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RESEARCH ARTICLE

Profiling of anthocyanidins against transcriptional activities of steroid and nuclear receptors

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ABSTRACT

The aim of current study was to evaluate the effect of the most common anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, and peonidin) on the transcriptional activity of steroid and nuclear receptors. The activities of steroid receptors – progesterone receptor (PR), estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), and nuclear receptors – vitamin D receptor (VDR), retinoid X receptor (RXR), retinoic acid receptor (RAR), pregnane X receptor (PXR), and thyroid receptor (TR) were assessed using either stable transfected luciferase gene reporter cell lines or transiently transfected cell lines. The cytotoxicity assays and gene reporter assays were performed after the 24-h treatment of cells with increasing range of concentrations (10 nM to 50 μM) of selected anthocyanidins. The results of experiments indicate that none of the examined anthocyanidins in all tested concentrations caused remarkable changes of transcriptional activity of studied steroid receptors, but their increasing concentrations slightly inhibited transcriptional activity of nuclear receptors induced by model agonists.

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Anthocyanidins; nuclear receptors; steroid receptors; transcriptional activity

Introduction

Anthocyanidins are the naturally occurring plant pigments responsible for typical coloration of many flowers, fruits and vegetables. They belong to the widespread group of phenolic compounds collectively termed flavonoids. Anthocyanidins represent the aglycon (sugar-free) backbones of anthocyanins, which are usually bound to various sugar residues (Welch *et al*. 2008). The most abundant anthocyanidins in plants are cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin (Ghosh and Konishi 2007). Anthocyanidins possess pharmacological properties that can influence human organism. Besides the well-known anti-oxidant effect, anti-inflammatory, anti-obesity and anti-mutagenic effects of anthocyanidins have been documented (Kong *et al*. 2003; Prior and Wu 2006; Kruger *et al*. 2014). Consummation of anthocyanidins, as the natural constituents of common diet or in the form of dietary supplements, can potentially lead to food–drug interactions, pharmacokinetic and toxicokinetic interactions between the food and drugs that change their activity. Food–drug interactions proceed either by inhibition or induction of drug metabolizing enzymes. These enzymes are transcriptionally regulated by the nuclear, steroid or xeno-receptors. It has been proved that the activity of some of these receptors can be affected by anthocyanidins. Jia *et al*. (2013) described that cyanidin is an agonistic ligand for peroxisome proliferator-activated receptor-alpha. We have described an activation of aryl hydrocarbon receptor and induction of CYP1A1 by pelargonidin and in lesser extent by cyanidin. In the same study we also described an inhibition of CYP1A1 catalytic activity by pelargonidin and delphinidin (Kamenickova *et al*. 2013b). Also, anthocyanins pelargonidin-3-O-rutinoside and cyanidin-3,5-O-diglucoside were identified as the activators of human AhR (Kamenickova *et al*. 2013a). Differential effects of anthocyanidins on the expression of xenobiotic metabolism phase II enzymes (Dvorak *et al*. 2014), organic anion transporting peptides OATP (Riha *et al*. 2015), and CYP2A6 (Srovnalova *et al*. 2014) were also described. The aim of current study was to investigate larger number of nuclear and steroid receptors to bring more complex information about the effect of anthocyanidins on their transcriptional activity.

Material and methods

Chemicals

The anthocyanidins: cyanidin chloride, delphinidin chloride, malvidin chloride, pelargonidin chloride and peonidin chloride were purchased from Extrasynthese (Lyon, France). The dimethylsulfoxide (DMSO), hygromycin B, dexamethasone (DEX), 3,3,5-triiodo-L-thyronine (T3), 5α-dihydrotestosterone (DHT), rifampicin (RIF), 17β-estradiol (E2), and progesterone (P4) were purchased from Sigma-Aldrich (Prague, Czech Republic). The 9-*cis*-retinoic acid (cisRA), all-*trans*-retinoic acid (transRA) and 1,25-dihydroxy vitamin D3 (VD3) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The FuGENE HD transfection reagent and reporter lysis buffer were from Promega (Madison, WI). All other chemicals were of the highest commercially available quality.

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Cell cultures

The HEK293 (ECACC no. 85120602), HeLa (ECACC no. 93021013), HepG2 (ECACC no. 85011430), LS180 (ECACC No. 87021202), MCF-7 (ECACC no. 86012803) cell lines and stably transfected lines AZ-GR (Novotna *et al*. 2012) and PZ-TR (Illes *et al*. 2015) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. The 22Rv1 (ECACC No. 105092802) cell line and stably transfected cell line AIZ-AR (Bartonkova *et al*. 2015) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 4 mM L-glutamine, and 1 mM sodium pyruvate. All cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

Plasmids

The Cignal PR reporter assay kit (catalog no. 336841 CCS-6043 L) and Cignal ER reporter assay kit (catalog no. 336841 CCS-005 L) were purchased from Quiagen (Manchester, UK). The reporter plasmids pDR1-Luc (PathDetect DR1 cis-Reporting System, catalog no. 240113) and pDR5-Luc (PathDetect DR5 cis-Reporting System, catalog no. 240119) were purchased from Agilent Technologies (Santa Clara, CA). The pCYP24-luc reporter plasmid containing 5' flanking region (−1200/−22) of human *CYP24* gene cloned into pGL3 plasmid upstream of firefly luciferase reporter gene was kindly provided by Dr. J.M. Pascussi (INSERM U632, Montpellier, France). The p3A4-luc reporter plasmid containing the basal promoter (−362/+53) with proximal PXR response element and the distal xenobiotic responsive enhancer module (−7836/−7208) of human *CYP3A4* gene 5' flanking region inserted into pGL3 plasmid was obtained from Dr. P. Pavek (Charles University, Hradec Kralove, Czech Republic). The expression plasmid for human PXR receptor, pSG5-hPXR, was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX) and the expression plasmid pSG5-hRXRα encoding human RXRα was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland).

Transient transfection and stably transfected cell lines

Transient transfections with appropriate reporter plasmids were performed in 96-well plates, using the FuGENE HD transfection reagent according to the manufacturer's standard protocol. For the assessment of transcriptional activity of selected receptors, following transfection reactions were carried out: estrogen receptor – transfection of MCF-7 cells with the Cignal ER reporter assay kit (MCF-7-ER), progesterone receptor – transfection of 22Rv1 cells with the Cignal PR reporter assay kit (22Rv1-PR), retinoic acid receptor – transfection of HEK293 cells with the pDR5-Luc reporter plasmid (HEK293-RAR), retinoid X receptor – co-transfection of HEK293 cells with the pDR1-Luc reporter plasmid and the pSG5-hRXRα expression plasmid (HEK293-RXR), vitamin D receptor – transfection of LS180 cells with the pCYP24-luc reporter plasmid (LS180-VDR), pregnane X receptor – co-transfection of LS180 cells with the p3A4-luc reporter plasmid and the pSG5-hPXR expression plasmid (LS180-PXR). For the determination of transcriptional activity of glucocorticoid receptor, androgen receptor and thyroid receptor, stably transfected cell lines AZ-GR, AIZ-AR and PZ-TR were used. The use of GMO at the Faculty of Science, Palacky University Olomouc, was approved by the Ministry of the Environment of the Czech Republic (Ref. 91997/ENV/10).

Cytotoxicity assay and gene reporter assay

All cell lines were seeded into 96-well tissue culture plates at different density depending on cell type in 0.2 mL of culture medium supplemented with 10% charcoal-stripped fetal bovine serum. After 16 h of stabilization, the cells were treated for 24 h with tested anthocyanidins at the concentrations of 10 nM, 100 nM, 1 μM, 5 μM, 10 μM, and 25 μM, or vehicle (0.1% DMSO, v/v). During the treatment, cells were cultivated in the presence (antagonist mode) or absence (agonist mode, cytotoxicity) of receptors model ligands DEX (100 nM; AZ-GR), T3 (10 nM; PZ-TR), DHT (100 nM; AIZ-AR), RIF (10 nM; LS180-PXR), VD3 (50 nM; LS180-VDR), E2 (100 nM; MCF-7-ER), P4 (100 nM; 22Rv1-PR), cisRA (1 μM; HEK293-RXR), and transRA (5 μM; HEK293-RAR). Later on, the cells were lysed in reporter lysis buffer and the luciferase activity was measured using a Tecan Infinite M200 Pro plate reader (Tecan, Grödig, Austria). In parallel, cell viability was determined by conventional MTT test.

Data and statistical analyses

The results are representatives of at least three independent experiments. The data correspond to the means ± standard deviation (SD) of triplicate measurements. Statistical significance was tested by Student's *t*-test. Differences were considered significant at $p < 0.05$. The dose–response curve fits values were calculated using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA).

Results

Cytotoxicity of anthocyanidins on human cell lines

As a first step, the cytotoxicity of anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin) on the HEK293, HeLa, HepG2, LS180, MCF-7, and 22Rv1 cell lines was examined. After the 24-h treatment of the cells with increasing range of concentrations (10 nM to 50 μM) of studied anthocyanidins and vehicle (0.1% DMSO, v/v) as a negative control, a conventional MTT test was performed. We found that none of the examined compounds had cytotoxic effect on the cell lines used in our study (Figures 1 and 2).

