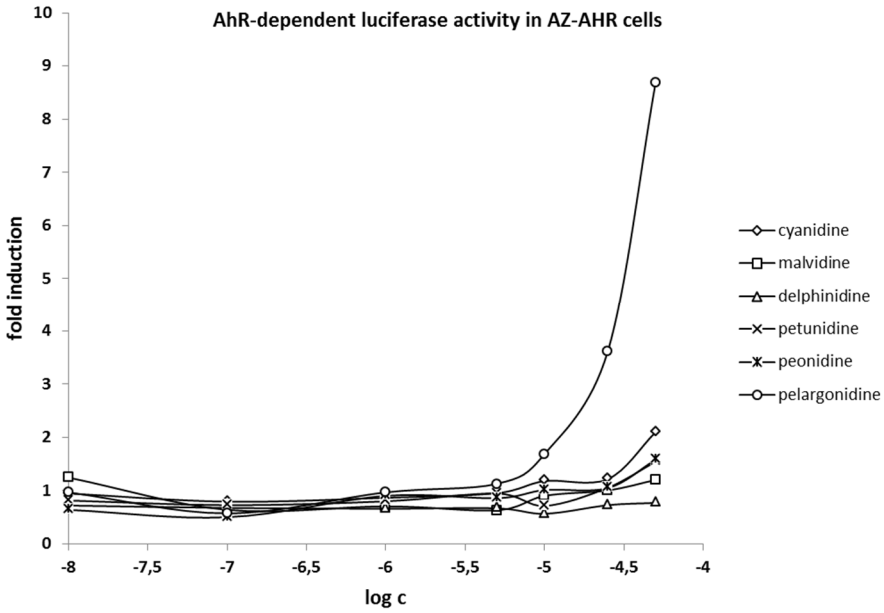

Figure 9. Effects of anthocyanidins on transcriptional activity of AhR in AZ-AHR reporter cell line.

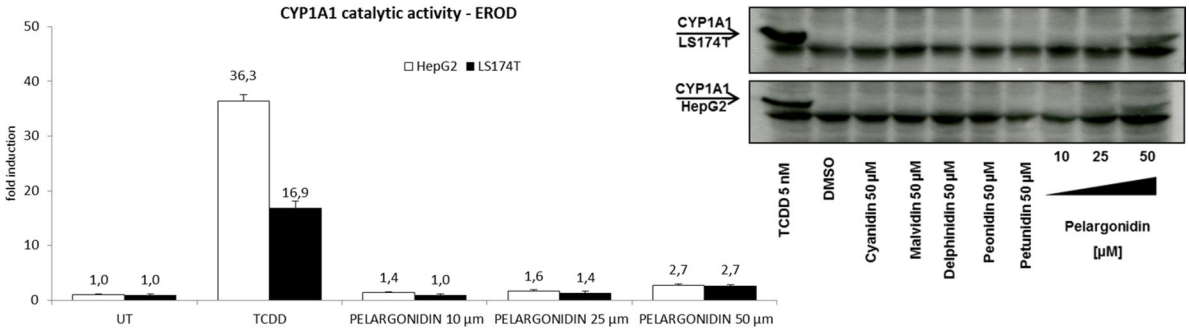

Figure 10. Catalytic activity of CYP1A1 measurement and protein expression in cell lines.

