BIOCHEMICAL PHARMACOLOGY 73 (2007) 1622–1634

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β-Naphthoflavone and 3′-methoxy-4′-nitroflavone exert ambiguous effects on Ah receptor-dependent cell proliferation and gene expression in rat liver 'stem-like' cells

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ARTICLE INFO

Article history:
Received 19 December 2006
Accepted 24 January 2007

Keywords:
Aryl hydrocarbon receptor
Flavones
Cell proliferation
Liver epithelial cells
Xenobiotic-metabolizing enzymes

ABSTRACT

Both natural and synthetic flavonoids are known to interact with the aryl hydrocarbon receptor (AhR); however, their agonist/antagonist properties *in vitro* have been so far studied mostly in the context of cytochrome P450 1A1 gene (*Cyp1a1*) regulation. We investigated effects of two synthetic flavones known either as AhR agonist (β-naphthoflavone; BNF) or antagonist (3′-methoxy-4′-nitroflavone; 3M4NF), using an *in vitro* model of liver 'stem-like' cells, on expression of various AhR target genes and AhR-dependent cell proliferation. We found that the presumed antagonist 3M4NF induces a partial nuclear translocation and activation of AhR. Although inhibiting the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced *Cyp1a1* expression, 3M4NF alone induced a minor increase of CYP1A1 mRNA and protein. However, 3M4NF did not induce AhR binding to synthetic dioxin response elements (DRE). In contrast to *Cyp1a1*, 3M4NF induced a marked expression of other AhR-regulated genes, such as *Cyp1b1* and *Nqo1*, as well as transcriptional repression of *Cdh13* gene, confirming that its effects may be promoter-context specific. Like BNF, 3M4NF induced AhR-dependent cell proliferation of contact-inhibited rat liver 'stem-like' WB-F344 cells, associated with a marked upregulation of Cyclin A, as well as the downregulation of proteins involved in formation of cell–cell contacts. Based on these experimental findings, we conclude that partial agonists/antagonists of AhR can increase cell proliferation rate and AhR-dependent genes expression in both cell type- and gene-specific manner. The specificity of effects of flavones on diverse AhR targets should be taken into account, when studying AhR signaling using presumed AhR antagonists.

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Abbreviations: 3M4NF, 3′-methoxy-4′-nitroflavone; AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; ANF, α-naphthoflavone; BNF, β-naphthoflavone; CYP, cytochrome P450; DRE, dioxin responsive elements; EMSA, electrophoretic mobility shift assay; FITC, fluorescein-isothiocyanate; NQO1, NAD(P)H-quinone dehydrogenase 1; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP)-ribose polymerase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, short interfering RNA; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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doi:10.1016/j.bcp.2007.01.032

1. Introduction

Flavonoids are a major group of polyphenolic plant secondary metabolites, commonly found in fruits and vegetables, which are being used as antioxidants, chemopreventive or anti-inflammatory agents, drugs preventing heart disease and immunomodulators [1,2]. They have been implicated in prevention of liver diseases associated with genotoxic and oxidative damage to DNA, proteins or cell membranes [2]. Because of their anti-proliferative and pro-apoptotic properties, flavonoids have been suggested to be used as a part of anti-cancer therapy [3,4]. Flavonoids interact with a number of intracellular receptors, including the aryl hydrocarbon receptor (AhR), and synthetic flavonoids are being used as model AhR ligands [5–9].

The Ah receptor, a member of the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factor family, is a ligand-activated transcription factor known to mediate toxic effects of various persistent organic pollutants [10]. Prior to ligand binding, the AhR is localized in cytoplasm in complex with heat-shock protein 90 (hsp90), immunophilin-like X-associated protein 2/AhR associated protein 9/AhR interacting protein 1 (XAP2/ARA9/AIP1) and interacting protein p23 [10]. After ligand binding, the AhR translocates into the nucleus, dimerizes with aryl hydrocarbon nuclear translocator (ARNT) and together bind to DNA at dioxin responsive elements (DRE). The AhR-regulated target genes include both Phase I (*Cyp1a1*, *Cyp1a2*, *Cyp1b1*) and Phase II (*Nqo1*, *Ugt1a6*) xenobiotic-metabolizing enzymes [11]. Apart from enzyme expression, the AhR may also regulate cell cycle and apoptosis in a cell-specific manner [12–15]. However, inhibition of cell cycle progression by synthetic flavonoids has been postulated to be AhR-independent [3].

