Induction of expression of aryl hydrocarbon receptor-dependent genes in human HepaRG cell line modified by shRNA and treated with β-naphthoflavone

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Abstract The aryl hydrocarbon receptor (AhR) mediates a variety of biological responses to ubiquitous environmental pollutants. In this study, the effects of administration of β-naphthoflavone (BNF), a potent AhR ligand, on the expression of AhR-dependent genes were examined by microarray and qPCR analysis in both, differentiated and undifferentiated HepaRG cell lines. To prove that BNF-induced changes of investigated genes were indeed AhR-dependent, we knock down the expression of AhR by stable transfection of HepaRG cells with shRNA. Regardless of genetical identity, our results clearly demonstrate different expression profiles of AhR-dependent genes between differentiated and undifferentiated HepaRG cells. Genes involved in metabolism of xenobiotics constitute only minute fraction of all genes regulated by AhR in HepaRG cells. Participation of AhR in induction of expression of genes associated with regulation of apoptosis or involved in cell proliferation as well as AhR-dependent inhibition of genes connected to cell adhesion could support suggestion of involvement of AhR not only in initiation but also in progression of carcinogenesis. Among the AhR-dependent genes known to be involved in metabolism of xenobiotics, cytochromes P4501A1 and 1B1 belong to the most inducible by BNF. On the contrary, expression of GSTA1 and GSTA2 was significantly inhibited after BNF treatment of HepaRG cells. Among the AhR-dependent genes that are not involved in metabolism of xenobiotics SERPINB2, STC2, ARL4C, and TIPARP belong to the most inducible by BNF. Our results imply involvement of Ah receptor in regulation of CYP19A1, the gene-encoding aromatase, and an enzyme responsible for a key step in the biosynthesis of estrogens.

Keywords AhR • CYP1A1 • CYP1A2 • CYP1B1 • CYP19A1 • SERPINB2 • STC2 • ARL4C • TIPARP • GSTA1 • GSTA2

Abbreviations

- AhR: Aryl hydrocarbon receptor
- ARNT: AhR nuclear translocator
- AhRR: AhR repressor
- CYP1A1: Cytochrome P450 1 family, member A1
- CYP1A2: Cytochrome P450 1 family, member A2
- CYP1B1: Cytochrome P450 1 family, member B1
- ER: Estrogen receptor
- STC2: Stanniocalcin 2
- ARL4C: ADP-ribosylation factor-like 4C
- TIPARP: TCDD-inducible poly(ADP-ribose) polymerase
- CCNE2: Cyclin E2
- IL8: Interleukin 8
- NQO1: NAD(P)H quinone oxidoreductase 1
- GSTA2: Glutathione transferase A2
- SLC: Solute carrier family
SCG5 Secretogranin V (7B2 protein)
TMEM156 Transmembrane protein 156
ALDH3A1 Aldehyde dehydrogenase 3 family, member A1
UGT1A1 UDP-glucuronosyltransferase 1 family, member A1
BNF β-Naphthoflavone
3-MC 3-methylcholanthrene
TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin
PAH Polycyclic aromatic hydrocarbon

Introduction

The aryl hydrocarbon receptor is a ligand-dependent transcription factor that mediates a variety of biological responses to ubiquitous environmental pollutants such as polycyclic aromatic hydrocarbons (PAH) and chlorinated dibenzo-p-dioxins [1]. Despite the variability observed between experiments aiming to discover AhR-dependent genes, a small subset of AhR target genes, including CYP1A1, CYP1A2, CYP1B1, NQO1, ALAHS1A1, UGT1A1, and GSTA1, are commonly upregulated following AhR activation. These genes encode phase I and phase II xenobiotic-metabolizing enzymes, which function to metabolize activating compounds and thus provide a vital role in the detoxification of xenobiotics [2–5]. These enzymes metabolize many of their substrates to more soluble and excretable products, but as the classic example of benzo[a]pyrene shows, the same enzymes are also responsible for activation of substrates to ultimate carcinogenic metabolites. This leads to DNA adducts formation, induction of sister chromatid exchanges and carcinogenesis [6–9]. Experiments with knockout animals revealed that PAH-induced carcinogenicity is lost in AhR-deficient mice [10]. Moreover, functional analysis of AhR knockout mice revealed that AhR is involved in lethality, teratogenesis, immunotoxicity, hepatotoxicity, and tumor promotion caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [11–15].

AhR resides in the cytoplasm as a complex with chaperone proteins: HSP90, XAP2, and p23 [16]. The receptor binds to xenobiotics such as β-naphthoflavone (BNF), benzo[a]pyrene, 3-methylcholanthrene, or TCDD with high affinity. Subsequently, the AhR ligand complex translocates to the nucleus, where after dissociation of chaperone proteins, it binds to AhR nuclear translocator (ARNT) protein [17, 18]. Then, the liganded AhR/ARNT heterodimer binds to xenobiotics responsive element sequences (XRE), which constitute enhancer DNA elements present in the 5’-flanking region of target genes. Elevated expression of target genes leads to altered metabolism, which often results in enhanced carcinogenesis and toxicity [19]. Activation of procarcinogenic PAHs to ultimate carcinogens by AhR regulating enzymes is traditionally considered as the first step in tumor initiation. On the other hand, numerous studies have shown that the AhR plays a role not only in tumor initiation but also in promotion and progression [20, 21]; however molecular mechanisms involved in these processes are not fully understood. Some pleiotropic effects of AhR activation could be partially explained by cross-talk with other signal transduction pathways. The ability of AhR agonists to interfere with multiple signal transduction pathways, including those regulated by nuclear receptors, has been reported by many laboratories and involves multiple mechanisms [22–26]. Although the best studied AhR-responsive genes produce enzymes involved in xenobiotics metabolism, gene expression profiling studies have identified a large number of other genes that are induced or repressed in an AhR- and ligand-dependent manner [27–33]. But a majority of discussed studies were accomplished with application of laboratory animals. However, substantial differences in regulation of AhR-dependent genes between human and mouse were reported [34].

The present study was therefore designed to investigate the role of AhR in BNF-regulated gene expression in HepaRG cells. BNF, a well-known AhR agonist [35], is a widely used inducer of phase I and phase II enzymes in xenobiotic metabolism which is considered as not being carcinogenic unlike majority of other PAHs [36, 37]. BNF has been also shown to suppress chemical carcinogenesis at numerous sites in mice [38]. In this report, we examined the microarray-based expression profiles of AhR-dependent genes. HepaRG cells, derived from a human hepatocellular carcinoma, exhibit unique features: when seeded at low density, they acquire an elongated undifferentiated morphology and actively divide, but after having reached confluency in the presence of DMSO, they form typical hepatocyte-like colonies surrounded by biliary epithelial-like cells. Moreover, contrary to other human hepatoma cell lines, HepaRG cells express various CYPs and the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) at levels comparable to those found in cultured primary human hepatocytes. They also express other genes with various functions, such as phase 2 enzymes, solute carrier transporters, albumin, haptoglobin as well as aldolase B that is a specific marker of adult hepatocytes [39]. The expression of AhR-dependent genes was found to be similar in highly differentiated HepaRG cells and in primary human hepatocytes [40]. Likewise, our earlier experiments revealed a diverse expression of some AhR-dependent genes in undifferentiated and differentiated HepaRG cells [41]. It was demonstrated that AhR was highly expressed in developing fetal liver of mouse embryo and presumably involved in liver development [42]. However, different cell types were involved in AhR-dependent development of liver and in AhR-dependent hepatotoxicity [43]. It seems to be plausible
that undifferentiated HepaRG cells are equivalent to cells from fetal liver of mouse embryo, whereas differentiated ones resemble matured hepatocytes. Therefore, HepaRG cell line gives us opportunity to investigate AhR-dependent regulation of genes in the cells with identical DNA but committed to diverse development programs. Consequently, we decided to compare the expression profiles of AhR-dependent genes in both stages of HepaRG cell differentiation. To prove that BNF-induced changes of investigated genes were indeed AhR-dependent, we knocked down the expression of AhR by stable transfection of HepaRG cells with shRNA. Quantitative PCR of the most interesting candidate genes was performed to validate the microarray results.