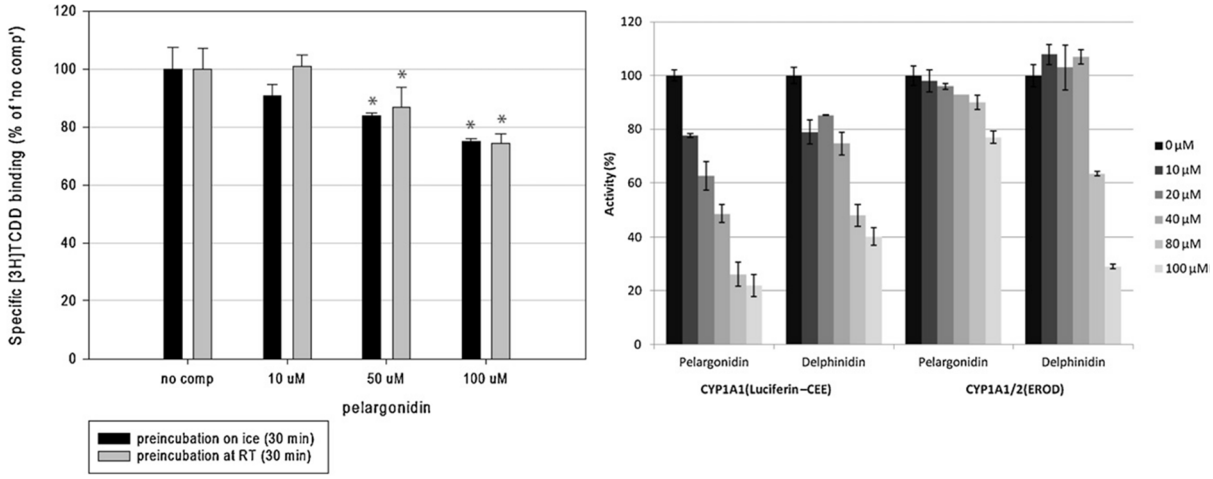

Figure 11. Ligand binding assay and and catalytic activity of CYP1A1/2 measurement.

4.4 Effects of anthocyanins on AhR-CYP1A1 signaling pathway

An ongoing study, published in *Toxicology Letters* in 2013, with Professor Denison investigated the effects of 21 major anthocyanins and 3 extracts from food supplements containing anthocyanins. The amounts of anthocyanins and other phenolics in food supplements are sometimes so high that their consumption results in intestinal or plasma concentrations an order of magnitude much higher than those attained by consumption of the fresh fruits. Indeed, one food supplement SUP-2 (Urinal Akut®, Tablets as 36 mg of proanthocyanidins Walmark a.s., Czech Republic), used in the current study, contains in one tablet, an amount of extract equivalent to 338,400 mg of fresh cranberries, as declared by the manufacturer. The data show that only two anthocyanins of the 21 tested, displayed any activity toward the AhR–CYP1A1 signaling pathway. This was the case of pelargonidin-3-O-rutinoside (PEL-2) and cyanidin-3,5-O-diglucoside (CYA-3), which dose-dependently activated the AhR, as revealed by gene reporter assay (Figure 14). In addition, these two compounds induced CYP1A1 mRNA (Figure 12) but not protein in HepG2 and LS174T cells. The effects of PEL-2 and CYA-3 on the AhR occurred by ligand-dependent and ligand-independent mechanisms, respectively, as demonstrated by ligand binding assay (Figure 15). Neither compound nor extract from food supplements induced CYP1A1 mRNA (Figure 13) or protein in four different primary human hepatocytes cultures (data not shown). This is an important finding given that examination in primary human hepatocytes comprises both maternal compounds and metabolites (Vanzo et al., 2011). In line with these results, CYP1A1 enzyme activity was not prominently influenced by tested anthocyanins. Inhibition of CYP1A1 was concentration-dependent; however, it did not reach 50% even at the highest anthocyanin concentration, i.e. 100 μM (Figure 15). Conjugation with sugar part, however, lowered the inhibition of enzyme activity as shown in this study and led to less prominent activation of the AhR. Collectively, tested anthocyanins and the extracts from food supplements that contain anthocyanins possessed very low, if any, potential for food–drug interactions with respect to the AhR–CYP1A1 pathway. This result is – taken from the consumer's point of view – important for the safety of their use.