Flavonoids may act either as AhR agonists, e.g. synthetic β-naphthoflavone (BNF), or its partial/full antagonists. Their agonistic/antagonistic properties seem to be species- and cell type-specific [5,8,9,16,17], and concentration-dependent. Many flavonoids function as AhR antagonists at lower concentrations, while at higher levels they behave as AhR agonists [16,18,19]. One of the most potent AhR antagonists identified to date is 3′-methoxy-4′-nitroflavone (3M4NF) [20], which is suggested to act through direct competition with AhR agonists for binding to the AhR ligand-binding site, thereby preventing the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced AhR nuclear translocation and activation, hence inhibiting the TCDD-caused toxic outcomes [7]. In one study, 3M4NF has been shown to act as a weak AhR agonist increasing *Cyp1a1* expression in mouse hepatoma cells [21]. However, our current knowledge of agonistic/antagonistic nature of interactions of flavonoids with AhR is almost exclusively based on regulation of *Cyp1a1* expression, while little is known about their effects on other AhR-regulated genes or AhR-dependent regulation of cell proliferation.

The liver contains multiple cell types, of which hepatocytes and hepatoma cell lines are by far the best characterized ones regarding the AhR activation. A sustained activation of AhR has been shown to inhibit hepatocyte proliferation and to delay liver regeneration in response to hepatectomy [22]. However, the response to AhR activation might be different in other liver cell populations, such as in liver progenitor cells. The oval cell (or progenitor cell) compartment in liver, which is activated during liver injury, has been implicated both in the liver regeneration and during hepatocarcinogenesis [23]. The rat liver epithelial WB-F344 cell line is a model of rat liver progenitor cells [24], which has been used, among others, to study proliferative effects of AhR ligands in contact-inhibited cells [25]. However, nothing is currently known about the impact of synthetic flavones on AhR signaling and regulation of cell proliferation in liver progenitor cells.

In the present study, we compared the effects of 3M4NF on AhR-signaling in WB-F344 cells, as well as on induction of model target genes and modulation of cell proliferation/contact inhibition, using AhR ligand models. The experiments reported here suggest that AhR antagonists/partial agonists can induce AhR-dependent cell proliferation, and that there are significant differences between effects of 3M4NF on regulation of *Cyp1a1* gene expression and other AhR-regulated genes, involved either in regulation of cell proliferation or in metabolism of xenobiotics.

2. Materials and methods

2.1. Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Cambridge Isotope Laboratories (Andover, MA). β-Naphthoflavone (BNF) and α-naphthoflavone (ANF) were purchased from Sigma–Aldrich (Prague, Czech Republic). 3′-Methoxy-4′-nitroflavone (3M4NF) was kindly provided by Thomas A. Gasiewicz (University of Rochester School of Medicine and Dentistry, NY) and Josef Abel (Heinrich Heine University, Düsseldorf, Germany). All other chemicals and cell culture media were obtained from Sigma–Aldrich.

2.2. Cell culture and treatment

The rat hepatic oval WB-F344 cells were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham medium supplemented with sodium pyruvate (110 mg/ml), gentamycin (50 mg/l) and 5% heat-inactivated fetal bovine serum. The cells were incubated in a humidified atmosphere of 5% CO_2/95% room air at 37 °C. Cells were maintained in 75 cm^2 flasks and sub-cultured twice a week. Only cells from passage numbers 15–22 were used throughout the study.