Effect of anthocyanidins on transctriptional activity of steroid receptors PR, ER, AR and GR in human cell lines

Transcriptional activity of glucocorticoid receptor (GR) and androgen receptor (AR) was assessed in the stably transfected

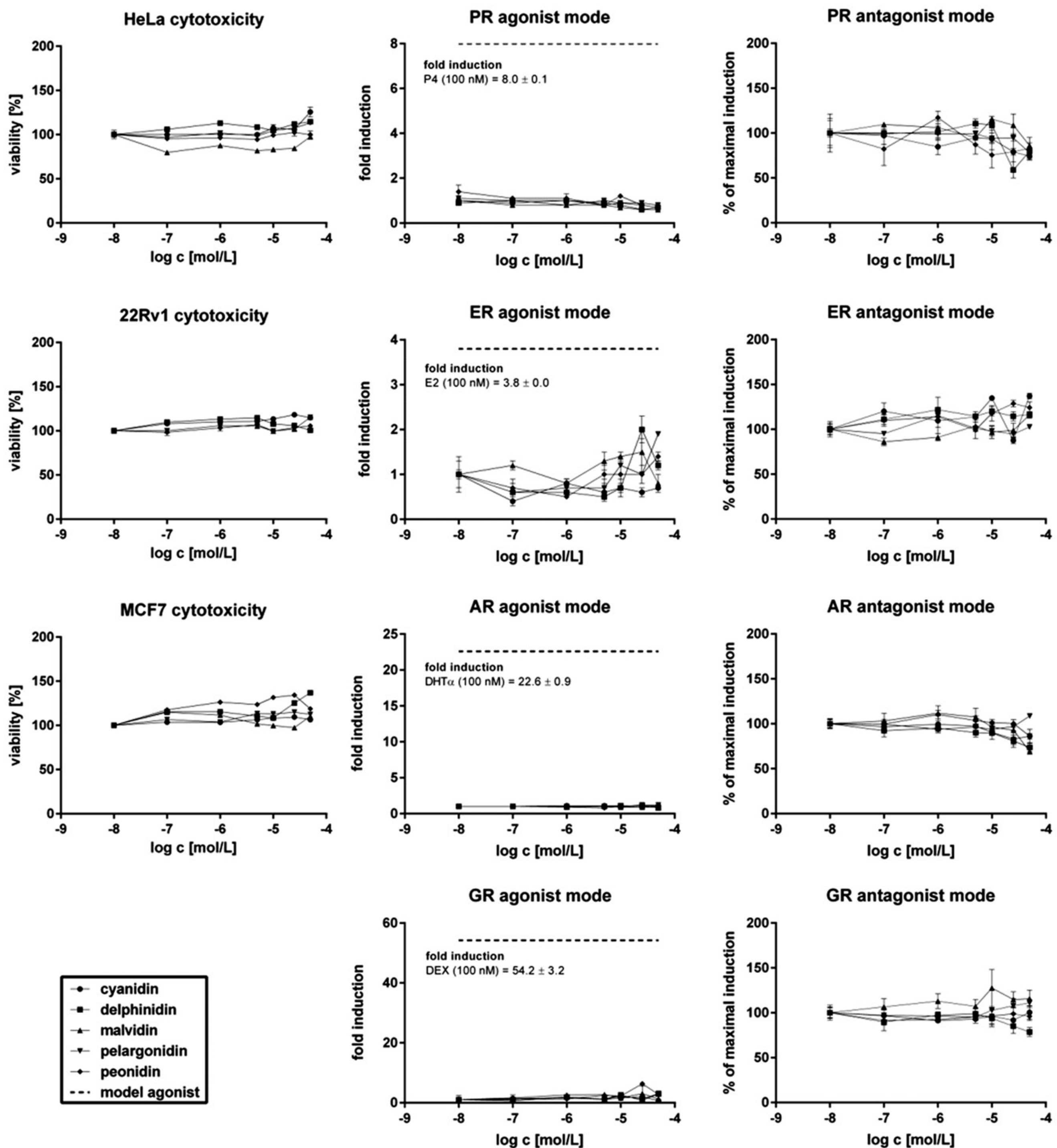

Figure 1. Effect of anthocyanidins on the transcriptional activity of steroid receptors. The line charts show cytotoxicity of anthocyanidins on MCF-7, 22Rv1 and HeLa cells (left column), luciferase activity of cells transfected with reporter plasmids for progesterone (PR), estrogen (ER), androgen (AR) and glucocorticoid receptors (GR) in agonist (middle column) and antagonist (right column) mode. The cells were treated with cyanidin, delphinidin, malvidin, pelargonidin, and peonidin (10 nM to 50 μM) for 24 h in the absence (cytotoxicity, agonist mode) or presence (antagonist mode) of model agonists P4 (100 nM), E2 (100 nM), DHT (100 nM), and DEX (100 nM), for PR, ER, AR, and GR, respectively. The data represent the mean ± SD of triplicate measurements and are expressed either as a percentage of viability (cytotoxicity), or as a fold induction over DMSO-treated cells (agonist mode), or as a percentage of the fold induction by model agonists (antagonist mode).

reporter gene lines AZ-GR and AIZ-AR, respectively. The transiently transfected cell lines MCF-7-ER and 22Rv1-PR were used for the assessment of estrogen receptor (ER) and progesterone receptor (PR) transcriptional activity, respectively. In the agonist mode of experiments (Figure 1), the cell lines were treated for 24 h with anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin) in concentrations ranging from 10 nM to 50 μM. The model agonists P4, E2, DHT and DEX were used as positive controls for PR, ER, AR and GR, respectively and the vehicle (0.1% DMSO, v/v) as a negative control. In the 22Rv1-PR cells treated with 100 nM P4, induction of luciferase activity reached 8-fold. None of the

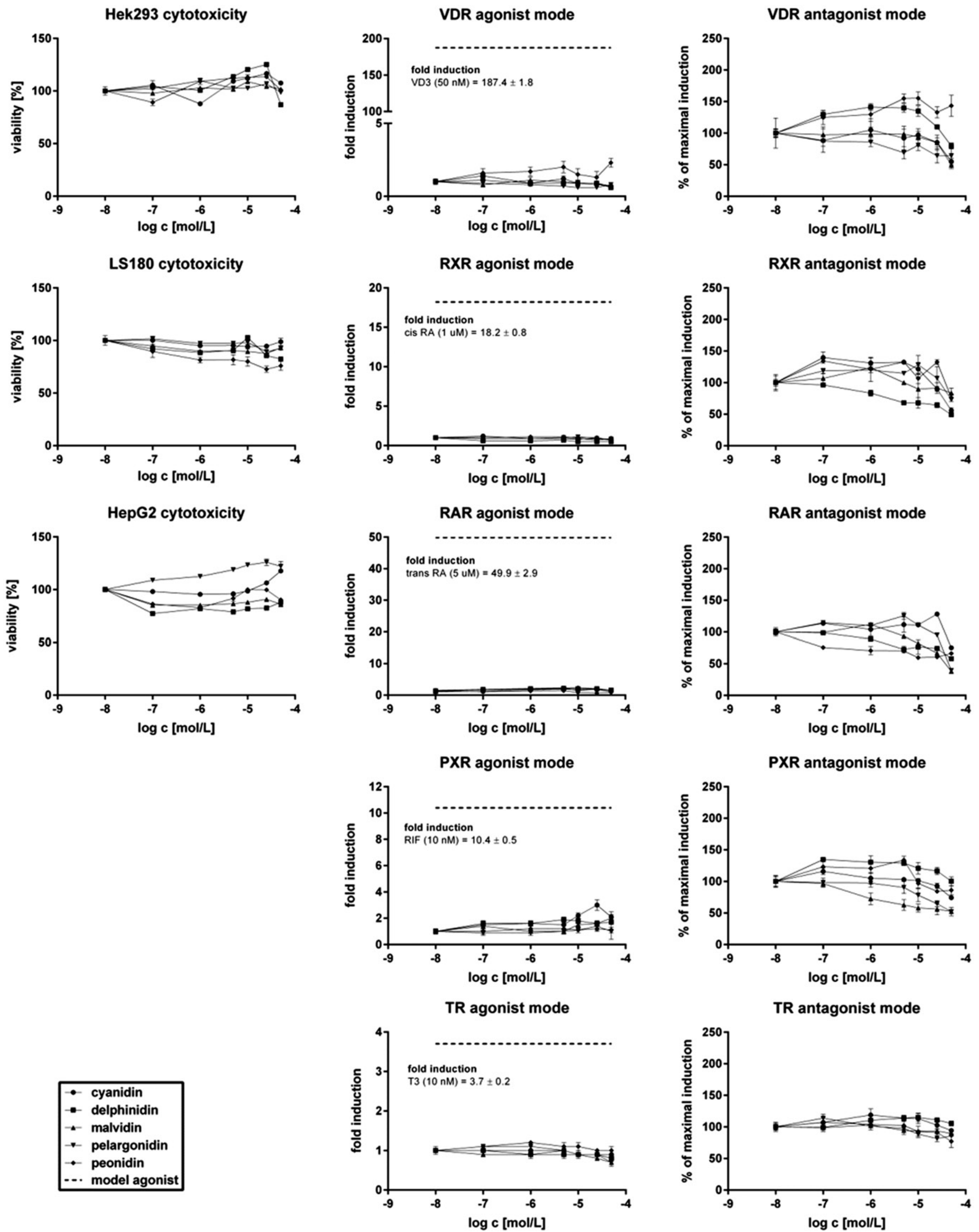

Figure 2. Effect of anthocyanidins on transcriptional activity of nuclear receptors. The line charts show cytotoxicity of anthocyanidins on HEK293, LS180 and HepG2 cells (left column), luciferase activity of cells transfected with reporter plasmids for vitamin D (VDR), retinoid X (RXR), retinoic acid (RAR), pregnane X (PXR) and thyroid receptors (TR) in agonist (middle column) and antagonist (right column) mode. The cells were treated with cyanidin, delphinidin, malvidin, pelargonidin, peonidin (10 nM to 50 μM) for 24 h in the absence (cytotoxicity, agonist mode) or presence (antagonist mode) of model agonists VD3 (50 nM), cisRA (1 μM), transRA (5 μM), RIF (10 nM), and T3 (10 nM) for VDR, RXR, RAR, PXR, and TR, respectively. The data represent the mean ± SD of triplicate measurements and are expressed either as a percentage of viability (cytotoxicity), or as a fold induction over DMSO-treated cells (agonist mode), or as a percentage of the fold induction by model agonists (antagonist mode).

tested anthocyanidins caused relevant induction of luciferase activity in these cells. Increase of luciferase activity after the treatment of the MCF-7-ER cells with 100 nM E2 was 3.8-fold. Exposure of the MCF-7-ER cells to examined anthocyanidins induced only moderate changes of luciferase activity, differently for all particular anthocyanidins, ranging from 0.4-fold (100 nM cyanidin) to 2.0-fold (25 μM delphinidin). No significant induction of luciferase activity in the AIZ-AR cells was observed for any of the anthocyanidins compared to 100 nM DHT that induced luciferase activity to 22.6-fold. Similarly, the treatment of the AZ-GR cells with examined anthocyanidins did not result in changes of luciferase activity, while 100 nM DEX increased induction of luciferase activity by 54.2-fold.