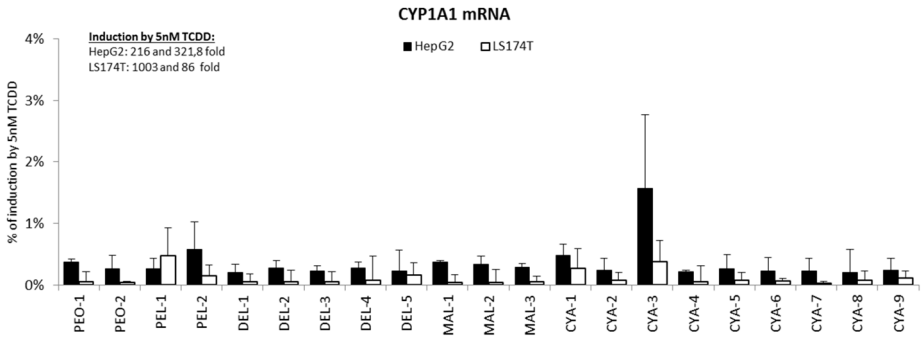

Figure 12. Effects of anthocyanidins on CYP1A1 mRNA expression in cell lines.

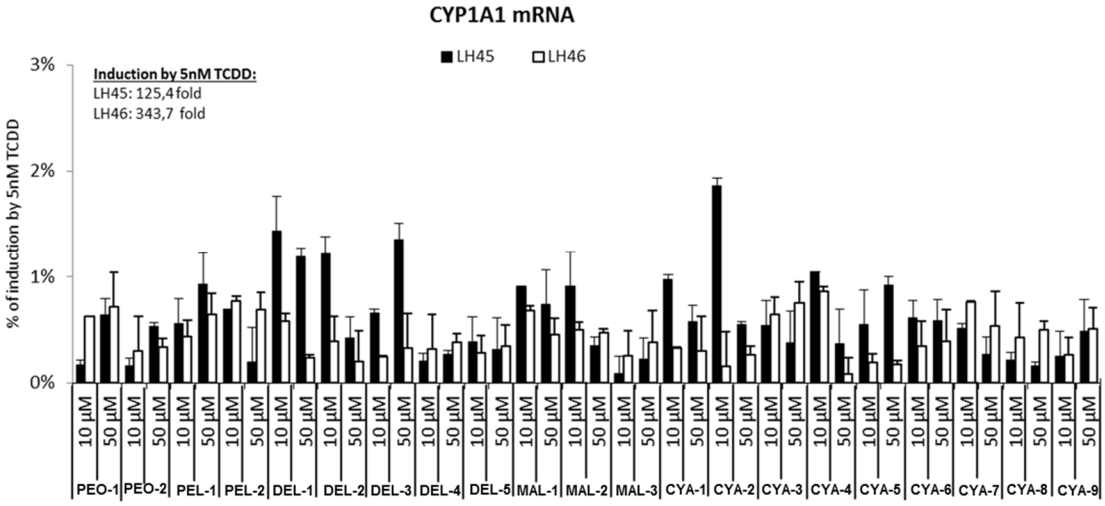

Figure 13. Effects of anthocyanidins on CYP1A1 mRNA expression in human hepatocytes.

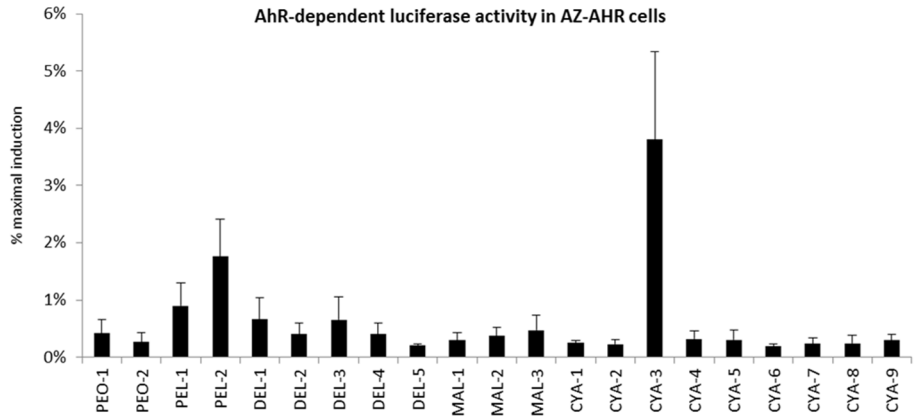

Figure 14. Effects of anthocyanins on transcriptional activity of AhR in AZ-AHR reporter cell line.