2.3. Cell proliferation assay and cell cycle analysis

WB-F344 cells were seeded at concentration of 30,000 cells/cm^2 in 35-mm cell culture dishes (Nunc, Roskilde, Denmark) and grown until confluency. The medium was changed, and after another 24 h of incubation, the tested compounds were applied. For cell cycle analysis, cells were collected after 24 h by trypsinization, washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4 °C overnight. Fixed cells were then washed once with PBS and stained with propidium iodide as described previously [26]. Cells were then analyzed on FACSCalibur, using 488 nm (15 mW) air-cooled argon-ion laser for propidium iodide excitation (Becton Dickinson, San Jose, CA) and a minimum of 15,000 cells was collected per

sample. Data were analyzed using ModFit LT software (Verify Software House, Topsham, ME). In order to determine the effects of tested compounds on cell numbers, confluent cells were grown for 72 h in the presence of tested compounds, trypsinized and counted with Coulter Counter (Model ZM, Coulter Electronics, Luton, UK) as described previously [26].

2.4. Indirect immunofluorescence

To investigate the effects of tested compounds on AhR-translocation, WB-F344 cells were grown on glass cover slips in four-well cell-culture plates until they reached confluency. The medium was changed and 24 h later the tested compounds were added for 30 min. Cells were washed with PBS (4 °C) and fixed with cold methanol:acetone mix (1:1) for 15 min at −20 °C. Cells were then washed three times with wash buffer (see Section 2.8), and incubated 2 h with the anti-AhR antibody (Biomol, Butler Pike, PA). Cells were again washed three times and incubated for 1 h with the anti-rabbit fluorescein-isothiocyanate (FITC)-conjugated antibody (GE Healthcare, Little Chalfont, UK). Cells were washed once with wash buffer containing RNAse (20 μg/ml) and once with wash buffer without RNAse, and mounted in 5 μl of Mowiol (Calbiochem, San Diego, CA) solution (10% Mowiol 4–88 was prepared in 25% glycerol, 100 mM Tris–HCl, pH 8.5) for visualization using a Olympus IX70 fluorescence microscope.

2.5. RNA isolation and real-time RT-PCR

Total RNA was isolated using NucleoSpin RNA II Purification Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The sequences of primers and probes for rat *Cyp1a1*, *Cyp1b1*, *PBGD* have been either published previously [26], or are listed in Table 1 (*Ccna2*, *Cdh13*, *Nqo1*). The amplifications of the samples were carried out in a final volume of 20 μl in a reaction mixture containing 10 μl of QuantiTect Probe RT-PCR Master Mix, 0.2 μl of QuantiTect RT Mix (Qiagen, Valencia, CA), 2 μl of solution of primers and probe (Generi-Biotech, Hradec Králové, Czech Republic), 5.8 μl of water and 2 μl of RNA sample. The final concentration of each primer was 0.4 μM and probe was 0.1 μM. The amplifications were run on the RotorGene3000 with RotorGene Real-Time Analysis Software (Corbett Research, Sydney, Australia), using the following program: 50 °C for 30 min for reverse transcription and 95 °C for 15 min for denaturation of cDNA, followed by cycling (30–40 repeats) 94 °C for 15 s and 60 °C for 60 s acquiring fluorescence. Optimalized RT-PCR conditions for *PBGD* detection corresponded with the recommendations of the producer of primers and probes (Generi Biotech, Hradec Králové, Czech Republic)—cycling mode 94 °C for 10 s and 60 °C for 40 s. All PCR reactions were performed in triplicates and changes in gene expression were calculated using the comparative threshold cycle method [27].

2.6. Preparation of nuclear extracts

Cells were treated with TCDD, 3M4NF, BNF or their combination for 1 h prior to harvest. Cells were washed and lysed with lysis buffer (Tris 10 mM, KCl 60 mM, EDTA 1.2 mM, (D, L)-1,4-dithiothreitol [DTT] 1 mM; pH 8.0), supplemented with 100 μM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Nonidet P-40 (NP-40), for 10 min on ice and centrifuged. Pellets were rinsed with lysis buffer without PMSF and NP-40, centrifuged and incubated for 25 min in 50 μl of nuclear extraction buffer (Tris 20 mM, NaCl 420 mM, $MgCl_2$ 0.7 mM, EDTA 0.25 mM with glycerol 25%, v/v) on ice. After final centrifugation, supernatants were collected and stored frozen in aliquots at −80 °C. The amount of proteins in nuclear extracts was quantified using the Bradford assay [28].