In the parallel antagonist experiments (Figure 1), the cell lines were treated for 24 h with anthocyanidins (10 nM to 50 μM) in combination with model agonists P4, E2, DHT and DEX corresponding to each receptor (PR, ER, AR, and GR, respectively). Moderate alterations of luciferase activity ranging from 58.9% (25 μM delphinidin) to 117.2% (1 μM peonidin) of activity induced by 100 nM P4 were observed in the 22Rv1-PR cells after the treatment with anthocyanidins. In the MCF-7-ER cells, the induction of luciferase activity was not markedly changed by any of the examined anthocyanidins. The values ranged from 86.0% (10 μM malvidin) to 137% (50 μM cyanidin) of maximal luciferase activity obtained by 100 nM E2. The induction of luciferase activity in the AIZ-AR cells was slightly reduced by increasing concentrations of anthocyanidins to the lowest value of 68.9% (50 μM malvidin), compared to 100 nM DHT. The highest value of lucifarese activity was measured at 1 μM malvidin (111.8%). Similarly, only the slight changes of luciferase activity representing 78.6% (50 μM delphinidin) to 127.6% (malvidin 10 μM) of maximal luciferase activity induced by 100 nM DEX were observed in AZ-GR cells exposed to examined anthocyanidins.

Effect of anthocyanidins on transctriptional activity of nuclear receptors VDR, RXR, RAR, PXR and TR in human cell lines

The stably transfected gene reporter cell line PZ-TR was used for assessment of thyroid receptor (TR) transcriptional activity. The transcriptional activities of all other investigated nuclear receptors – vitamin D receptor (VDR), retinoid X receptor (RXR), retinoic acid receptor (RAR), and pregnane X receptor (PXR) were evaluated using the transiently transfected cell lines LS180-VDR, HEK293-RXR, HEK293-RAR, and LS-180-PXR, respectively. In the agonist experiments (Figure 2), the cells were treated for 24 h with anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin) in concentrations of 10 nM to 50 μM. The model agonists VD3, cisRA, transRA, RIF, and T3 were used as positive controls for VDR, RXR, RAR, PXR, and TR, respectively, and the vehicle (0.1% DMSO, v/v) as a negative control. The treatments of the transiently and stably transfected cell lines with model agonists resulted in the following inductions of luciferase activity: LS180-VDR (50 nM VD3) – 187.4-fold, HEK293-RXR (1 μM cisRA) – 18.2-fold, HEK293-RAR (5 μM transRA) – 49.9-fold, LS180-PXR (10 nM RIF) – 10.4-fold, and PZ-TR (10 nM T3) – 3.7-fold, over the vehicle-treated cells. After the treatments with all examined anthocyanidins, any increase of luciferase activity was not observed. The only exception was the LS180-PXR cells treated with cyaniding, where the luciferase activity rose up to 3-fold at 25 μM concentration. Slight increase of luciferase activity was measured also in the LS180-VDR cells after the treatment with peonidin (2.3-fold induction at 50 μM concentration). But, compared to the model agonist-treated cells, induction of luciferase activity was not significantly relevant, as well.

In the parallel antagonist experiments (Figure 2), the cell lines were treated for 24 h with anthocyanidins (10 nM to 50 μM) in combination with model agonists VD3, cisRA, transRA, RIF, and T3 adequate to each receptor (VDR, RXR, RAR, PXR, and TR, respectively). In general, none of the examined anthocyanidins caused dramatic changes of luciferase activity in all studied cell lines, only the slight inhibition of luciferase activity was observed. With increasing concentrations of anthocyanidins, the induction of luciferase activity slightly dropped down in LS180-VDR cells. The minimal value of 49.4%, compared to luciferase activity obtained by 50 nM VD3, was measured at 50 μM malvidin. Similarly, slight decrease of luciferase activity was observed in the HEK293-RXR cells after the treatment with anthocyanidins, reaching minimum at 49.3% of luciferase activity induced by model agonist (1 μM cisRA) at 50 μM delphinidin. In the HEK293-RAR and LS180-PXR cells, application of anthocyanidins resulted in moderate decrease of luciferase activity with the strongest effect induced by 50 μM malvidin and 50 μM pelargonidin. Both of them reduced luciferase activity in the LS180-PXR cells to 52% of 10 nM RIF-induced maximal activity. In the HEK293-RAR cells, 50 μM malvidin decreased induction of luciferase activity to 38% and 50 μM pelargonidin to 39.4%, respectively, compared to luciferase activity induced by 5 μM transRA. Finally, the slightest inhibition of luciferase activity after the exposure of cells to anthocyanidins was observed in the PZ-TR cells. The values ranged from 77.2% (50 μM malvidin) to 118.8% (1 μM cyanidin) of maximal luciferase activity obtained by 10 nM T3.

Discussion

It has been demonstrated that transcriptional activity of some receptors can be affected by anthocyanidins (Jia *et al*. 2013; Kamenickova *et al*. 2013a, 2013b), but complex study describing larger scale of receptors is still missing (Pastorkova and Dvorak 2015). Therefore, the main aim of current study was to examine potential effects of the most common anthocyanidins on the transcriptional activities of two groups of receptors – steroid and nuclear receptors. Using the stable and transient luciferase gene reporter systems, it was revealed that in the agonist mode none of the examined anthocyanidins had remarkable influence on the induction of luciferase activity in all reporter cell lines, for both, steroid and nuclear receptors (Figures 1 and 2). Similarly, in antagonist mode of experiments investigating steroid receptors, anthocyanidins did not dramatically changed luciferase activity induced by model ligands, even though some fluctuations of luciferase

activities were observed (Figure 1). In the case of nuclear receptors, moderate decrease of luciferase activity after application of anthocyanidins was measured in the antagonist mode (Figure 2). Decrease of luciferase activity was concentration-dependent with the values dropping at the highest concentrations of anthocyanidins below the half of the activity induced by model ligands. These findings indicate that the most commonly occurring anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, and peonidin) do not affect transcriptional activity of both, steroid and nuclear receptors, in a substantial way. Only the exception is the potential inhibition of transcriptional activity of nuclear receptors induced by their natural ligands. PubMed search performed on 12 March 2017 yielded no results for putative effects of anthocyanidins on RXR, RAR, PR, GR, and PXR, of the receptors under investigation in this current study. Several reports describe beneficial roles for anthocyanidins against prostate cancer, comprising various AR-independent mechanisms. Cyanidin down-regulated cyclooxygenase 2 expression, which resulted in attenuation of prostaglandins production (Munoz-Espada and Watkins 2006). Delphinidin induced apoptosis of both androgen-independent and androgen-refractory human prostate cancer cells *via* activation of caspases and in addition, this effect might be due to the inhibition of NFkappaB signaling (Bin Hafeez *et al*. 2008). Cyanidin-3-glucoside and pelargonidin-3-glucoside decreased cyclin D1 levels and inhibited prostate cancer cells growth in the G1 phase (Long *et al*. 2013). Protective effects of Blue honeysuckle extract against chemically-induced hyperthyroidism in rats were described, which tentatively implies the interference between anthocyanidins and TR signaling (Park *et al*. 2016). Delphinidin and cyanidin were identified as weak ligands for VDR and also weak activators of VDR in transiently transfected keratinocytes (Hoss *et al*. 2013; Austin *et al*. 2014), which is consistent with our findings that peonidin slightly increases luciferase activity in our assays. There are numerous reports on estrogenic activities of anthocyanidins, however, binding affinity to the estrogen receptor-alpha was 10 000- to 20 000-fold lower than that of the endogenous estrogen estradiol (Schmitt and Stopper 2001). Estrogenic, resveratrol-independent (Klinge *et al*. 2003), and alcohol-independent (Simoncini *et al*. 2011) activity in white and red wine extracts was described. Involvement of ER in the effects of anthocyanidins is, however, controversial. While some studies demonstrate that anthocyanidins bind to and activate ER (Chalopin *et al*. 2010; Nanashima *et al*. 2015), other studies shown ER-independent estrogenic effects through activation of AMP-kinase (Park *et al*. 2015).

Disclosure statement

No potential conflict of interest was reported by the authors.

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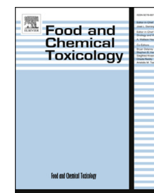

Hydroxystilbenes and methoxystilbenes activate human aryl hydrocarbon receptor and induce CYP1A genes in human hepatoma cells and human hepatocytes

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Hepatocytes

A B S T R A C T

Natural polyphenol resveratrol (trihydroxystilbene) is a partial agonist of human aryl hydrocarbon receptor AhR, thereby, displaying a plethora of biological effects. Biological activities of metoxylated and hydroxylated stilbenes were studied in the past. The aim of the current study was to describe the effects of 13 different hydroxy- and methoxystilbenes, including their *cis/trans* isomers on the transcriptional activity of AhR and the expression of CYP1A genes in hepatic cancer cells HepG2 and in primary human hepatocytes. Techniques of gene reporter assays, qRT-PCR, Simple Western blotting by Sally Sue™ and electrophoretic mobility shift assay EMSA were employed. All compounds activated AhR, but their efficacies, potencies and dose-response profiles differed substantially. The strongest activators of AhR and inducers of CYP1A1 in HepG2 cells were DMU-212 ((E)-3,4,5,4′-tetramethoxystilbene), *trans*-piceatannol, *cis*-piceatannol, *trans*-trismethoxyresveratrol and *trans*-pinostilbene. While DMU-212 and *trans*-trismethoxyresveratrol also induced CYP1A1 and CYP1A2 in primary human hepatocytes, the effects of *trans*-piceatannol, *cis*-piceatannol and *trans*-pinostilbene weaned off. On the other hand, *trans*-4-methoxystilbene was strong CYP1A inducer in hepatocytes but not in HepG2 cells. Differences between effects of stilbenes in HepG2 cells and human hepatocytes are probably due to the extensive phase I and phase II xenobiotic metabolism in human hepatocytes. The data obtained may be of toxicological relevance.