Materials and methods

Chemicals

BNF, SYBR® Green I (10,000× concentration), agarose, JumpStart Taq DNA polymerase, Enhanced Avian RT first-strand synthesis kit (STR-1), GenElute™ PCR Clean-Up Kit, GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit, PCR Low Ladder Marker Set, guanidine thiocyanate, ammonium thiocyanate, Williams’ E medium, LB broth, and LB agar were supplied by Sigma-Aldrich Co (St. Louis, MO, USA). Fluorescin was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Restriction endonucleases were purchased from Fermentas International Inc. (Burlington, Canada). Deoxyribonucleotide triphosphates such as dATP, dGTP, aCTP, and dTTP were provided by Roche Diagnostics (Mannheim, Germany). PCR primers were provided by Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland (oligo.pl), and Genomed, Poland. Agilent RNA 6000 Reagents were provided by Agilent Technologies (Santa Clara, CA, USA). Affymetrix Human Genome U219 Array Strip, GeneChip 3’IVT Express Kit and GeneAtlas Hybridization, and Wash and Stain Kit for 3’IVT Arrays were provided by Affymetrix (Santa Clara, CA, USA). GeneClip™ U1 Hairpin Cloning System—Neomycin Vector and antibiotic G418 (Geneticin)—was provided by Promega (Madison, WI, USA). Lipofectamine 2000 and Opti-MEM® I Reduced Serum Medium was provided by Invitrogen (Carlsbad, CA, USA). All the other compounds were readily available as commercial products.

HepaRG cell line and BNF treatment

HepaRG cells were obtained from Biopredic Ltd. (Rennes, France). The procedures of plating and maintaining HepaRG cells were described previously [44]. In brief, HepaRG cells were cultured in 25-cm² flasks (37 °C, 5% CO₂) either in Williams’ E medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine, and 5 × 10⁻⁵ M hydrocortisone hemisuccinate (undifferentiated cells) or, after reaching full confluence in differentiation medium corresponding to the above one, but supplemented with 2% of DMSO (differentiated cells).

HepaRG cell line was treated with BNF dissolved in DMSO to a final concentration of 100 μM in medium (8 μl 50 mM BNF/4 ml medium in 25-cm² flask; 0.2% of DMSO) for 24 h. Appropriate amount of solvent (DMSO) were added to control, untreated cells.

GeneClip hairpin oligonucleotide design and transformation of Escherichia coli

Selection of siRNA hairpin target sequence to AhR mRNA (NM_001621) was achieved by using siDESIGN Center (http://dharmacon.gelifesciences.com/design-center/?redirect=true) and the rules from technical manual from GeneClip™ U1 Hairpin Cloning System (Promega). We have designed four different siRNA targets, from which the best one was selected for the subsequent analysis. Selected oligonucleotides (siRNA target underlined) purchased from “oligo.pl” were as follows: forward-5’-TCTCGAACAGAGCATTTACGAAATTCAGATTTCGTAATGCTCTGTTCCT-3’ and reverse-5’-CTGCAGGACACAGTCACTTTACGAAATTCCTTGATACCTCTGTTC-3’. Sequences were hybridized and ligated to pGeneClip neomycin vector construct according to the manufacturer’s instruction. Further, One Shot® TOP10 competent E. coli cells (Invitrogen) were transformed with the vector and cloned. The pGeneClip vector was isolated back from bacteria by GenElute™ HP endotoxin-free plasmid maxiprep kit and digested with PstI to confirm the presence of an appropriate insert.

As a negative control for RNA interference, a non-specific target sequence (scrambled) was used. Non-specific sequence was designed, ligated to plasmid, and multiplied into bacteria in the same way as presented above. Sequences of non-specific oligonucleotides were as follows: forward-5’-TCTCGATAGGCGATGACATATTTCGAGAAATATGTCGATGCCTACTACCT-3’ and reverse-5’-CTGCAGGATGCTGCTGGACTTTGAATATGTCGATGCCTACTAC-3’. This non-specific sequence was not complementary to any known human, rat, and mouse sequence.

Transfection of pGeneClip vector to HepaRG cells

Stable transfection of pGeneClip neomycin vector construct to HepaRG cells was carried out according to the
suggestions from GeneClip™ U1 Hairpin Cloning System’s technical manual (Promega). We have applied lipofection with Lipofectamine 2000 (Invitrogen) as transfection method. Cell line cultures were grown as monolayer in 500 μl of Williams’ E medium supplemented with 10% FCS (without standard antibiotics) to reach about 90% confluence of flask bottom (2 cm²). Liposomes were prepared by mixing 0.8 g plasmid DNA diluted in 50 μl Opti-MEM medium with 2 μl of Lipofectamine 2000 in 50 μl of Opti-MEM. Following incubation for 20 min, DNA-lipid complexes (100 μl) were added to HepaRG cells. Cells were passaged at 1:10 dilution into fresh growth medium with standard antibiotics 24 h after transfection. Selective antibiotic G418 was added to the medium 3–4 days later, when cells reached about 50% confluence.

RNA isolation

Total RNA was isolated directly from monolayer cells in culture dish as described before [45]. The extracted total RNA dissolved in water was quantified spectrophotometrically at 260 nm (A260; NanoDrop). The A260/280 ratio > 1.9 was considered as an acceptable measurement of RNA purity. RNA integrity was estimated by BioAnalyzer 2100 analysis (Agilent, RIN: 9.40–9.80). The amount of cDNA synthesized in a single reaction was sufficient to PCR-amplify all genes.

Microarray-based gene expression analysis and statistics

Expression analysis was performed using Human Genome U219 array (Affymetrix) in duplicate biological replicates of each sample type.

RNA preprocessing

cDNA was synthetized in two steps, namely first-strand synthesis and second-strand synthesis, respectively, using Affymetrix GeneChip® 3’IVT Express Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s instructions. Biotin-labeled cRNA synthesis (IVT Labeling) and cRNA fragmentation were performed by Affymetrix GeneChip® kit reagents according to the procedure described in the Affymetrix GeneAtlas™ 3’IVT Express Kit’s technical manual.