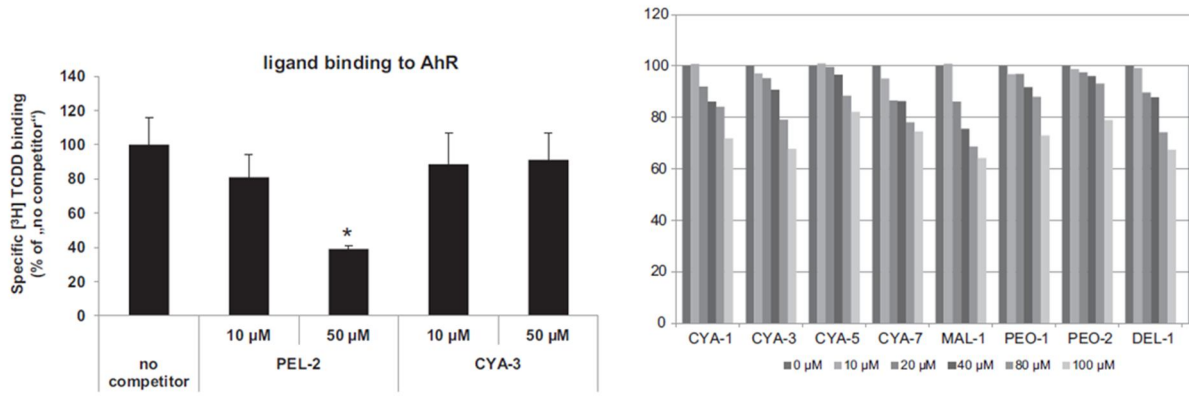

Figure 15. Ligand binding assay and and catalytic activity of CYP1A1/2 measurement.

4.5 Effects of artificial sweeteners on AhR-CYP1A1 signaling pathway

In the study published in *Toxicology In Vitro* in 2013, the most common artificial sweeteners (saccharin, aspartame, cyclamate and acesulfame K) were tested for their potential effects on AhR-CYP1A1 signaling pathway. The studied sweeteners are components of regularly used food and beverages and were chosen for analysis for this reason. Sweeteners were tested in concentrations up to those occurring in non-alcoholic beverages (Bergamo et al., 2011). We found that the tested sweeteners did not influence the basal and ligand-inducible transcriptional activity of AhR, as was revealed by gene reporter assay. The expression of CYP1A1 mRNA and protein was not induced by any of the sweeteners tested in primary human hepatocytes or in human intestinal LS174T and hepatic HepG2 cancer cells (Figure 16). These results suggest that the use of aspartame, acesulfame, cyclamate and saccharin in foods may be considered safe, with regard to effects on CYP1A1 induction and activation of the AhR receptor.