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1. Introduction

Stilbene is 1,2-diphenylethene, which exists as two possible isomers known as *(E)*-stilbene (*trans*-stilbene) and *(Z)*-stilbene (*cis*-stilbene). Stilbenoids are hydroxylated derivatives of stilbenes, belonging to the family of phenylpropanoids that share biosynthetic pathways with chalcones. The most known stilbenoid is resveratrol, which is structurally 3,5,4′-trihydroxy-*trans*-stilbene. It is natural phenolic compound produced by several plants as phytoalexin in response to injury or microbial attack. The richest sources of resveratrol and other stilbenoids are grapes, nuts and berries (Riviere et al., 2012). Resveratrol has been extensively studied as evidenced by 9160 search results yielded in PubMed on January 2017. Biological activity of resveratrol comprises the wide array of effects, including anti-hypertensive (Huang et al., 2013), anti-apoptotic (Renaud et al., 2014; Xia et al., 2011; Zhou et al., 2014), anti-proliferative (Cui et al., 2010; De Leo et al., 2011), anti-inflammatory (Tao et al., 2016; Rangarajan et al., 2016), anti-cancer (Chimento et al., 2016), anti-diabetic (Oyenihi et al., 2016; Bagul and Banerjee, 2015), cardio-protective (Yang et al., 2016) and many others (Erdogan and Vang, 2016). Consistently with pleotropic biological effects of resveratrol, its cellular targets are also numerous, involving AMP-activated protein kinase [AMPK] (Wang et al., 2016), nuclear factor kappa B (Kubota et al., 2009), sirtuins (Howitz et al., 2003), mitogens-activated protein kinases p38 (Yuan et al., 2016) and [ERK] (Cao et al., 2016), estrogen receptor (Gehm et al., 1997) and other targets (Britton et al., 2015; Kulkarni and Canto, 2015). Myriads of resveratrol derivatives,

Abbreviations: AhR, aryl hydrocarbon receptor; AMPK, AMP-activated protein kinase; ARNT, AhR nuclear translocator; DMSO, dimethylsulfoxide; ERK, extracellulary regulated kinase; GAPDH, glyderaldehyd-3-phosphate dehydrogenase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element.

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both natural and synthetic were tested in biological assays and clinical trial to date. Briefly, methylation of resveratrol enhanced its anti-proliferative activity, and isomerization (E/Z) of resveratrol and its derivatives changed that activity (Cardile et al., 2007; Roberti et al., 2003). Methoxylation of hydroxyl groups at resveratrol molecule resulted in better bioavailability and enhanced bioactivity (Walle et al., 2007; Mazue et al., 2010; Aldawsari and Velazquez-Martinez, 2015).

The aryl hydrocarbon receptor [AhR] is a ligand-activated transcriptional factor belonging to the family of basic helix-loop-helix transcription factors. In its inactive form, AhR resides in cytoplasm in complex with chaperone proteins. Upon ligand binding, AhR translocates to the nucleus where it heterodimerizes with AhR nuclear translocator [ARNT]. Heterodimer AhR/ARNT produces changes in genes transcription by binding to xenobiotic responsive element [XRE] (Denison et al., 2002). AhR is involved in many cellular and biological processes, including regulation of the cell cycle, DNA repair, immune response, apoptosis, xenoprotection etc. (Abel and Haarmann-Stemmann, 2010; Esser et al., 2013; Bruhs et al., 2015; Dittmann et al., 2016). Exogenous activators of AhR comprise environmental pollutants (e.g. polycyclic aromatic hydrocarbons, dibenzodioxins, dibenzofurans), drugs (e.g. omeprazole (Quattrochi and Tukey, 1993), ketoconazole (Korashy et al., 2007; Novotna et al., 2014), primaquine (Fontaine et al., 1999)), synthetic compounds (e.g. SP600125 (Dvorak et al., 2008), TSU-16 (Matsuoka-Kawano et al., 2010), U0126 (Andrieux et al., 2004)) and natural compounds (e.g. berberine (Vrzal et al., 2005), curcumin (Rinaldi et al., 2002), genistein, quercetin (Zhang et al., 2003)). Endogenous ligands for AhR include indirubin, bilirubin, tryptamine, indoyl acetic acid, prostaglandings etc (Denison and Nagy, 2003; Stejskalova et al., 2011).

There are numerous reports on the interactions between stilbenoids and the AhR-CYP1A signaling pathway. Many stilbenoids, including resveratrol (Potter et al., 2002; Piver et al., 2004), *trans*-3,4,5,4'-tetramethoxystilbene [D] (Mikstacka et al., 2012; Piotrowska-Kempisty et al., 2016), 2,3′,4,5'-tetramethoxystilbene [E] (Chun et al., 2001), pterostilbene and pinostilbene (Mikstacka et al., 2007), were described as substrates and inhibitors of CYP1A1, CYP1A2 and CYP1B1 enzymes. Resveratrol was demonstrated as a modulator of AhR transcriptional activity by multiple mechanisms. Dioxin-inducible CYP1A1 expression in HepG2 cells was inhibited by resveratrol. While binding of TCDD to AhR was not influenced by resveratrol, the transformation of AhR to its DNA binding form was blocked by this compound (Ciolino et al., 1998). Casper et al. described that resveratrol is a competitive antagonist of dioxin and other AhR ligands in HepG2 and T47D cells and that it promotes AhR translocation to the nucleus and binding to the DNA at dioxin-responsive elements but subsequent transactivation does not take place (Casper et al., 1999). Consistently, Beedanagari et al. showed that resveratrol inhibits dioxin induction of the CYP1 genes either by directly or indirectly inhibiting the recruitment of AhR and ARNT to the xenobiotic response elements of these genes (Beedanagari et al., 2009). Lee et al. proposed that resveratrol down-regulates CYP1A1 in T47D and MCF7 cells *via* an AhR-independent post-transcriptional mechanism (Lee and Safe, 2001). Indirect mechanism of AhR activation by resveratrol was proposed, involving inhibition of metabolic turn-over of FICZ, an endogenous AhR ligand (Mohammadi-Bardbori et al., 2012). Gouédard et al. observed AhR-dependent induction of *paraoxonase* gene in HuH7 hepatoma cells by resveratrol, which they explained by agonist effect of resveratrol that was specific for *paraoxonase* gene but not for classical AhR responsive elements (Gouedard et al., 2004). Resveratrol was an effective AhR antagonist, but it induced CYP1A1 in low doses in primary trout hepatocytes, indicating partial agonist activity (Aluru and Vijayan, 2006). Indeed, we have also described partial agonist activity of resveratrol in human hepatoma cells and in human hepatocytes (Dvorak et al., 2008). Various natural and synthetic stilbenoids were demonstrated to inhibit or activate AhR pathway (Licznerska et al., 2016; de Medina et al., 2005; Guyot et al., 2012).

In the current study, we have evaluated the effects of 13 different hydroxystilbenes and methoxystilbenes, including their *cis/trans* isomers on the transcriptional activity of AhR and the expression of CYP1A genes in hepatic cancer cells HepG2 and in primary human hepatocytes.

2. Materials and methods

2.1. Chemicals

Trans-resveratrol, *cis*-resveratrol, *trans*-trismethoxyresveratrol, *cis*-trismethoxyresveratrol, *trans*-piceatannol, pinostilbene, pterostilbene, *trans*-2′,3,4′,5-tetramethoxystilbene and oxyresveratrol were from Bertin Pharma (Montigny le Bretonneux, France). *Trans*-4-methoxystilbene, *trans*-3,4,5,4′-tetramethoxystilbene, dimethylsulfoxide [DMSO] and hygromycin B were from Sigma-Aldrich (Prague, Czech Republic). The a,b-dihydroresveratrol, *cis*-piceatannol and *cis*-resveratrol were from Santa Cruz Biotechnology (Santa Cruz, USA). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] was from Ultra Scientific (RI, USA). Reporter lysis buffer was from Promega (Madison, USA). All other chemicals were of the highest commercially available quality.

2.2. Cancer cell lines

Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC no. 85011430) and a stably transfected line AZ-AhR (Novotna et al., 2011) were cultivated in Dulbecco's modified Eagle's medium DMEM supplemented with 10% fetal bovine serum, 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.

2.3. Primary cultures of human hepatocytes

Human hepatocytes were obtained from two sources. (i) Hepatocytes were isolated from human liver resected from three adult multiorgan donors: HH65 (male, 34 years), HH66 (male, 65 years) and HH71 (male, 58 years). The tissue acquisition protocol was in accordance with the requirements stated by the local ethical commission in the Czech Republic. The hepatocytes were isolated from the (HTK, Koehler Chemie, Germany) prewashed liver using two-step collagenase perfusion (Pichard et al., 1990). Yield and viability of cells were assessed by the trypan blue exclusion test. Hepatocytes were re-suspended in medium, consisting of a 1:1 mixture of Ham F12 and Williams'E, supplemented with additives as follows: glucose (7 mM), glutamine (2.4 mM), penicillin (100 U/mL), streptomycin (10 mM), sodium pyruvate (0.4 mM), dexamethasone (1.8 mM), holo-transferrin (5 mg/l), ethanolamine (1 mM), insulin (350 nM), glucagon (0.2 mg/l), linoleic acid (11 mg/l), ascorbic acid (15 mg/l), amphotericin B (1.4 mg/l), pH 7.2 (Isom et al., 1985). Cells were seeded on collagen-coated plates at a density of 1.25×10^5 cells/cm^2. For the first 4 h, culture medium was enriched with 5% foetal calf serum to improve cell attachment. Cultures were allowed to stabilize for 48 h prior to the treatments and were maintained at 37 °C and 5% CO_2 in a humidified incubator. (ii) Human hepatocytes in monolayer batch Hep200525 (male, 44 years) and batch Hep200529 (female, 26 years) were purchased from Biopredic International (Rennes, France). Cells were cultured according to manufacturers' instructions.

2.4. Cytotoxicity and gene reporter assay

The stably transfected gene reporter cell line AZ-AhR derived from human hepatoma cells HepG2 transfected with a construct containing several AhR binding sites upstream of a luciferase reporter gene (Novotna et al., 2011) was used for the valuation of transcriptional activity of AhR. Cells were seeded into 96-well tissue culture plates and incubated for 24 h with tested compounds and vehicle (DMSO; 0.1% v/v) in the presence (agonist mode) or in the absence (antagonist mode) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM). Afterwards the cells were lysed and luciferase activity was measured on Tecan Infinite M200 Pro plate reader (Schoeller Instruments, Czech Republic).