Target hybridization and scanning

Biotin-labeled and fragmented target cRNA samples were loaded into Affymetrix GeneChip® (Human Genome U219) Array Strip together with control cRNAs and oligo B2. Hybridization procedure was conducted at 45 °C, for 16 h in AccuBlock™ Digital Dry Bath (Labnet international, Inc.) hybridization oven. Washing and staining procedure was performed in Affymetrix GeneAtlas™ Fuvides Station according to the instructions in the technical manual. Affymetrix GeneAtlas™ Imaging Station was used for scanning the arrays.

Data analysis and preparation of gene lists

Preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. The quality of gene expression data was checked according to quality control criteria provided by the software. Then, Partek® Express™ Software (Partek, Inc., Chesterfield, MO, USA) was used for further data analysis and evaluation. Using quality control checkpoints and statistical analysis of gene fold-change significances, table of the most important changes in gene expression was created. Next, generated table was imported to Pathway Studio® Explore (Ariadne Genomics, Rockville, MD, USA) where right statistical analyses were made. To evaluate the P value indicating the significance of the enrichment score, nonparametric Mann–Whitney statistical test was used (α = 0.05). Venn diagrams were calculated and drawn using online software tool—(http://bioinformatics.psb.ugent.be/webtools/Venn/). Selected list of genes was further analyzed by online DAVID functional annotation tools (https://david.ncifcrf.gov/home.jsp) [46].

Real-time PCR-based gene expression analysis and statistics

Expression analysis was performed using two-step quantitative real-time PCR with SYBR® Green I chemistry in triplicate biological replicates of each sample type.

cDNA synthesis for real-time PCR

Eight micrograms of total RNA was reverse-transcribed to cDNA in a total volume of 40 μl, using Enhanced Avian RT first-strand synthesis kit (Sigma-Aldrich Co., St. Louis, MO, USA) according to the manufacturer’s instruction. Random nonamers were used as primers of the reaction. The amount of cDNA synthesized in a single reaction was sufficient to PCR-amplify all genes (targets and standards).

Primer design for real-time PCR

PCR primers were designed according to published genes sequences (GenBank, accession numbers in Table 1) with the Beacon Designer™ software (PRIMER Biosoft International) and their specificity was verified with BLAST alignment search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Primers and/or amplicons were designed to cross the exon/exon boundaries (Table 1). To confirm amplification of the expected size fragment, amplification products were characterized by agarose gel electrophoresis. Identity of amplicons was further verified by the analysis of digestion products generated by restriction endonucleases (not shown). Primers for reference genes and other genes used herein were published earlier [41].

Real-time PCR
cDNA of investigated genes was amplified by real-time PCR in the iCycler iQ5 real-time PCR detection system with iQ5 optical system software 2.0 (Bio-Rad Laboratories; Hercules, CA, USA) using SYBR® Green I as the detection dye. Amplification was carried out in a total volume of 20 μl containing 0.2x SYBR® Green I, PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 3.5 mM MgCl2, 10 nM fluorescein, 0.2 μM each primer, 0.2 mM each dNTPs, 0.5 U JumpStart Taq DNA polymerase, and 0.4 μl cDNA (undiluted reverse-transcription product derived from 8 μl RNA in 40 μl reaction). The reactions were cycled 40 times using the following parameters: 95 °C for 10 s, 52–60 °C for 5–15 s (Table 1 plus [41]), and 72 °C for 15–20 s during which the fluorescence data were collected. At the end of the PCR, a melting curve was generated by heating the samples from 50 to 95 °C in 0.5 °C increments with a dwell time at each temperature of 10 s to verify the specificity of the product. Nontemplate controls were run with every assay and no indication of PCR contamination was observed. Lack of PCR products from the nonreverse transcribed RNA control indicated that possible contamination of the genomic DNA has not served as an amplification template.

Quantitative PCR data analysis and statistics
Expression levels of the target genes were normalized with respect to two reference genes, β-actin and ARNT, using relative quantification method. The β-actin expression was reported as not affected by treatment of rats with TCDD [47]. The ARNT mRNA levels were not altered as the result of in vivo treatment of rats with TCDD, 3-MC, and BNF [48]. Similar observation was reported by Franc et al. [49] with rats exposed to TCDD. All calculations were performed using Gene Expression Macro™ 1.10 software (Bio-Rad Laboratories, CA, USA).

To determine the limit of detection and the efficiency of PCR amplification of reference and target genes, dilution series (1:5 dilution) of PCR products were prepared. PCR products (about 200 μl) were purified using EZ-10 Spin Column PCR purification Kit (Bio Basic Inc., Canada). Concentration of DNA was determined spectrophotometrically (NanoDrop) and the number of copies of a template was calculated using online software (http://www.uri.edu/research/gsc/resources/cndna.html). Molecular biology-

### Table 1 Sequence of primers used in real-time PCR, amplicon sizes, annealing temperatures, and the amplification efficiencies

| Target’s Accession No. | Sequences | Amplicon length (bp) | Annealing Tm(°C)/t(s) | PCR efficiency |
|------------------------|-----------|---------------------|-----------------------|---------------|
| SERPINB2 NM_002575     | F: 5’ GAAGCTGTCTACTTCAA 3’<br>R: 5’ TCTTCTATGTATCCAATGTT 3’  | 147-i  | 55/15  | 0.99  |
| SLC7A5 NM_003486       | F: 5’ G-GTGATGTGTCCCAATCTA 3’<br>R: 5’ AAGTAATTC-CATCCTCCATA 3’  | 116  | 56/15  | 1.00  |
| SLC14A1 NM_015865      | F: 5’ GACATTACAATCCATTCT 3’<br>R: 5’ ATTACACAGCCCATAGT 3’  | 140-i  | 52/15  | 0.96  |
| CCNE2 NM_057749        | F: 5’ GTCTCTCTACTTATTCTCT 3’<br>R: 5’ AGACACCTGACATTTCT 3’  | 114-i  | 55/10  | 0.98  |
| TIPARP NM_015508       | F: 5’ CTGTCTTGCCATATCATT 3’<br>R: 5’ ATTCTGTGCC-TCCATACT 3’  | 144  | 55/10  | 0.96  |
| STC2 NM_003714         | F: 5’ CAACTCTTTGAGATCCG 3’<br>R: 5’ TACATTTCGCCGCTTCT 3’  | 110-i  | 58/15  | 0.91  |
| SLC14A1 NM_003486      | F: 5’ G-TTCTCTACTTATTCTCT 3’<br>R: 5’ TTCTTATCCTCTCAGA 3’  | 138-i  | 56/15  | 0.93  |
| TMEM156 NM_024943      | F: 5’ G-TTCTATACGGAGGAGAT 3’<br>R: 5’ ATGCAGGGTAGGTATGTTATCC 3’  | 123  | 56/15  | 0.94  |
| GSTA2 NM_000846        | F: 5’ CCCTACTCCATTATGAC 3’<br>R: 5’ CCATCAATCTCAACCATT 3’  | 165-i  | 58/15  | 0.94  |

The hyphen in the primer sequence denotes the exon/exon boundary. Letter “i” after the amplicon length indicates that exon/exon boundary was inside the amplified sequence.
grade tRNA from *E. coli* (100 ng/µl) was used as a carrier during dilutions. Each dilution was amplified in triplicate by real-time PCR and the obtained quantification cycle (Cq) values were used to construct a graph Cq vs. log10 of the number of template copies. The slope of the graph was used to determine the reaction efficiency according to the formula: Efficiency = \[10^{(-1/\text{slope})} - 1\]. The efficiencies of target and reference genes amplification are shown in Table 1. Limit of detection defined as minimal number of DNA copies that can be detected with reasonable certainty was estimated as 10 copies per single PCR reaction (Cq about 33–36) for reference and target genes. We were capable to observe amplification even in a single template copy PCR reaction, but due to stochastic processes such detection was rather qualitative than quantitative.