Table 1 – Real-time RT-PCR primers and probes

Gene	Oligonucleotide	Accession no.
Ccna2 (Cyclin A2)		
F603[a]	5′-CACGTACCTTAGGGAAATG-GAGGTTA-3′	XM_342229
R727	5′-ATTCTTCTCCCACTTCAAC-TAGCCAG -3′	
P699	5′-CACAAGGATGGCCCGCA-TACTGTTAGTG-3′	
Nqo1 (NQO1)		
F374	5′-TCAGTTCCCATTG-TATTGGTTTGG-3′	NM_017000
R509	5′-AAGCAAGGTCTTCTTATTCTG-GAA-3′	
P400	5′-TGCCCGCCATTCT-GAAAGGCTGGTTT-3′	
Cdh13 (T-cadherin)		
F1951	5′-CCCTGATAAAGTCTGGAAGA-3′	NM_138889
R2021	5′-GGTTGTAGTTCGCCTTGTT-3′	
P1984	5′-CAACACCCACGCCCTCGT-GAGCCT-3′	

[a] The primers and probes are identified by letters designating the forward (F), the reverse (R) primer or the probe (P), and a number corresponding to the position of the base at the 5′ end of the positive strand of primer or probe in the reference sequence, according to GenBank accession number.

2.7. Electrophoretic mobility shift assay (EMSA)

DNA probe was labeled with ^{32}P-γ-ATP (MP Biomedicals, Illkirke, France) by T4-polynucleotide kinase (New England BioLabs, Ipswich, MA) and purified by Mini QuickSpin columns (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. Nuclear extracts (11 μg per sample) were pre-incubated for 10 min at room temperature in binding buffer (Tris 50 mM, EDTA 6 mM, DTT 0.5 mM, with glycerol 50%, v/v) with 0.017 units of polydI.dC (Roche Diagnostics). Binding reactions were then performed for 25 min at room temperature with 1 μl of [γ^{32}P]-ATP labeled DRE-DNA (DNA 25 ng/μl; 40,000 cpm). To confirm the specificity of binding to the oligonucleotide, parallel samples were also incubated with excess of unlabeled wild-type DRE probe (competitive control), with nonspecific DNA (mutated DRE), or with labeled wild-type DRE probe and 1 μg of anti-ARNT1 antibody (BD Biosciences; San Jose, CA) in supershift sample. For the DRE sequences used see [29]. AhR/ARNT1/DRE complexes were separated under non-denaturing conditions on 6% polyacrylamide gel. The gels were dried and the DNA–protein complexes were visualized using the STORM phosphoimager (GE Healthcare, Little Chalfont, UK).

2.8. Western blots

Whole cell lysates were prepared using SDS sample buffer (1% SDS, 10% glycerol, 100 mM Tris, pH 7.4) and protein concentration was estimated using Bio-Rad DC Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA). The total cell lysates or nuclear extracts were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore, Prague, Czech Republic) and blocked with nonfat dry milk. The proteins were detected with following antibodies: anti-AhR (Biomol, Butler Pike, PA), anti-Cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CYP1A1 (Daiichi Pure Chemicals Co., Tokyo, Japan), anti-β-actin (Sigma–Aldrich), anti-E-cadherin and anti-γ-catenin (Transduction Laboratories, BD Biosciences; San Jose, CA). Anti-rabbit, anti-mouse and anti-goat horse radish peroxidase-conjugated secondary antibodies and ECL-Plus reagent (GE Healthcare) were used for visualization of selected proteins according to manufacturer's instructions.

2.9. Cell transfections with short interfering RNA (siRNA)

Cells were plated at a density of 20,000 cells/cm^2 in 24-well plates in medium without antibiotics. After 24 h cultivation, transfections were performed, using the previously described siRNA duplexes directed against rat AhR mRNA sequence or control siRNA directed against mRNA encoding the red fluorescence protein DsRed [15]. The transfections were carried out in a total volume of 600 μl containing 200 pmol siRNA and 1 μl of Lipofectamine 2000 (Invitrogene, Carlsbad, CA), according to the manufacturer's instructions. Transfection mix was removed 24 h later and cells were cultivated for another 24 h in DMEM/F12 medium with antibiotics, followed by exposure to 3M4NF for 48 h.