Cell viability was determined by conventional MTT test; briefly: Cells were incubated for 24 h with the tested compounds, using 96-well plates. In parallel, the cells were treated with vehicle (DMSO; 0.1% v/v) and Triton X-100 (1% v/v) to assess the minimal (i.e. positive control) and maximal (i.e. negative control) cell damage, respectively. The cells were incubated with MTT for 3–4 h; after removing the medium and washing the cells with phosphate buffer solution, formazan dye was dissolved in DMSO containing 1% ammonia. The MTT assay absorbance was measured spectrophotometrically at 540 nm on Tecan Infinite M200 Pro plate reader (Schoeller Instruments, Czech Republic). The data were expressed as a percentage of viability, where 100% and 0% represent the treatments with DMSO and Triton X-100, respectively.

2.5. mRNA determination and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The total RNA was isolated by TRI Reagent® (Sigma-Aldrich, USA) and cDNA was synthesized according to the common protocol from 1000 ng of total RNA using M-MuLV Reverse Transcriptase (New England Biolabs, USA) at 42 °C for 60 min in the presence of random hexamers (New England Biolabs, USA). The levels of *CYP1A1, CYP1A2* and *glyderaldehyd-3-phosphate dehydrogenase [GAPDH]* mRNAs were determined using the Light Cycler® 480 II apparatus (Roche Diagnostic Corporation, Czech Republic), as described elsewhere (Vrzal et al., 2013). Measurements were carried out in triplicates. Gene expression was normalized *per GAPDH* as a housekeeping gene. Treatments did not systematically influence the expression of GAPDH. The data were processed by the delta-delta method.

2.6. Simple western blotting by Sally Sue™

The total protein extracts were prepared as decribed elsewhere (Vrzal et al., 2013). Reagents used for *simple western by Sally Sue™*, including antibody diluent, streptavidin, goat anti-mouse secondary antibody, goat anti-rabbit secondary antibody, capillaries and 384-well plates were obtained from ProteinSimple (San Jose, California) and samples were prepared according to the recommended ProteinSimple manual (http://www.proteinsimple.com/sally_sue.html). CYP1A1 (rabbit polyclonal, sc-20772, H-70), CYP1A1 (goat polyclonal, sc-9828, G-18), CYP1A2 (mouse monoclonal, sc-53614, 3B8C1) primary antibodies and rabbit anti-goat secondary antibody (sc-2768) were purchased from Santa Cruz Biotechnology Inc. β-actin (mouse monoclonal; 3700S, 8H10D10) primary antibody was from Cell Signaling Technology (Denvers, Massachusetts, USA). Target proteins were identified using primary antibodies and immunoprobed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified by the Compass Software version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the CYPs signals were normalized *per* β-actin as a loading control. Treatments did not systematically influence the expression of beta-actin.

2.7. Electrophoretic mobility shift assay (EMSA) AhR-DRE

MCF-7 were treated for 2 h with tested compounds, DMSO (0.1% v/v) and dioxin (TCDD; 5 nM). Nuclear fractions were isolated from cells using Nuclear extract kit (Active Motif) according to manufacturer's protocol. EMSA binding assay of AhR-DRE, using non-radioactive detection, was performed as we recently described elsewhere (Grycova et al., 2015).

2.8. Statistics

Student's t-test, One-way ANOVA followed by Dunnett's post test as well as calculations of EC50 and IC50 values were calculated using GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla, California, USA.

3. Results

3.1. Effect of stilbenoids on transcriptional activity of aryl hydrocarbon receptor

Prior to the gene reporter assays, we performed a conventional MTT test in AZ-AhR cells incubated with tested compounds for 24 h. Based on cytotoxicity data we determined maximal concentration of 50 μM for next *in vitro* experiments (Fig. 2A).

The effects of stilbenoids on the transcriptional activity of AhR were studied in human gene reporter cell line AZ-AhR. For this purpose, the cells were incubated for 24 h with tested compounds in concentrations ranging from 10 nM to 50 μM in the absence (agonist mode) or in the presence (antagonist mode) of AhR model ligand (TCDD; 5 nM). Average induction of luciferase activity by 1953-fold was attained by 5 nM TCDD. All tested compounds activated AhR, but their potency (EC_{50}), efficacy (fold induction) and dose-response profiles differed substantially (Fig. 2B). According to the magnitude of the induction by tested compounds in 50 μM concentration, we may sort out these compounds into three groups by comparative efficacy; strong activators (fold induction > 100; D, G, H, J, M), medium activators (10 < fold induction < 100; A, B, I, K) and weak activators (fold induction < 10; C, E, F, L). Half maximal effective concentrations (EC_{50}) ranged from 3.4 μM to 28 μM. In general, there was an inverse correlation between potency and efficacy, which is common for partial agonists. The majority of compounds caused dose-dependent increase of AhR activity, reaching a plateau (B, F, I, J, K, L) or being still ascending (A, D, G, H, M) in 50 μM concentration. Compound E activated AhR only in 5 μM concentration (25-fold induction), while compound C had negligible activity (2-fold induction). All stilbenoids antagonized AhR in experiments where AZ-AhR cells were co-incubated with tested compounds and TCDD (Fig. 2C). Half maximal inhibitory concentrations (IC_{50}) ranged from 1 μM to 25 μM. Depending on particular compound, the dose-response profiles displayed sigmoid profile (A, B, C, F, H, J, L, M), inversed U-shaped profile (I, K) or atypical profile (D, G, E), revealing about combined agonist and antagonist effects of the compound.

3.2. Effects of stilbenoids on the expression of CYP1A1 in HepG2 cells

HepG2 cells were incubated for 24 h (mRNA analyses) and for 48 h (protein analyses) with tested compounds (50 μM), vehicle (DMSO; 0.1% v/v), and dioxin (TCDD; 5 nM). Average induction of *CYP1A1* mRNA in three consecutive cell passages by TCDD was 605-

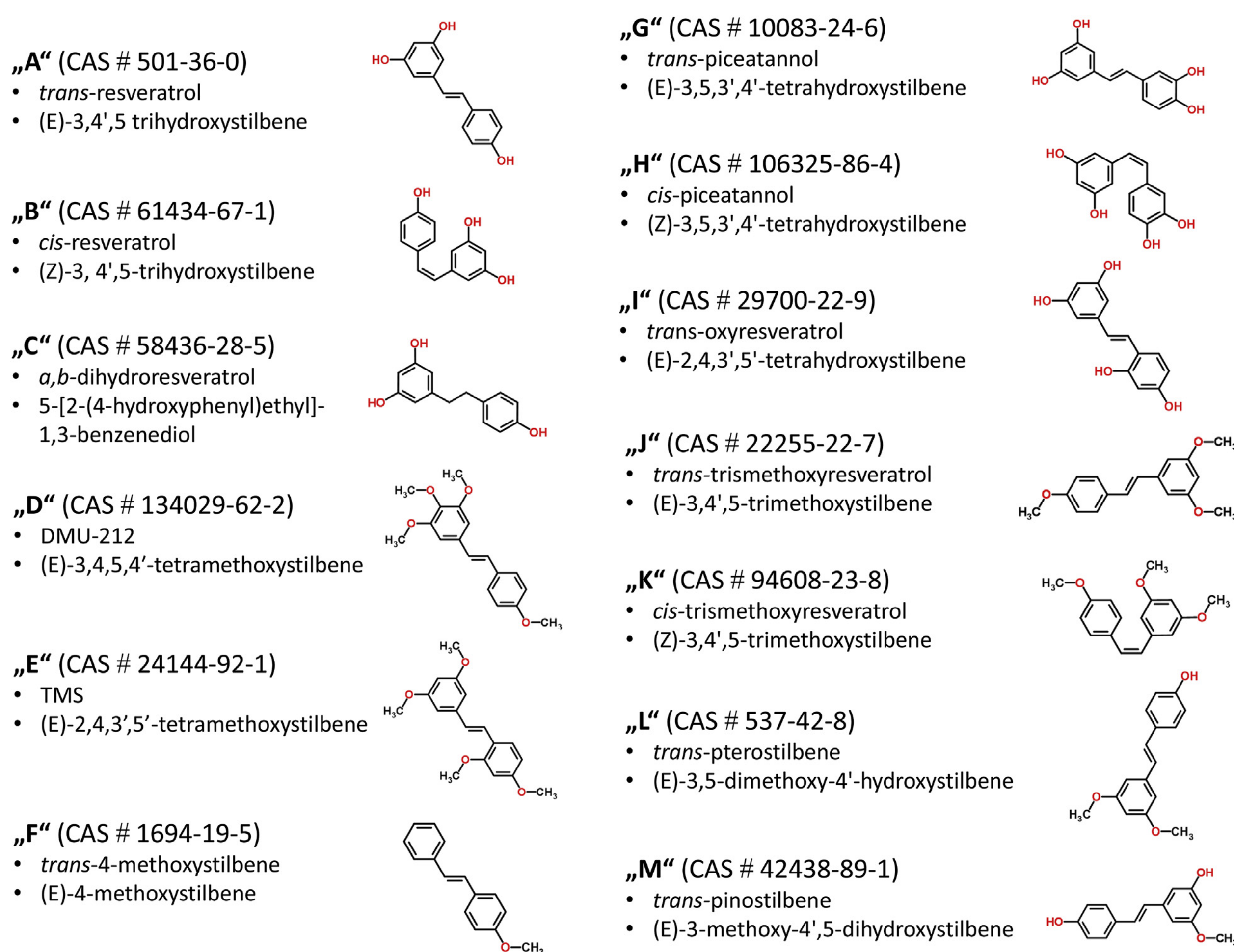


Fig. 1. Chemical structures, CAS numbers, trivial and structural names of tested stilbenes.

fold (Fig. 3; upper panel). According to the magnitude of the induction by tested compounds in 50 μM concentration, we may distinguish strong inducers (fold induction > 100; D, G, H, J) and weak inducers (10 < fold induction < 100; B, F, I, L, M) of CYP1A1 mRNA. Average induction of CYP1A1 protein in three consecutive cell passages by TCDD was 13-fold (Fig. 3; lower panel). Significant induction of CYP1A1 protein was achieved by G (13-fold), H (9-fold) and J (2.5-fold).