The relative gene expression was calculated for the triplicate samples derived from each RT reaction by Gene Expression MacroTM 1.10 (Bio-Rad) software. The average of the three values was carried forward as the value to be entered into calculation of the mean ± SD for each treatment group.

Statistical significance of differences was assessed by one-way ANOVA followed by Tukey–Kramer post-test (Table 8). All calculations were done using GraphPad Prism for Windows version 6.01 (GraphPad Software, San Diego CA); \(P \leq 0.05\) was considered statistically significant.

### Experimental design

We have used the expression microarrays and real-time PCR to study the transcriptional response to 100 µM BNF in transfected control and AhR(−) HepaRG cells. Control HepaRG cell line was stably transfected with unspecific construct, whereas AhR(−) HepaRG line was transfected with construct silencing *AhR* expression by RNA interference. Experiments were performed on both, undifferentiated and differentiated HepaRG cell lines (Table 2). The effects of BNF on the expression of AhR-dependent genes were evaluated 24 h after administration of BNF. Solvent-treated, AhR(−)-transfected HepaRG cells, both undifferentiated and differentiated, were analyzed only by real-time PCR.

### Results

**Effects of AhR silencing on BNF-induced mRNA expression of target genes in undifferentiated and differentiated HepaRG cells**

AhR-dependent induction of expression of numerous genes was observed after BNF treatment of HepaRG cells. A list of 20 most inducible genes by BNF treatment, as determined by microarray-based gene expression analysis, in HepaRG undifferentiated and differentiated cells is presented in Tables 3 and 4, respectively. Only AhR-dependent effects of BNF treatment were further analyzed in presented publication, i.e., such effects which were significantly suppressed after reduction of *AhR* expression by RNA interference. Quantitative PCR analysis revealed that *AhR* mRNA expression was reduced after transfection of the silencing vector by about 77 and 89% in undifferentiated and differentiated HepaRG cells, respectively (Table 8). Similar results can be calculated from microarrays (see supplementary material). It is always a matter of investigator’s arbitrary choice, how strong effect should be to balance the specificity and the sensitivity of the analysis. Statistically significant induction of expression of 66 genes in undifferentiated cells and induction of expression of 40 genes in differentiated cells was observed after BNF treatment, when twofold effect was chosen as cutoff point (full list of induced genes in supplementary material). The resulting gene sets were compared using Venn diagram analysis in order to examine overlaps among the different gene sets, indicating on 21 genes mutually induced twofold or more in undifferentiated as well as differentiated HepaRG cells (Fig. 1). However, such stringent criterion eliminated most of genes involved in metabolism of xenobiotics and previously recognized as AhR-dependent. The expression of only two genes from classical AhR-dependent battery of genes, namely genes encoding cytochromes P450- *CYP1A1* and *CYP1B1*, were induced more than twofold by BNF treatment. When cutoff point was reduced to 1.5-fold, 188 and 154 genes were upregulated in undifferentiated and differentiated HepaRG cells, respectively. Only 70 genes coexist on both lists (Fig. 1 and supplementary). However, most of genes from AhR-dependent battery of genes are presented on the list this time.

### Table 2 Summary of the experimental design applied to analyze gene expression by microarray and qPCR

| Type of cells | Transfected vector | Treatment |
|--------------|--------------------|-----------|
| HepaRG undifferentiated | Negative control | DMSO       |
| HepaRG undifferentiated | Negative control | BNF       |
| HepaRG undifferentiated | *AhR*(−) | DMSO       |
| HepaRG undifferentiated | *AhR*(−) | BNF       |
| HepaRG differentiated | Negative control | DMSO       |
| HepaRG differentiated | Negative control | BNF       |
| HepaRG differentiated | *AhR*(−) | DMSO       |
| HepaRG differentiated | *AhR*(−) | BNF       |

The negative control shRNA is a scrambled artificial sequence which does not match any human gene. The *AhR*(−) shRNA is a sequence which decreases the expression of *AhR* mRNA by RNA interference. Specimens in italic were analyzed only by qPCR.
Further reduction of cutoff point, below 1.5-fold effect, resulted in substantial increase of casual results, and so was generally omitted in data analysis. It is worth to notice that the expression of another gene from cytochrome P450 family, namely \textit{CYP19A1}, encoding aromatase, was induced more than 1.5-fold by BNF treatment, but only in undifferentiated HepaRG cells (supplementary material). The induction was AhR-dependent. Nonetheless, the expression of \textit{CYP19A1} was slightly or not induced after BNF treatment of differentiated HepaRG cells. But alike as in undifferentiated cells, the expression of \textit{CYP19A1} mRNA in differentiated ones was significantly reduced after silencing of AhR (see supplementary material). Functional analysis of genes selected by Venn diagram was performed using DAVID online tools. From 70 genes induced by BNF treatment in AhR-dependent way in both—differentiated and undifferentiated HepaRG cells—as much as ten appear to have connection with regulation of apoptosis and seven is involved in cell proliferation. It is worth to mention that the expression of as much as five genes from solute carrier family of transporters (SLC) was induced by BNF treatment in AhR-dependent manner. The remaining genes are involved in numerous different biological pathways, without obvious domination of one of them.

### Effects of AhR silencing on BNF-inhibited mRNA expression of target genes in undifferentiated and differentiated HepaRG cells

A list of 20 most inhibited genes by BNF treatment, as determined by microarray-based gene expression analysis, in HepaRG undifferentiated and differentiated cells is presented in Tables 5 and 6, respectively. Statistically significant reduction of expression of 76 genes in undifferentiated cells and reduction of expression of 65 genes in differentiated cells was observed after BNF treatment, when twofold effect was chosen as cutoff point (Fig. 2, full list of inhibited genes in supplementary material). Expression of 25 genes was reduced simultaneously in undifferentiated as well as in differentiated cells. When cutoff point was reduced to 1.5-fold, 255 and 198 genes