2.10. Statistical analysis

The mRNA data were analyzed by nonparametric Mann–Whitney *U*-test or ANOVA followed by Tukey test. Cell proliferation data were expressed as averages ± standard deviation of at least three independent repeats and, similar to flow cytometry data, these were analyzed by nonparametric Mann–Whitney *U*-test. A *P* value of less than 0.05 was considered significant.

3. Results

3.1. Activation of AhR

Since flavones can act both as agonist and antagonist in cell type- and concentration-dependent manner [9], we selected two model concentrations (1 and 10 μM) of 3M4NF and BNF. The concentrations were optimized according to our preliminary experiments, where both flavones, as well as α-naphthoflavone (ANF), were found to induce cell proliferation in contact-inhibited WB-F344 cells in a concentration-dependent manner (data not shown). Both concentrations of 3M4NF that were used in the present study have been previously shown to inhibit the TCDD-induced AhR-binding to DRE and induction of *Cyp1a1* expression [7,20]. We have compared the effects of flavones in WB-F344 cells with model AhR ligand TCDD as a positive control, as well as with a combined impact of TCDD and flavones.

3M4NF has been previously reported to prevent the TCDD-induced AhR nuclear translocation [7]. Therefore, we first performed immunocytochemical detection of AhR cellular localization in methanol-acetone fixed cells following 30 min exposure, as well as Western blotting of AhR protein in nuclear extracts of WB-F344 cells treated with tested compounds for 1h. Both 3M4NF and BNF induced translocation of AhR from cytoplasm to the nucleus at both selected concentrations, similar to TCDD (Fig. 1A). This was confirmed by detection of AhR in nuclear extracts from cells treated with 3M4NF and BNF (Fig. 1B). However, in case of 3M4NF, a significant part of total AhR pool was detected in cytoplasm, suggesting that 3M4NF induces only a partial translocation of AhR into nucleus (Fig. 1A; data not shown). Similar to this, 3M4NF induced a partial retention of AhR in cytoplasm of TCDD-treated cells, but its significant portion was also found in nuclear extracts (Fig. 1B; data not shown).

The activation of AhR is known to lead to ubiquitination and degradation of AhR via 26S proteasome pathway, modulating both the duration and magnitude of gene regulation mediated by AhR/ARNT complex [30]. Therefore, we determined the levels of AhR in total cell lysates after 6 h and 48 h incubations. While both TCDD and BNF induced a strong AhR-degradation after 6 h, 3M4NF caused only a partial reduction of AhR protein levels (Fig. 2). The higher concentration of 3M4NF induced a more pronounced degradation of AhR; however, it partially prevented the TCDD-induced decrease of total AhR protein. The metabolism of flavones in liver progenitor cells probably affects their impact on AhR degradation, as 1 μM concentration of BNF only transiently reduced AhR levels after 48 h. Both tested concentrations of 3M4NF (1 and 10 μM) were able to significantly inhibit the TCDD-induced AhR degradation at this time-point (Fig. 2). Taken together, these results showed that 3M4NF induced a partial AhR nuclear translocation and degradation in WB-F344 cells, suggesting that it might modulate expression of AhR target genes in this model of liver progenitor cells.

3.2. Effects of flavones on expression of model AhR-target gene *Cyp1a1*

A previous study suggested that 3M4NF may behave as a weak AhR agonist inducing *Cyp1a1* expression. Because, AhR/ARNT complex binds to DRE in enhancer/promoter region of model AhR-target gene, *Cyp1a1* [31], the ability of model compounds to induce AhR/ARNT binding to synthetic DRE-containing oligonucleotides derived from rat *Cyp1a1* enhancer region was investigated by EMSA. One hour after treatment of WB-F344 cells with DMSO, TCDD, BNF (1 and 10 μM), 3M4NF (1 and 10 μM), shifted bands were detected only in TCDD- and BNF-treated samples, while 3M4NF failed to induce AhR-DNA binding (Fig. 3). Unlike BNF, 3M4NF inhibited the TCDD-induced binding of AhR/ARNT1 complexes to DRE (Fig. 3).