3.3. Effects of stilbenoids on the formation of AhR-DNA complex

Electrophoretic mobility shift assay (EMSA) was employed to test whether the studied compounds transform AhR into its DNA-binding form. Strong formation of AhR-DNA complex was observed in nuclear extracts from cells incubated with compounds K, G, H, M, I and F. Weak activity was observed for compounds B, C and E (Fig. 4).

3.4. Effects of stilbenoids on the expression of CYP1A1 and CYP1A2 in primary human hepatocytes

We used primary human hepatocytes obtained from five different liver tissue donors. Primary cultures were incubated with tested compounds, vehicle and TCDD for 24 h (mRNA analyses) and 48 h (protein analyses). Induction of *CYP1A1/CYP1A2* mRNAs in human hepatocytes cultures HH65, HH66, HH71, Hep200525 and Hep200529 by TCDD was 43/54-fold, 189/179-fold, 1020/122-fold, 702/461-fold and 454/202-fold, respectively (Fig. 5A). Effects of tested compounds on the expression of *CYP1A* genes displayed inter-individual variability, depending on particular human hepatocyte culture. Stilbenoids A, C and M did not induce *CYP1A* genes in any hepatocyte culture. Compounds B, G, I and H weakly induced *CYP1A* genes, but only in 100 μM concentration and only in one human hepatocyte culture. Weak induction of *CYP1A* in at least 3 human hepatocytes cultures was observed for derivatives E, K and L. Strong, dose-dependent induction of *CYP1A1* and *CYP1A2* mRNAs was caused by stilbenoids D, F and J. Analyses of CYP1A1 and CYP1A2 proteins were performed in three human hepatocytes cultures. Dioxin increased the levels of CYP1A1/CYP1A2 proteins by 22/18-fold, 30/45-fold and 37/22-fold, in human hepatocytes cultures HH71, Hep200525 and Hep200529, respectively (Fig. 5B). Significant induction of CYP1A1 and CYP1A2 proteins was achieved by compounds D, F and J, which is consistent with mRNAs data.

4. Discussion

In the current paper we have described the effects of 13 different stilbenoids (for structures see Fig. 1) on transcriptional activity of AhR and the expression of CYP1A genes in human hepatic cells. In gene reporter assays, the majority of compounds displayed dose-dependent activation of AhR. While activation of AhR by stilbenoids B, F, I, J, K and L reached a plateau, dose-response curve remained still ascendant by compounds A, D, G, H and M in highest tested concentration. In either case, the high (~10^{-5} M) half-

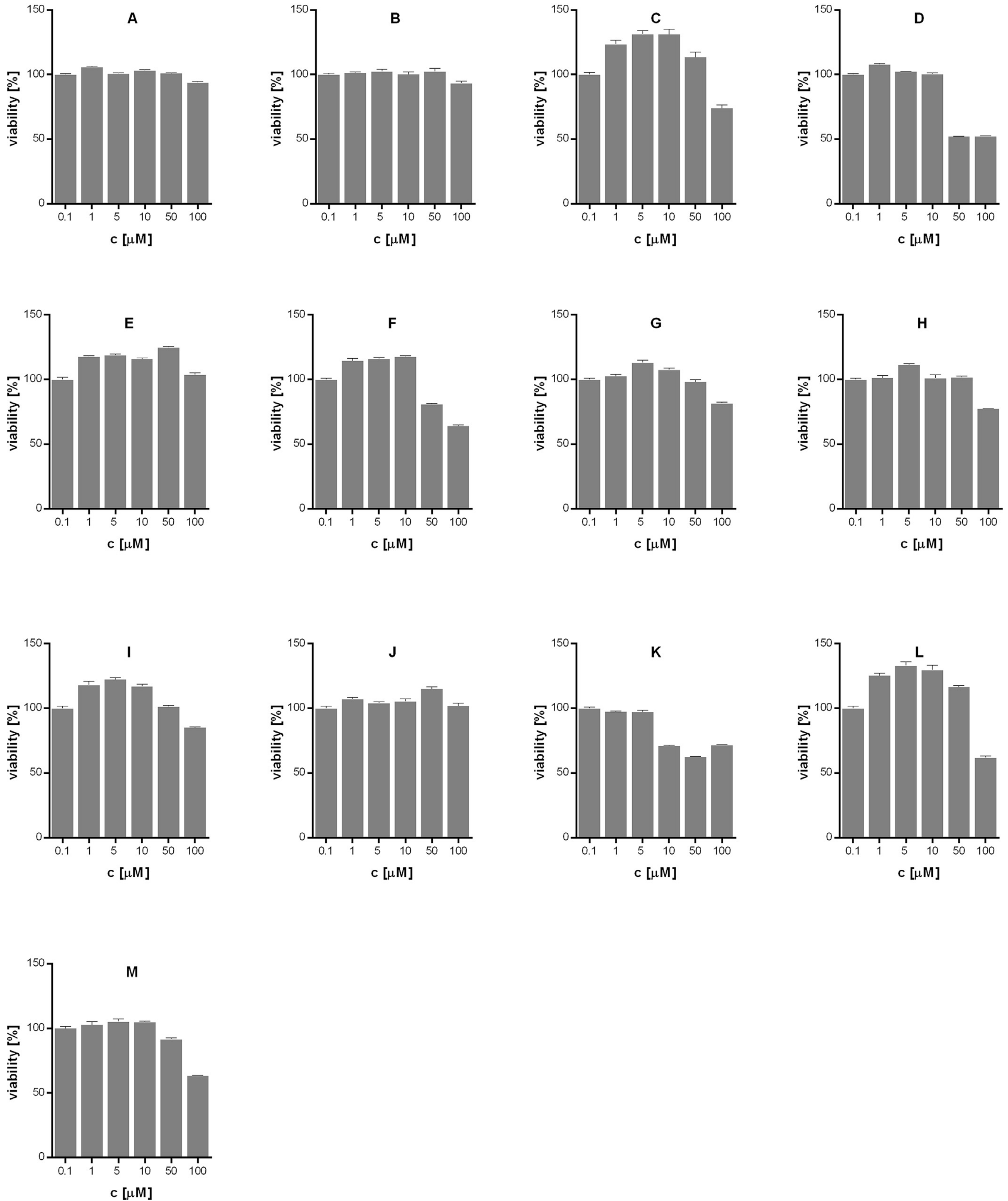
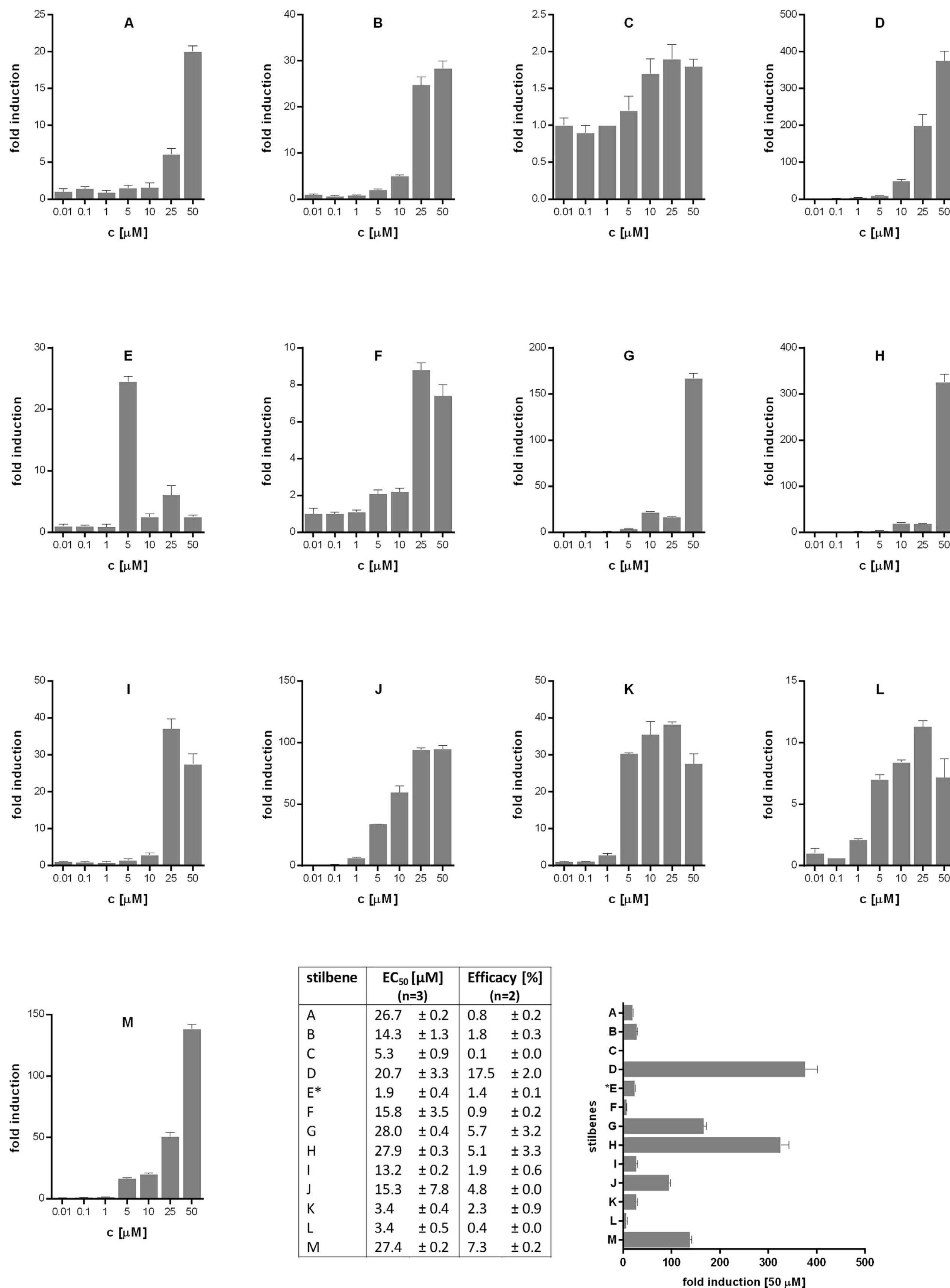
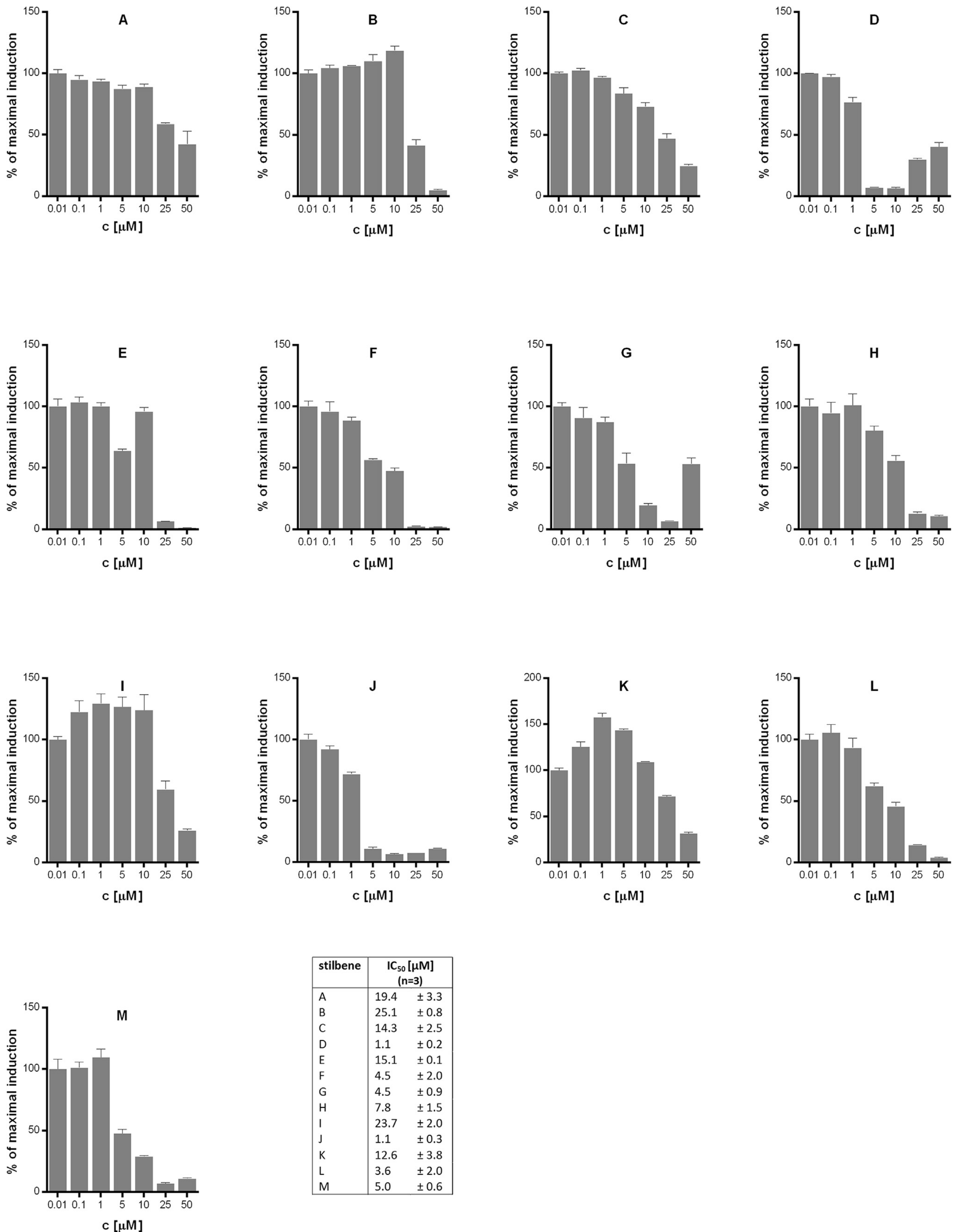