| Table 3 Top 20 most inducible by BNF treatment and AhR-dependent genes as determined by microarray-based gene expression analysis in HepaRG undifferentiated cells |
|-----------------------------------------------|
| Gene (symbol) | Accession No. | BNF induction (contr.BNF/contr. DMSO) | AhR knockdown (AhR(−) BNF/contr. BNF) |
|-----------------------------------------------|
| CYP1A1 | NM_000499 | 84.4479 | 1.96E-09 | −2.26041 | 4.43E-05 |
| SERPINB2 | NM_002575 | 38.9277 | 5.37E-10 | −1.74217 | 3.88E-05 |
| TMEM156 | NM_024943 | 17.0552 | 1.31E-06 | −4.25365 | 6.58E-05 |
| CYP1B1 | NM_000104 | 9.99312 | 6.71E-06 | −4.8724 | 0.000114 |
| TIPARP | NM_015508 | 9.97469 | 6.29E-07 | −3.38236 | 2.62E-05 |
| TAC1 | NM_003182 | 7.52859 | 2.97E-06 | −5.1163 | 1.03E-05 |
| SLC7A11 | NM_014331 | 6.84783 | 9.66E-07 | −1.89915 | 0.000527 |
| SLC7A5 | NM_003486 | 6.65738 | 5.53E-06 | −5.4473 | 6.80E-06 |
| IGFBP1 | NM_000596 | 6.23676 | 7.86E-08 | −1.89982 | 3.77E-05 |
| SCG5 | NM_003020 | 5.92127 | 5.02E-06 | −5.22126 | 7.71E-06 |
| SLC37A2 | NM_198277 | 5.91685 | 2.40E-06 | −2.79372 | 5.83E-05 |
| AMIGO2 | NM_181847 | 5.36008 | 1.15E-05 | −3.69017 | 4.90E-05 |
| SLC14A1 | NM_015865 | 5.29511 | 1.02E-05 | −17.1535 | 4.38E-07 |
| EREG | NM_001432 | 4.41706 | 2.30E-05 | −1.89436 | 0.002332 |
| ARL4C | NM_005737 | 3.87399 | 3.59E-07 | −1.30965 | 0.003173 |
| PXK | NM_017771 | 3.8716 | 1.52E-07 | −2.37851 | 2.14E-06 |
| HMGA2 | NM_003484 | 3.81293 | 1.82E-07 | −1.44529 | 0.000326 |
| HK2 | NM_000189 | 3.63351 | 4.47E-05 | −2.41403 | 0.000378 |
| STC2 | NM_003714 | 3.52418 | 8.44E-07 | −1.67544 | 0.00015 |
| KYNU | NM_003937 | 3.39497 | 2.82E-06 | −1.89998 | 0.000117 |

Information of all additional genes out of top 20 is available in the supplementary material accompanying of the manuscript (Supplementary 2—induction)

Fold-change value that was less than 1 has been replaced by the negative of its inverse (for example, 0.1 was replaced by −10)

\textit{Contr.} HepaRG cells transfected with control plasmid, \textit{AhR}− HepaRG cells transfected with plasmid knocking down Ah receptor
were downregulated after BNF treatment in undifferentiated and differentiated HepaRG cells, respectively. Expression of 94 of them was reduced concomitantly in both stages of HepaRG differentiation (Fig. 2 and supplementary). Interestingly, the expression of GSTA1 and GSTA2 was downregulated after BNF treatment in AhR-dependent manner (Table 6). Functional analysis of 94 genes selected by Venn diagram revealed that ten of

Table 4 Top 20 most inducible by BNF treatment and AhR-dependent genes as determined by microarray-based gene expression analysis in HepaRG differentiated cells

| Gene (symbol) | Accession No. | BNF induction (contr.BNF/contr. DMSO) | AhR knockdown (AhR(−) BNF/contr. BNF) |
|---------------|---------------|--------------------------------------|----------------------------------------|
|               |               | Fold-change | P value     | Fold-change | P value     |
| SERPINB2      | NM_002575     | 19.263      | 1.93E-09    | −11.1499    | 6.56E-09    |
| CYP1A1        | NM_000499     | 10.9253     | 7.89E-08    | −2.76109    | 1.25E-05    |
| STC2          | NM_003714     | 7.26696     | 3.70E-07    | −1.98847    | 0.000175    |
| ARL4C         | NM_005737     | 6.70831     | 4.72E-08    | −3.32309    | 7.33E-07    |
| TIPARP        | NM_015508     | 6.0173      | 2.73E-06    | −6.40034    | 2.24E-06    |
| SCG5          | NM_003020     | 5.95549     | 4.92E-06    | −14.4314    | 4.56E-07    |
| CYP1B1        | NM_000104     | 4.76405     | 6.34E-05    | −9.085      | 8.60E-06    |
| SLC37A2       | NM_198277     | 4.33837     | 4.18E-07    | −4.1171     | 5.19E-07    |
| SLC7A5        | NM_003486     | 3.50711     | 3.89E-05    | −3.25045    | 5.57E-05    |
| BMPER         | NM_133468     | 3.4992      | 4.40E-05    | −5.24619    | 8.70E-06    |
| SYNJ2         | NM_003898     | 3.41386     | 1.11E-06    | −2.14635    | 1.79E-05    |
| SLC14A1       | NM_015865     | 2.61341     | 0.000938    | −19.6687    | 1.51E-06    |
| GDF15         | NM_004864     | 2.5802      | 0.000948    | −3.18849    | 0.000321    |
| KIFC3         | NM_005550     | 2.5573      | 8.61E-06    | −1.62367    | 0.000369    |
| UGC6          | NM_003358     | 2.55685     | 1.05E-05    | −2.60471    | 9.33E-06    |
| ATF3          | NM_004024     | 2.52517     | 3.11E-05    | −1.33103    | 0.013789    |
| PXK           | NM_017771     | 2.51771     | 7.21E-07    | −3.20378    | 1.82E-07    |
| MYADM         | NM_138373     | 2.5164      | 2.96E-05    | −1.36498    | 0.009073    |
| SSH1          | NM_018984     | 2.49408     | 0.000607    | −2.37291    | 0.000818    |
| IL8           | NM_000584     | 2.47773     | 1.87E-05    | −1.80874    | 0.000211    |

Information of all additional genes out of top 20 is available in the supplementary material accompanying of the manuscript (Supplementary 2—induction)

Fold-change value that was less than 1 has been replaced by the negative of its inverse (for example, 0.1 was replaced by −10)

Contr. HepaRG cells transfected with control plasmid, AhR HepaRG cells transfected with plasmid knocking down Ah receptor

![Fig. 1 Venn diagram representation](image)
them appeared to be connected with cell adhesion, five of them are engaged in formation of anchoring junction, and another five are connected with response to steroid hormone stimulus. The remaining genes are dispersed between numerous different biological pathways.