In the next step, *Cyp1a1* gene expression at both mRNA and protein levels was investigated. As expected, there was a strong increase of CYP1A1 mRNA levels in both TCDD- and

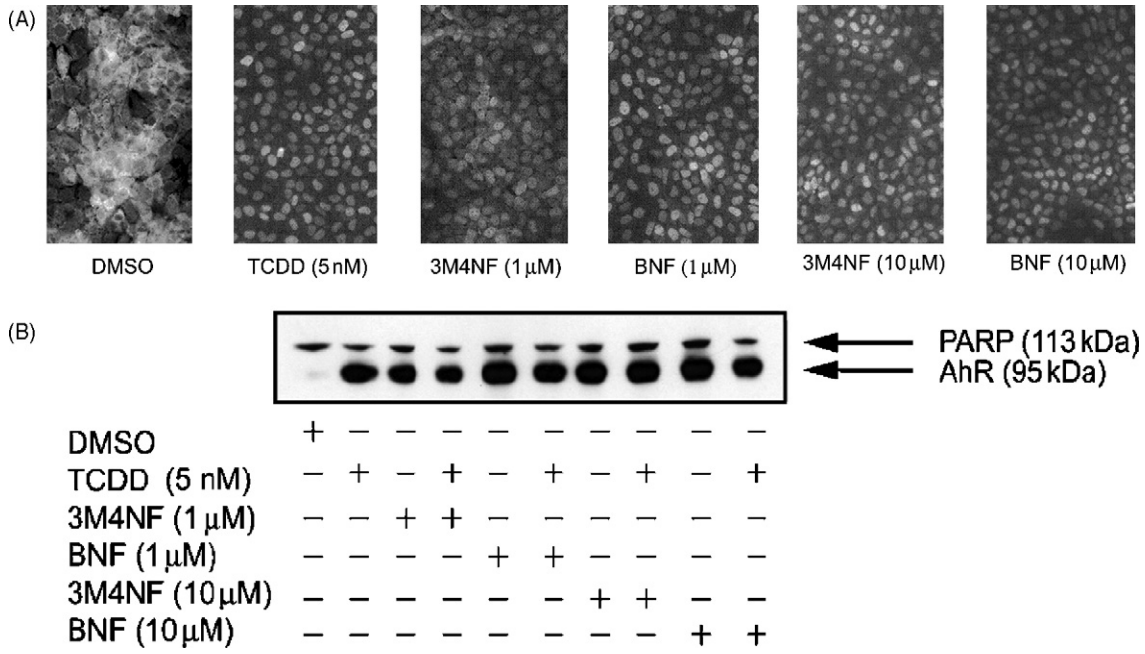

Fig. 1 – Both 3M4NF and BNF flavones induced cytosol-nuclear translocation of AhR in WB-F344 cells. (A) Localization of Ah receptor in confluent WB-F344 cells was assessed by indirect immunofluorescence using anti-AhR antibody and FITC-conjugated secondary antibody. TCDD, 3M4NF and BNF after 30 min treatment. WB-F344 cells were grown on cover slips, treated, fixed and labeled as described in Section 2. DMSO (0.1%) was used as a vehicle control. (B) AhR detection by Western blotting was performed using nuclear extracts of WB-F344 cells treated for 1 h with TCDD, 3M4NF, BNF or their combinations. Poly(ADP)-ribose polymerase (PARP) was used as a loading control for nuclear extracts. The data are representative of three independent experiments.