Fig. 2. Effects of stilbenoids on transcriptional activity of human aryl hydrocarbon receptor. AZ-AHR cells were seeded in 96-well plates, stabilized for 16 h, and then incubated for 24 h with tested compounds (A-M) and vehicle (DMSO; 0.1% v/v), in the presence or in the absence of dioxin (TCDD; 5 nM). Treatments were performed in triplicates. The experiments were carried out in three consecutive passages of cells. *Panel A:* A conventional MTT test was performed and absorbance was measured at 540 nm. The data are expressed as a percentage of viability of control cells and they are the mean ± SD from a representative experiment (cell passage). *Panel B:* Agonist mode, i.e. incubations in the absence of TCDD. Cells were lysed and luciferase activity was measured. Data are expressed as a fold induction of luciferase activity over control cells and they are the mean ± SD from a representative experiment (cell passage). Half-maximal effective concentrations EC_{50} were calculated from three independent cell passages and they are shown in inserted table. Inserted horizontal bar graph shows comparative induction efficacies by tested compounds. *Panel C:* Antagonist mode, i.e. incubations in the presence of TCDD. Cells were lysed and luciferase activity was measured. Data are expressed as a percentage of maximal activation attained by TCDD and they are the mean ± SD from a representative experiment (cell passage). Half-maximal inhibitory concentrations IC_{50} were calculated from three independent cell passages and they are shown in inserted table.



stilbene	EC50 [μM] (n=3)		Efficacy [%] (n=2)	
A	26.7	± 0.2	0.8	± 0.2
B	14.3	± 1.3	1.8	± 0.3
C	5.3	± 0.9	0.1	± 0.0
D	20.7	± 3.3	17.5	± 2.0
E*	1.9	± 0.4	1.4	± 0.1
F	15.8	± 3.5	0.9	± 0.2
G	28.0	± 0.4	5.7	± 3.2
H	27.9	± 0.3	5.1	± 3.3
I	13.2	± 0.2	1.9	± 0.6
J	15.3	± 7.8	4.8	± 0.0
K	3.4	± 0.4	2.3	± 0.9
L	3.4	± 0.5	0.4	± 0.0
M	27.4	± 0.2	7.3	± 0.2

Fig. 2. (*continued*).



stilbene	IC_{50} [μM] (n=3)	
A	19.4	± 3.3
B	25.1	± 0.8
C	14.3	± 2.5
D	1.1	± 0.2
E	15.1	± 0.1
F	4.5	± 2.0
G	4.5	± 0.9
H	7.8	± 1.5
I	23.7	± 2.0
J	1.1	± 0.3
K	12.6	± 3.8
L	3.6	± 2.0
M	5.0	± 0.6

Fig. 2. (*continued*).

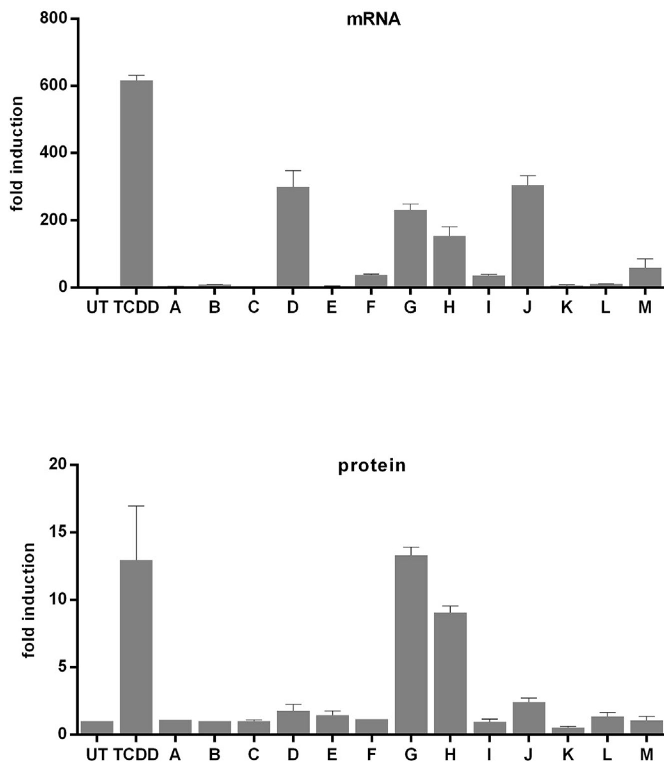


Fig. 3. Effects of stilbenoids on the expression of CYP1A1 in HepG2 cells. HepG2 cells were seeded in 6-wells plates, stabilized 16 h and treated for 24 h (mRNA analyses) and 48 h (protein analyses) with tested compounds (A-M) at concentration 50 μM, vehicle (DMSO; 0.1% v/v) and dioxin (TCDD; 5 nM). The data are the mean ± SD from experiments performed in two consecutive passages of cells and are expressed as a fold induction over DMSO-treated cells. Upper graph shows qRT-PCR analysis of *CYP1A1* mRNA. The data were normalized *per GAPDH* mRNA levels. Lower graph shows CYP1A1 protein analysis by Simple Western blotting by Sally Sue™. The data were normalized *per* β-actin level.

maximal effective concentrations (potencies; EC_{50}) indicate rather indirect effects of stilbenoids on AhR than direct consequence of receptor-ligand complex formation. All tested stilbenoids also displayed antagonist activity on AhR, but the dose-response profiles differed substantially, comprising sigmoid curve (A, B, F, H, J, L, M), inversed U-shaped curve (I, K) or atypical profile (D, G, E). Collectively, gene reporter assays revealed about combined agonist and antagonist effects of the stilbenoids, or their partial agonist effects. No activity was observed for "C", which was the only stilbenoid having saturated ethane backbone instead of unsaturated ethene one. Hence, it seems that the presence of ethene element in stilbenoids structure is necessary for activation of AhR. On the other hand, we did not observe systematic difference between *cis*- or *trans*-forms of stilbenes, with regard to their activity against AhR (isomers pairs A-B, G-H, J-K). Stilbenoid "E" activated AhR only in 5 μM concentration (25-fold induction), whereas "D" was the strongest AhR activator out of all stilbenoids tested. It is interesting, because both compounds are position isomers of *trans*-tetramethoxystilbene (E = 2,4,3′,5′-; D = 3,4,5,4′-). Finally, we observed much higher potency but not efficacy of methoxylated stilbenoids as compared to their hydroxylated counterparts. The examples are "A" *vs* "J" and "B" *vs* "K".