Diverse, dependent on the stage of cell differentiation, effects of AhR silencing and BNF treatment on mRNA expression of some target genes

If Ah receptor is responsible for BNF-related induction of appropriate genes, someone could expect that silencing of AhR would reduce such induction. Indeed, expression of most of the analyzed genes followed this pattern (Table 3). However, expression of several genes seems not to follow such simplified rules. Two examples of such genes are presented in Table 7. Expression of the first one, cyclin E2 \((CCNE2)\), was significantly induced by BNF treatment of undifferentiated HepaRG cells. However, silencing of Ah receptor not only did not counteract such induction, but also further increased the expression of AhR-silenced, BNF-treated cells as compared to control BNF-treated undifferentiated HepaRG cells. This paradoxical effect was statistically significant and indicates the involvement of AhR. Distinct to above, but consistent with expectation, pattern of \(CCNE2\) expression was observed in differentiated HepaRG cells. BNF treatment slightly induced \(CCNE2\) mRNA expression and AhR silencing significantly reduced this induction this time as well (Table 7). Two distinct, complementary to \(CCNE2\) mRNA probe sets are placed on Affymetrix U219 array chip. Each one consists of eleven 25 base oligomers spanning the region of 818–1299 bp (set 11728301_at) and 2137–2636 bp (set 11728300_at) of the reference mRNA sequence (NM_057749). The results of cDNA hybridization to both probe sets were consistent to one another (Table 7) and are also supported by qPCR expression analysis (Table 8). DNA region amplified by quantitative real-time PCR was localized between 888 and 1001 base of NM_057749 sequence. Likewise, the expression of the second depicted gene, interleukin 8 \((IL8)\), followed very similar pattern to \(CCNE2\) one. The results of cDNA hybridization to all three \(IL8\) probe sets were consistent to one another and indicated on differences between differentiated and undifferentiated HepaRG cells (Table 7).

### Table 5

Top 20 most inhibited by BNF treatment and AhR-dependent genes as determined by microarray-based gene expression analysis in HepaRG undifferentiated cells

| Gene (symbol) | Accession No. | BNF repression (contr.BNF/contr. DMSO) | AhR knockdown (AhR(−) BNF/contr. BNF) |
|--------------|--------------|--------------------------------------|---------------------------------------|
|              |              | Fold-change | \(P\) value | Fold-change | \(P\) value |
| KIAA1456     | NM_020844    | −4.45339 | 9.35E-05 | 2.06645 | 4.25E-03 |
| KDR          | NM_002253    | −4.13891 | 4.18E-06 | 1.5346 | 3.18E-03 |
| FGG          | NM_021870    | −3.98685 | 9.06E-07 | 2.38767 | 1.38E-05 |
| ART3         | NM_001179    | −3.83642 | 1.48E-06 | 9.44067 | 7.00E-08 |
| KCNB1        | NM_004975    | −3.65479 | 1.44E-05 | 3.2784 | 2.39E-05 |
| FAM65B       | NM_014722    | −3.559   | 1.79E-04 | 2.76605 | 5.99E-04 |
| PLCL1        | NM_006226    | −3.51205 | 7.21E-05 | 1.60191 | 1.12E-02 |
| MLIP         | NM_138569    | −3.50818 | 3.56E-07 | 3.30055 | 4.79E-07 |
| PPL          | NM_002705    | −3.47763 | 4.52E-05 | 4.68824 | 1.31E-05 |
| MCF2         | NM_005369    | −3.44442 | 1.67E-05 | 1.81629 | 9.73E-04 |
| CIDE2        | NM_022094    | −3.39535 | 5.04E-06 | 2.71645 | 1.63E-05 |
| PDE1A        | NM_005019    | −3.35762 | 5.66E-05 | 3.50297 | 4.65E-05 |
| CIDE2        | NM_022094    | −3.32232 | 2.55E-05 | 2.76084 | 6.65E-05 |
| PLCL1        | NM_006226    | −3.24423 | 8.63E-05 | 1.54723 | 0.013509 |
| SAA2         | NM_030754    | −3.19599 | 0.003267 | 3.18935 | 0.003296 |
| NRXN3        | NM_004796    | −3.15472 | 1.60E-05 | 4.53509 | 3.21E-06 |
| MUM1L1       | NM_152423    | −3.14915 | 6.06E-06 | 1.81139 | 0.000261 |
| FLRT3        | NM_013281    | −3.14526 | 2.78E-06 | 2.13495 | 3.09E-05 |
| SORBS1       | NM_006434    | −3.11927 | 0.000186 | 2.49061 | 0.000621 |
| FABP4        | NM_001442    | −3.0878  | 1.80E-05 | 25.5207 | 3.50E-08 |

Information of all additional genes out of top 20 is available in the supplementary material accompanying of the manuscript (Supplementary 3— inhibition)

Fold-change value that was less than 1 has been replaced by the negative of its inverse (for example, 0.1 was replaced by \(-10\)

Contr. HepaRG cells transfected with control plasmid, AhR− HepaRG cells transfected with plasmid knocking down Ah receptor
Real-time PCR validation of microarray-based genes expression data

The results of our qPCR experiments are presented as relative expression of the genes (Table 8). Expression of AhR mRNA was determined to demonstrate real effectiveness of our AhR silencing construct on mRNA level. On the other hand, expression of CYP1A1 mRNA, model gene regulated by Ah receptor, showed how changes of AhR mRNA translate to the receptor function. Genes such as...
SERPINB2, SLC7A5, SLC14A1, CCNE2, TIPARP, STC2, SCG5, and TMEM156, which expression was validated by real-time PCR, belonged to the most inducible by BNF genes but outside classical AhR-dependent genes battery, or as in the case of GSTA2, regulated in opposite direction as was expected. As it was written in the previous chapter, the expression of CCNE2 was determined by real-time PCR because microarray analysis suggested very strange and unexpected regulation of this gene expression by Ah receptor.

Comparison of two different treatments groups, both with very low gene expression, could result in multiplying stochastic errors. Thus, the knowledge of approximate level of particular gene expression appeared to be a valuable one. We did not determine efficiencies of reverse transcription of particular mRNAs; therefore, we could not present our results as "absolute" quantification, e.g., as exact mRNA copy number. However, to determine the limit of detection and the efficiency of PCR amplification, we have used calibration (dilution) curve from which we could anticipate the approximate copy number of particular cDNAs in our PCR reaction (see "Materials and methods" section). Thus, the values of 100.0 presented in Table 8 correspond to $2.34 \times 10^3$ molecules of CYP1A1 cDNA in 0.4 µl of undiluted reverse-transcription products, 159 molecules for GSTA2, $4.31 \times 10^3$ molecules for SERPINB2, 209 molecules for SLC7A5, 76 molecules for SLC14A1, 916 molecules for CCNE2, $2.63 \times 10^3$ molecules for TIPARP, 589 molecules for STC2, $4.57 \times 10^3$ molecules for SCG5, $1.45 \times 10^3$ molecules for TMEM156, and $19.9 \times 10^3$ molecules for AhR.