BNF-treated cells, while 3M4NF induced a minor increase of CYP1A1 mRNA (Fig. 4A). There were significant differences among the time- and concentration-dependent effects of individual AhR ligands and their combinations. TCDD induced the highest elevation of CYP1A1 mRNA (this was further used as a positive control, 100%), which was consistent through 48 h treatment. In contrast, levels of CYP1A1 mRNA increased by 92 and 27% upon 6 h treatment with BNF (1 and 10 μM, respectively), and by 4 and/or 55% after 48 h treatment (1 and 10 μM, respectively) (Fig. 4A). Co-treatment of cells with BNF and TCDD resulted in suppression of the TCDD-induced CYP1A1 mRNA to 53 and 20% after 6 h cultivation (1 and 10 μM concentrations). This effect of BNF was largely abrogated after 48 h treatment. These results suggested that also BNF may behave as partial AhR antagonist in WB-F344 cells, at least when considering *Cyp1a1* regulation. In case of 3M4NF, we detected elevation of CYP1A1 mRNA levels by 16 and 8% upon 6 h treatment with 1 and 10 μM concentrations, respectively. After 48 h treatment, the levels of CYP1A1 mRNA were 4 and 29%, relative to TCDD. 3M4NF reduced the TCDD-induced

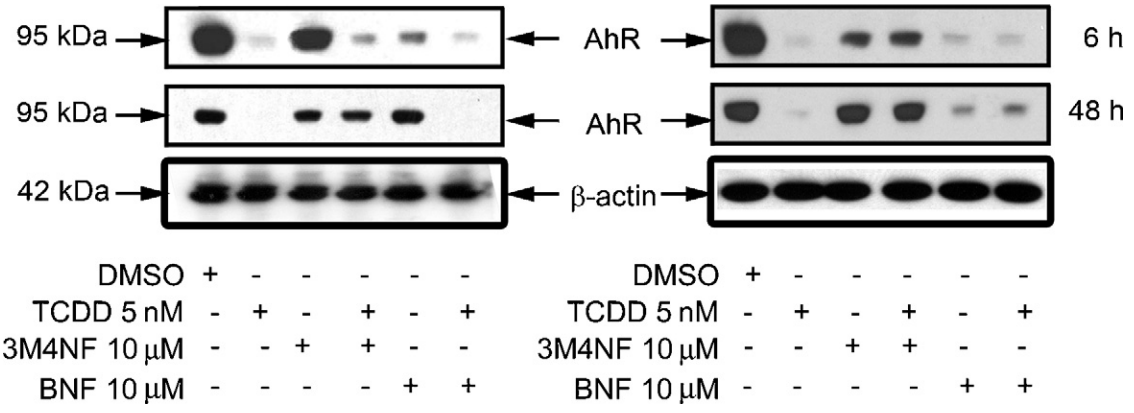

Fig. 2 – 3M4NF induced only a limited AhR protein degradation and prevented TCDD-induced AhR protein degradation. Total cell lysates were prepared and analyzed by Western blotting for AhR and β-actin (loading control). Cells were treated for 6 and 48 h with DMSO (0.1%), TCDD, 3M4NF and BNF or with combination of flavones and TCDD. The results are representative of three independent experiments.