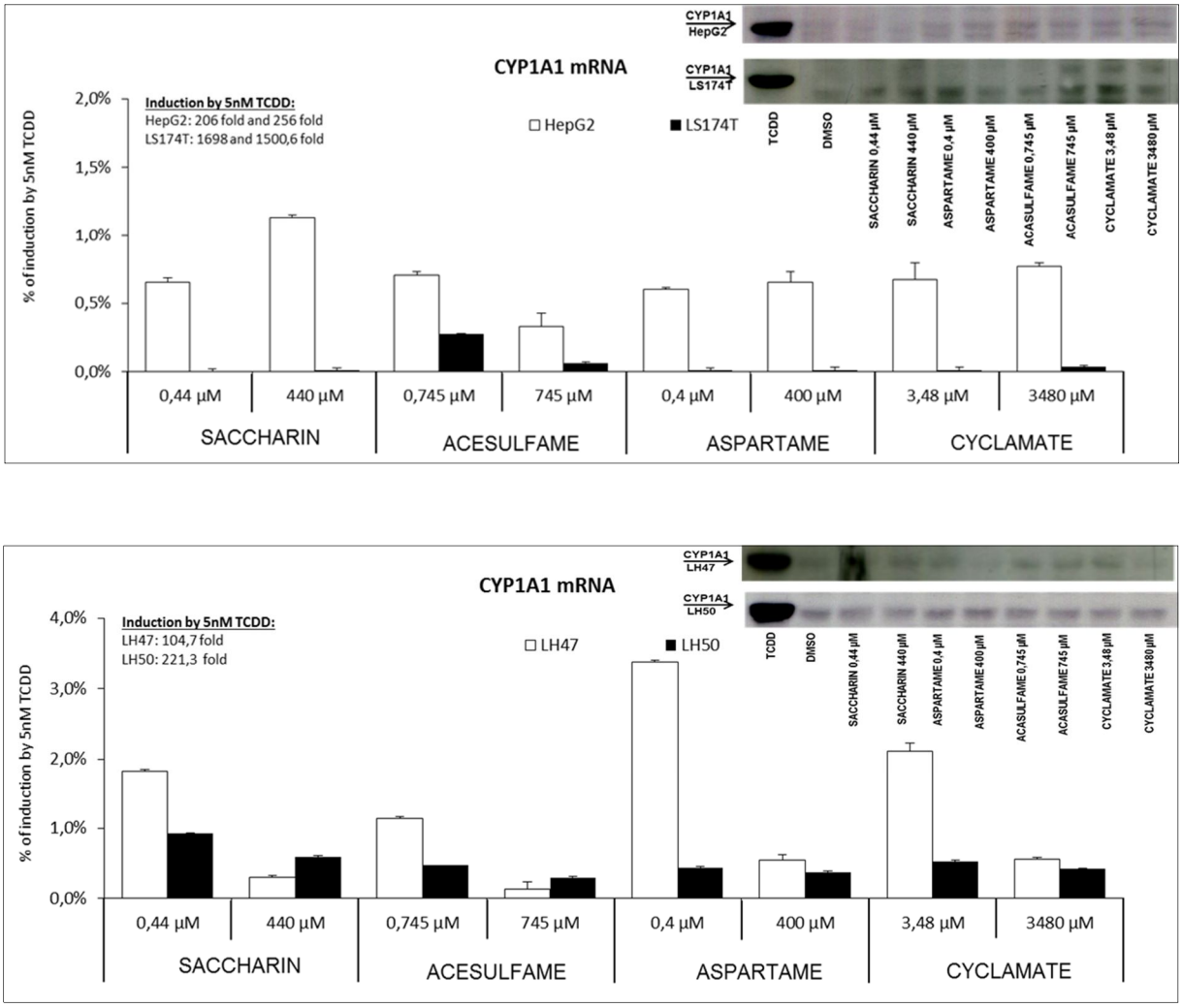

Figure 16. Effects of artificial sweeteners on CYP1A1 mRNA and protein expression.

5. CONCLUSION

The present thesis evaluated the effects on the AhR-CYP1A1 signaling pathway of 17 ready to drink teas, 28 mineral waters, 6 anthocyanidins, 21 anthocyanins and 4 artificial sweeteners were evaluated. *In vitro* models were used for studying the potential interaction of food constituents with the AhR receptor and CYP1A1/2 induction. The activity of AhR was increased by two ready to drink teas, four mineral waters, pelargonidin, pelargonidin-3-O-rutinosid, cyanidin-3,5-diglucoside, which was revealed by reporter gene assay. The RT-PCR analysis revealed increased induction of CYP1A/2 genes at the mRNA level by the same compounds and immunoblotting method confirmed the elevated level of CYP1A1 protein after treatment by these substances as well.

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