### Discussion

The aryl hydrocarbon receptor is a ligand-activated transcription factor involved in many physiological processes. In laboratory animals, genetic variations in the AhR lead to significant differences in sensitivity to biochemical and carcinogenic effects of PAHs, TCDD, and related compounds [50]. Since late fifties till the end of twentieth century, most aspects of AhR function were contributed to its ability to induce enzymes responsible for metabolism of xenobiotic, drugs, and carcinogens [1, 51]. The situation has changed together with the dawn of microarray era at the beginning of twentieth century. It was demonstrated by gene expression profiling studies that AhR is responsible for induction or repression of hundreds of other genes, supposedly not directly connected to metabolism of xenobiotics [21, 27, 31, 52–57]. Generally, our results confirm the above observations. From 21 genes induced more than twofold by BNF treatment in both, undifferentiated and differentiated HepaRG cells, only cytochromes CYP1A1 and CYP1B1 belonged to classical AhR-dependent battery of genes encoding enzymes involved in metabolism of xenobiotics. However, when stringency of cutoff criterion was reduced to 1.5-fold, AhR-specific induction of ALDH3A1, NQO1, and UGT1A1 expression by BNF treatment has been observed. However, to our surprise, we did not observe AhR-dependent induction of CYP1A2 expression after BNF treatment of the cells. Induction of CYP1A2 expression after treatment of animals or human cell lines with diverse AhR ligands was widely demonstrated in many publications in this field. It was observed also after treatment of HepaRG with either TCDD [40] or...
| Gene   | HepaRG undifferentiated | HepaRG differentiated |
|--------|-------------------------|------------------------|
|        | Relative expression ± SD|                        |
|        | Type of cells            | Treatment               | Solvent | BNF | Solvent | BNF | Solvent | BNF |
|        |                         |                        |         |     |         |     |         |     |
| CYP1A1 | 0.405 ± 0.2113           | 100.0 ± 57.28a         | 4.091 ± 2.582 | 49.51 ± 42.20 | 3.132 ± 1.569 | 32.22 ± 14.21a | 8.803 ± 6.778 | 14.67 ± 12.42 |
| GSTA2  | 70.44 ± 3.862            | 17.62 ± 5.749a         | 34.94 ± 3.833 | 13.91 ± 4.109a | 52.89 ± 33.33 | 13.75 ± 5.733 | 100.0 ± 80.27 | 30.93 ± 13.82 |
| SERPINE2 | 1.205 ± 0.6849         | 100.0 ± 10.22a         | 1.234 ± 1.032 | 20.73 ± 13.18b | 1.173 ± 0.1412 | 33.80 ± 11.94a | 0.2250 ± 0.1469 | 2.713 ± 1.336b |
| SLC7A5 | 6.274 ± 1.461            | 100.0 ± 22.94a         | 2.963 ± 0.5229 | 18.74 ± 10.17b | 2.547 ± 0.6880  | 89.42 ± 28.47a  | 1.687 ± 0.5035c | 7.641 ± 2.617ab |
| SLC14A1 | 11.15 ± 0.1337          | 100.0 ± 34.49a         | 2.307 ± 1.731 | 3.675 ± 3.376b | 52.47 ± 36.10 | 78.07 ± 19.72 | 4.562 ± 3.840 | 2.147 ± 0.7728b |
| CCNE2  | 18.77 ± 9.006            | 53.92 ± 16.49a         | 24.98 ± 11.91 | 100.0 ± 14.59ab | 52.82 ± 6.010 | 71.14 ± 11.51 | 35.74 ± 8.194 | 32.58 ± 17.95b |
| TIPARP | 13.22 ± 2.026            | 98.99 ± 10.46a         | 11.67 ± 1.943 | 18.42 ± 8.759b | 24.62 ± 2.026 | 100.0 ± 24.18a | 24.45 ± 3.926 | 24.22 ± 4.037b |
| STC2   | 35.39 ± 16.29            | 100.0 ± 22.33a         | 43.16 ± 21.43 | 62.09 ± 21.18 | 18.25 ± 12.41 | 92.41 ± 78.18 | 17.65 ± 3.669 | 57.64 ± 49.78 |
| SCG5   | 12.95 ± 8.600            | 100.0 ± 47.09a         | 1.514 ± 0.6559 | 17.69 ± 15.42b | 2.632 ± 1.659 | 9.453 ± 5.483 | 0.1560 ± 0.1291 | 0.9109 ± 0.6250b |
| TMEM156 | 12.75 ± 7.720            | 100.0 ± 22.84a         | 4.393 ± 1.505 | 21.13 ± 11.91b | 1.233 ± 0.0565 | 7.672 ± 0.4095a | 0.4391 ± 0.1383c | 1.158 ± 0.2322ab |
| AhR    | 37.51 ± 9.057            | 33.92 ± 4.433          | 7.732 ± 1.840c | 9.052 ± 3.721c | 100.0 ± 23.76 | 81.13 ± 10.21 | 11.11 ± 4.527b | 9.161 ± 0.9119f |

*a* Significantly different from solvent-treated cells

*b* Significantly different from BNF-treated negative control transfected cells

*c* Significantly different from negative control transfected cells
BNF [41]. Our earlier experiments with BNF treatment of HepaRG cells were performed in virtually identical conditions as performed herein [41], except one substantial difference—in our earlier work, we had used unmodified HepaRG cells, whereas in the present study, HepaRG cell line was stably transfected with either control or AhR(−) pGeneClip™ vectors. It is possible that transfected control vector interfered somehow with expression of CYP1A2 mRNA, either by accidental localization of the vector integration site nearby the gene’s locus or by interference of negative control shRNA with the gene’s RNA. However, the second case is unlikely, as negative control shRNA is a scrambled artificial sequence which does not match any human gene. Another possibility which cannot be excluded is some kind of interference between the expression of CYP1A2 and RNA transcribed from neomycin or ampicillin resistance genes present on shRNA plasmids.

Our results suggest the involvement of Ah receptor in the regulation of CYP19A1, another member of the cytochrome P450 superfamily as well. Protein product of CYP19A1 is known as aromatase is an enzyme responsible for a key step in the biosynthesis of estrogens. Cross-talk of Ah receptor and estrogen receptor 1 (ER) signaling pathways are well described, but the underlying molecular mechanisms have been largely elusive. Interactions between these two pathways have been proposed to be due to a combination of several different mechanisms including increased metabolism of estrogen mediated by the AhR-dependent expression of CYP1A1 and CYP1B1 [58], direct interaction between AhR and ER [59], synthesis of inhibitory factors [60], direct inhibition through inhibitory XREs located in estrogen-responsive gene promoters [61], and increased ER degradation [62]. AhR-dependent induction of CYP19A1 expression by BNF treatment of HepaRG cells could be considered as another mechanism of AhR and ER pathways intersection. Our results are consistent with earlier findings describing AhR-dependent regulation of CYP19A1 expression in mouse ovarian granulosa cells [63]. BNF-related induction of CYP19A1 expression could explain some estrogen-like effects of BNF treatment of ovariectomized rats as well [22]. However, hepatocytes surely are not a primary source of the aromatase activity.

The reactive metabolites formed from xenobiotics by cytochromes P450 are usually detoxified to more polar products by phase II conjugative enzymes, such as GSTA1 [5]. Rodent Gsta1(GstYa) is known to be a target gene of AhR [2, 64]. It was demonstrated that the expression of GstYa was induced in the liver after BNF treatment of rats [65]. Human GSTA1 and its paralog GSTA2 are the orthologs of rodent GstYa gene. Consequently, it should be expected that BNF treatment of human HepaRG cells would increase the expression of GSTA genes as well. Our previous results suggested that the expression of GSTA1 was regulated by AhR in unmodified HepaRG cells [41]. Present results did confirm this suggestion. Indeed, expression of both, GSTA1 and GSTA2, was regulated by AhR. However, instead of anticipated induction, we have noticed significant inhibition of GSTA1 and GSTA2 expression following BNF treatment of HepaRG cells and this effect was significantly reduced after AhR knockdown by means of RNA interference. In our previous work, we hypothesized that maybe the decrease of GSTA1 expression after BNF treatment is compensated by simultaneous induction of some other GST isoenzymes [41]. Our present results did not confirm the above hypothesis. Analysis of the expression of genes by microarrays indicated that none of the GST isoenzymes were induced by BNF treatment of HepaRG cells, at least in investigated time point. Additional studies, especially at different time points, are necessary to determine if AhR-dependent inhibition of GSTA1 and GSTA2 by BNF treatment of HepaRG cells depicts interspecies differences between human and rodents, is model specific confined only to HepaRG cell line, or maybe is a result of different timing’s or ligand’s specificity.