All stilbenoids, with exception of "C", induced CYP1A1 in human hepatoma cells HepG2, and the magnitude of *CYP1A1* mRNA expression was consistent with AhR activation in gene reporter assays. In contrast, induction of CYP1A1 and CYP1A2 in primary cultures of human hepatocytes did correspond with effects of stilbenoids in HepG2 and AZ-AhR cells only for some derivatives. There may be, in particular, two reasons for such inconsistencies: (i) Inter-individual variability – human hepatocytes primary cultures are prepared from liver tissue obtained from donors, therefore, differences in age, sex, race, epigenetic record, physiological and pathophysiological status apply here (Dvorak, 2016). The molecular basis then include differences in the expression and function of phase I/II enzymes, xenobiotics transporters and detoxification regulatory pathways. Concerning current study, the differences between CYP1A induction by stilbenoids are obvious. Therefore, we used five different human hepatocytes culture to ameliorate inter-individual variability and to reliably interpret the data. (ii)

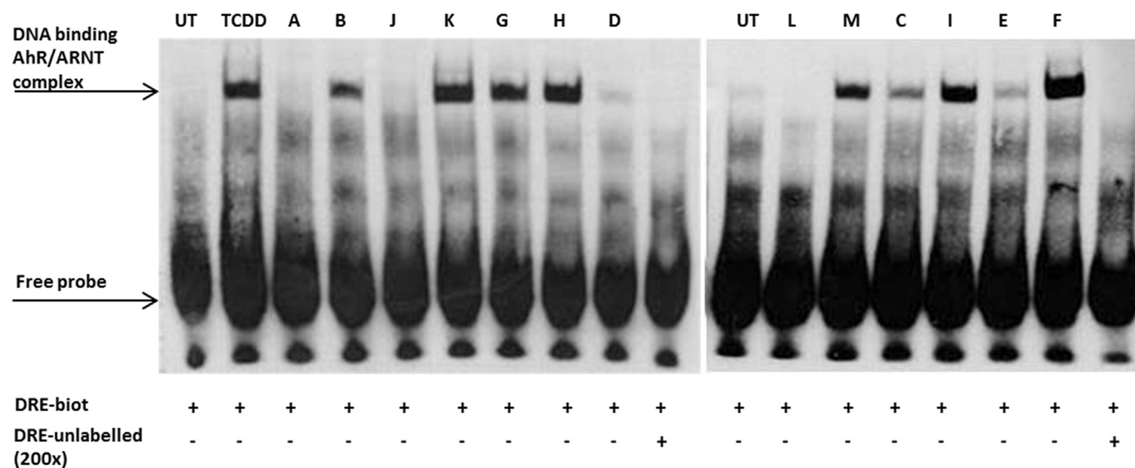


Fig. 4. Effects of stilbenoids on AhR binding to DNA - electrophoretic mobility shift assay. MCF-7 cells at 100% confluence were incubated for 2 h with studied compounds (A-M) at the concentration 50 μM, vehicle as a negative control (UT; 0.1% DMSO v/v) and dioxin as a positive control (TCDD; 5 nM). Nuclear extracts were incubated with a biotin-labeled probe containing dioxin-response element and electrophoresed on 5% polyacrylamide gel. The protein-DNA complexes were electro-blotted to positively charged nylon membrane and detected by streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate. Experiments were performed in four independent cell passages. Representative EMSA is shown.

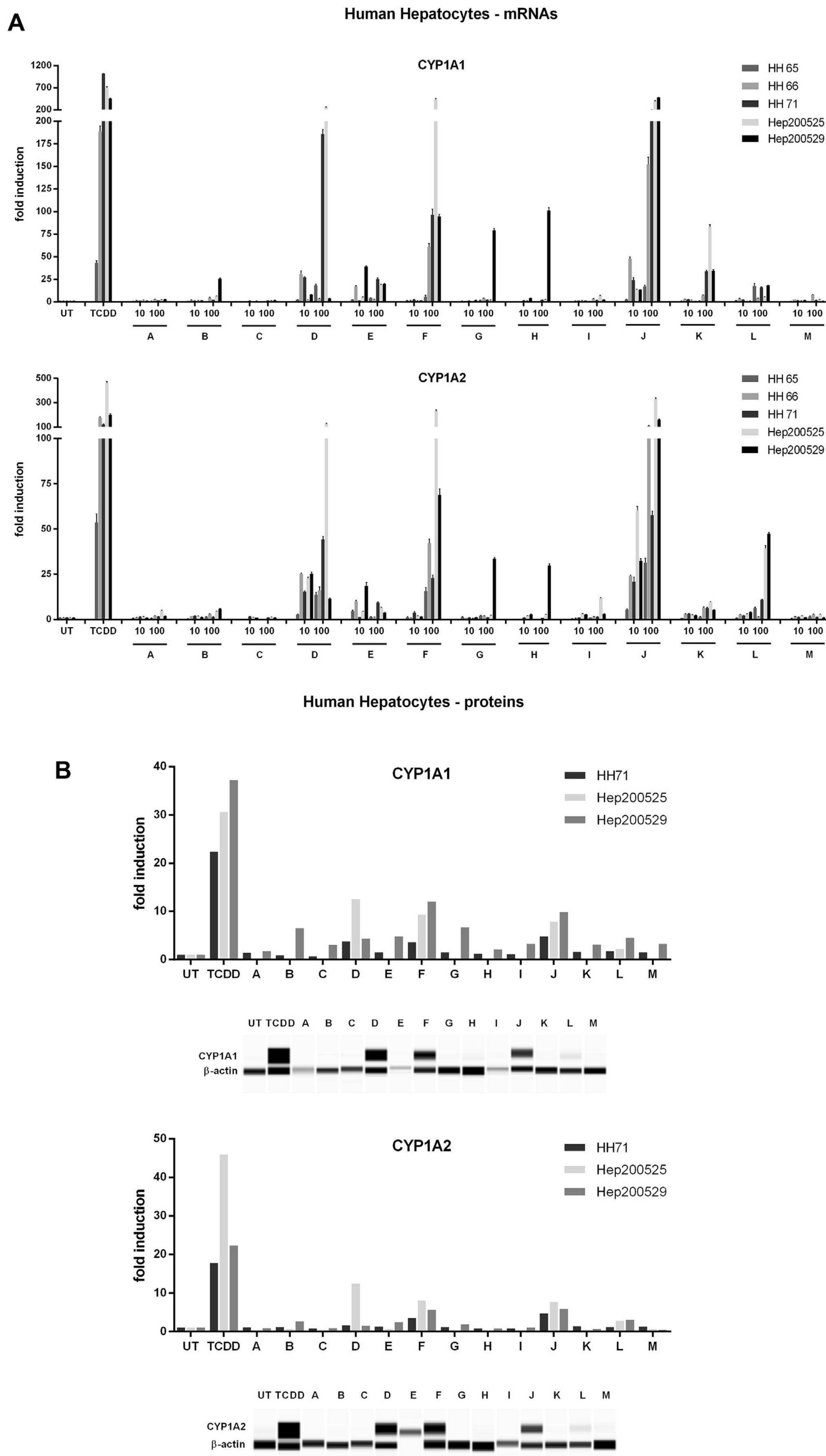


Fig. 5. Effects of stilbenoids on the expression of CYP1A1 and CYP1A2 in primary human hepatocytes. Human hepatocytes from five different liver donors (HH65, HH66, HH71, Hep200525, Hep200529) were used. Cells were incubated for 24 h (mRNA analyses) and 48 h (protein analyses) with tested compounds (A-M), vehicle (DMSO; 0.1% v/v) and dioxin (TCDD; 5 nM). *Panel A:* Bar graphs of qRT-PCR analyses of *CYP1A1* and *CYP1A2* mRNA expression are shown. The data are the mean ± SD from triplicate measurements in each culture and are expressed as a fold induction over DMSO-treated cells. The data were normalized *per GAPDH* mRNA. *Panel B:* Bar graphs show Simple Western blot of CYP1A1 and CYP1A2 proteins. The data are expressed as a fold induction over DMSO-treated cells and were normalized *per* β-actin levels. An illustrative record from SallySue apparatus is inserted in the Figure.

Metabolic equipment – unlike human hepatic cancer cell, primary human hepatocytes are endowed with fully functional and complex metabolic apparatus in terms of intermediary and xenobiotic metabolism. Therefore, molecular effects of tested compounds include also the effects of their phase I and phase II metabolites, which may lead both to activation or inactivation towards particular cellular target of interest (Dvorak, 2016). We observed some robust differences between cancer hepatoma cells and human hepatocytes. The strongest AhR activators and CYP1A1 inducers in AZ-AhR and HepG2 cells, respectively, were stilbenoids D, G, H and J. In human hepatocytes, "D" and "J" remained strong inducers of CYP1A1/CYP1A2, but the effects of "G" and "H" weaned off. Structurally, D/J are fully methoxylated stilbenoids while G/H are fully hydroxylated stilbenoids. The plausible explanation for diminution of G/H effects in human hepatocytes could be an inactivation of G/H by glucuronidation, which takes a place in human hepatocytes and which was described for "G" (Setoguchi et al., 2014). Compound "F" was weak activator of AhR and weak inducer of *CYP1A1* mRNA in HepG2 cells, but very strong inducer of CYP1A1 and CYP1A2 mRNAs and proteins in several human hepatocytes cultures. Structurally, "F" is the only mono-substituted stilbenoid out of 13 derivatives tested. Since hydroxylation of benzene ring is common metabolic pattern of aromatic compounds, it is likely that "F" became CYP1A inducer in human hepatocytes by metabolic activation involving aromatic hydroxylation of benzene rings in "F" molecule.

In conclusion, we demonstrated activation of AhR and induction of CYP1A genes by structurally different stilbenoids, including their *cis-/trans*-forms, hydroxylated and methoxylated derivatives. We observed differences in CYP1A induction in cancer HepG2 cells and in human hepatocytes, due to the metabolic incompetence and competence of the cells, respectively. While there were no systematic differences in effects of *cis-/trans*-forms of stilbenoids, the methoxylated derivatives were more potent AhR activators as compared to their hydroxylated counterparts.

Stilbenoids are food-born compounds that are contained in berries, nuts and grapes. Recent discoveries on the pivotal roles of AhR in human intestinal physiology and immunity (Schiering et al., 2017; Qiu et al., 2012; Li et al., 2011) imply that the data presented here might be of toxicological importance in food perspective and further profound the knowledge in the field.

Conflict of interest

The authors declare that they have no conflict of interest.

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