Differentiated and undifferentiated HepaRG cells are genetically identical but committed to diverse gene expression programs. Consequently, our results clearly demonstrate different gene expression profiles between differentiated and undifferentiated cells. Barely about 25% of AhR-dependent genes were mutually induced and roughly 26% of genes were mutually inhibited in undifferentiated as well as in differentiated HepaRG cells. Therefore, levels of cell differentiation followed by condition of cell culture appeared to be much more important than the genetic background for pattern of activity of AhR-dependent genes. The above-mentioned conclusion is consistent with our earlier findings where expression of some AhR-dependent genes was compared between both, differentiated and undifferentiated, unmodified HepaRG cells [41]. The conclusion is consistent also with the findings of involvement of AhR in development of fetal mouse liver [42] or control of expansion of human hematopoietic stem cells in culture [66]. It was demonstrated that different cell types were involved in AhR-dependent development of mouse liver and in AhR-dependent hepatotoxicity [43]. Taken together, as undifferentiated HepaRG cells could be considered as similar to cells from fetal liver or stem cells, whereas differentiated ones resemble matured hepatocytes, so both variants of cell differentiation stages generate distinct pattern of expression of AhR-dependent genes.

Likewise, analysis of effects of BNF treatment and AhR silencing on the expression of genes such as interleukin 8 (IL8) or cyclin E2 (CCNE2), indicated on predominant
influence of cell differentiation stages in AhR-dependent regulation of the gene expression. Expression of IL8 has been already reported as AhR-dependent [67–69]. However, induction of IL8 expression by AhR ligands is supposed to be mediated by different from classical mechanism. Instead ARNT, liganded AhR binds to RelB and such heterodimer activates RelB/AhR-responsive element of the IL-8 promoter. Postulated RelB/AhR-responsive element differs from classical XRE [69]. To our best knowledge, CCNE2 expression has been not connected to AhR yet. However, analysis of the promoter of CCNE2 indicated on 2 classical core XRE sequences localized -824 and -559 bp upstream to the transcription starting site. On the contrary to IL8, we did not found any RelB/AhR-responsive element in the promoter of CCNE2. Nevertheless, treatment with BNF significantly induced expression of both genes in undifferentiated HepaRG cells, but silencing of Ah receptor not only did not counteract of such induction, but also further increased the expression of both genes in AhR-silenced, BNF-treated cells as compared to control BNF-treated undifferentiated HepaRG cells. This paradoxical effect was statistically significant and indicated on involvement of AhR. It was observed only in undifferentiated HepaRG cells. As far as differentiated HepaRG cells concerned, AhR-dependent response of IL8 and CCNE2 expression to BNF treatment proceeded according to the expectations of investigators. In this case, BNF treatment of differentiated HepaRG cells resulted in significant induction of IL8 and CCNE2 expression, respectively, and the induction was significantly reduced after knocking down AhR. Attempts to explain above phenomenon are difficult and can be only speculative at present state of our knowledge. Differentiated HepaRG cells are committed to another gene expression program with different patterns of transcriptionally active chromatin than undifferentiated ones. Maybe some differentiation-dependent modifications of CCNE2 and IL8 gene promoters’ structure followed by diverse accessibility for transcription factors cooperating with AhR could explain discussed results. Additional studies are necessary to explain the observed phenomenon.

Direct comparison of our results with different microarray studies is difficult, as most of other studies used TCDD as an AhR ligand and substantial differences between diverse AhR ligands, including BNF and TCDD, were observed [31]. Likewise, substantial differences between different species [34], rat strains [56], and different mouse tissues [27] were reported. Similar to above, substantial differences in respect to expression of AhR-dependent genes encoding xenobiotic-metabolizing enzymes were observed in undifferentiated as compared to differentiated HepaRG cells after BNF treatment [41]. Nevertheless, some well-established, AhR ligands regulating genes were induced despite of different species, strains, ligands, and tissues. Apart from cytochromes P450, SERPINB2 and TIPARP belonged to the most inducible by BNF- and AhR-dependent genes in both undifferentiated and differentiated HepaRG lines. SERPINB2 was reported in different human cell lines as inducible by TCDD treatment [70–73]. We demonstrated that expression of SERPINB2 was induced by BNF treatment of HepaRG cells as well. As a matter of fact, SERPINB2 was the most inducible gene in differentiated and the second one after CYP1A1 in undifferentiated HepaRG cells, respectively. It is especially interesting as the precise role of SERPINB2 remains an enigma [74, 75] and its connection to cancer has been reported [76]. The expression of TIPARP was also reported to be regulated by TCDD via activation of the AhR [77, 78]. Our results demonstrate that BNF is also a potent inducer of TIPARP expression. Very efficient induction of TIPARP expression by BNF in HepaRG cells is somehow contradictory to identification of TIPARP as the gene that can mediate TCDD toxicity by suppression of hepatic gluconeogenesis [79]. In short-term toxicity studies in animals, the typical effects of exposition on TCDD were wasting syndrome and thymus atrophy [80]. To our knowledge, such effects were never observed after exposition of animals to BNF, even after the 9-dose treatment of rats with BNF, the treatment which according to intention of investigators was supposed to mimic the effect of exposition to persistent TCDD [22]. If elevated, the expression of TIPARP would be accountable for TCDD-mediated toxicity, it should be expected that BNF is not effective inducer of this gene. However, our results did not confirm the above expectation, at least in HepaRG cell line. In addition, it was reported that TIPARP is a repressor of AhR transactivation, revealing a new mechanism of negative feedback control in AhR signaling [81]. Such negative feedback control of AhR expression by TIPARP in HepaRG cell line could be of particular importance, as expression of AHRR, the other negative controller of AhR [82], appears to be on a very low level as observed herein and in our earlier study [41].

Functional analysis of genes induced or inhibited by BNF treatment of HepaRG cells revealed involvement of these genes in multiple biological pathways, not directly connected to metabolism of xenobiotics. As a matter of fact, genes involved in metabolism of xenobiotics constitute only minute fraction of all genes regulated by AhR. Participation of the aryl hydrocarbon receptor in induction of expression of genes connected to regulation of apoptosis or involved in cell proliferation from one side, and in inhibition of genes connected to cell adhesion from the other side could explain some results suggesting involvement of AhR not only in initiation but also in progression of cancer [83]. In agreement with above, novel physiological function for AhR has been proposed recently, as
regulator of self-renewal of hematopoietic stem cells [66] or in general, modulator of the balance between differentiation and pluripotentiality in normal and transformed tumor cells [84].

Current work in our laboratory aims to identify possible function of some AhR-dependent genes selected in this study.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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