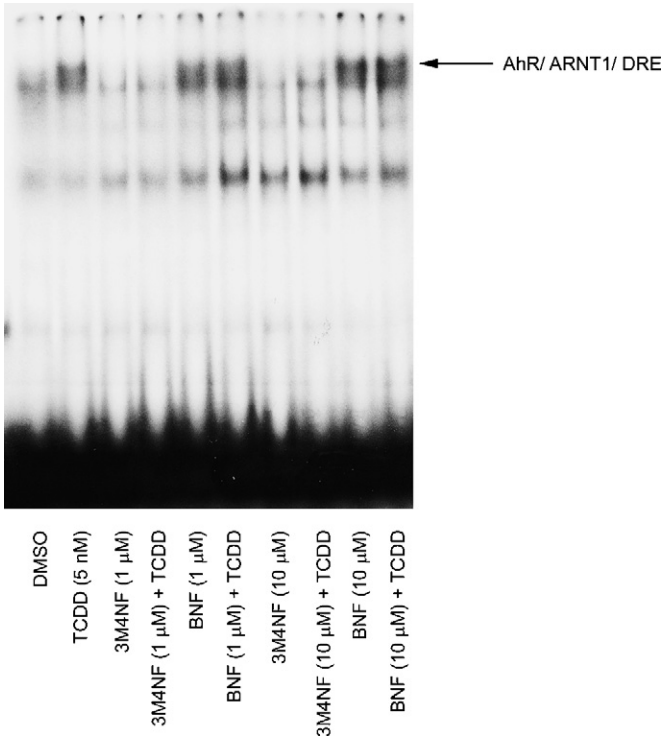

Fig. 3 – 3M4NF did not induce AhR binding to synthetic DRE. DNA binding assay was performed using nuclear extracts from WB-F344 cells treated for 1 h with DMSO, TCDD, 3M4NF and BNF, or with combination of TCDD and flavones. The mobility shift assay was performed with [γ^{32}P]-labeled oligonucleotide probe containing an AhR binding site from the enhancer region of rat *Cyp1a1* gene. The arrow denotes the position of the AhR/ARNT1/DNA complex. The results are representative of three independent experiments.

CYP1A1 mRNA to 31 and 7% after 6 h. After 48 h treatment, only the 10 μM concentration of 3M4NF was effectively suppressing CYP1A1 mRNA levels (32%). This was likely due to inactivation of 3M4NF in cells through its metabolism. The mRNA data largely corresponded with the results of detection of CYP1A1 protein levels (Fig. 4B) by Western blotting, with few notable exceptions. First, BNF seemed to be as potent CYP 1A1 protein inducer as TCDD. The inhibitory effect of 10 μM BNF on TCDD-induced CYP1A1 protein levels was not observed. Second, only 10 μM concentration of 3M4NF induced a sustained prevention of TCDD-induced CYP1A1 protein (Fig. 4B). The differences in mRNA and protein expression might be related to short half-life of CYP1A1 mRNA reported from other cell lines [32].

3.3. *Effects of flavones on other AhR-regulated genes—Cyp1b1, Nqo1, Cdh13*

Apart from *Cyp1a1* being a model AhR target gene, there are numerous AhR-regulated genes that are either directly or indirectly activated or repressed through AhR activation. The previous study of Zhou and Gasiewicz [21] suggested that the agonist/antagonist properties of flavones may depend on the promoter context of a particular gene, however they compared *Cyp1a1* regulation only with luciferase reporter constructs. Therefore, we selected three gene targets that are regulated to a different extent through AhR activation: (i) *Cyp1b1*, which relative magnitude of response to model AhR ligands is different in WB-F344 cells from that of *Cyp1a1*, and which can be regulated in cell-specific manner in different liver cell populations [26,33]; (ii) *Nqo1*, which can be regulated by AhR (both directly and indirectly through Nrf2 protein upregulation) and oxidative stress via antioxidant responsive elements [34,35]; (iii) *Cdh13* gene, which codes for T-cadherin protein, and which has been reported to be an AhR-repressed gene [36]. For this part of the study, we treated WB-F344 cells with test compounds for 24 h prior to determination of CYP1A1, CYP1B1 and NQO1 mRNA levels, and for 48 h to detect T-cadherin mRNA level (Fig. 4C). In TCDD-treated cells we detected 158-fold induction of CYP1A1 mRNA level, 23-fold induction of CYP1B1 mRNA, 3.5-fold induction of NQO1 mRNA, while T-cadherin mRNA was reduced to 90% of control levels. In 3M4NF-treated cells, we detected only 7-fold induction of CYP1A1 mRNA, which is less than 5% of TCDD induction. In contrast, 3M4NF elicited 11-fold induction of CYP1B1 mRNA, 3-fold induction of NQO1 mRNA and reduction of T-cadherin mRNA to 41% of control. BNF induced 104-fold induction of CYP1A1 mRNA, 21-fold induction of CYP1B1 mRNA, 4-fold induction of NQO1 mRNA and reduction of T-cadherin mRNA to 76% of control. Thus, 3M4NF induced both CYP1B1 and NQO1 expression in a manner similar to TCDD, and it was the most efficient agent in downregulation of *Cdh13* gene expression. These data suggested that it may have significant impact on expression of AhR-regulated genes other than *Cyp1a1*.

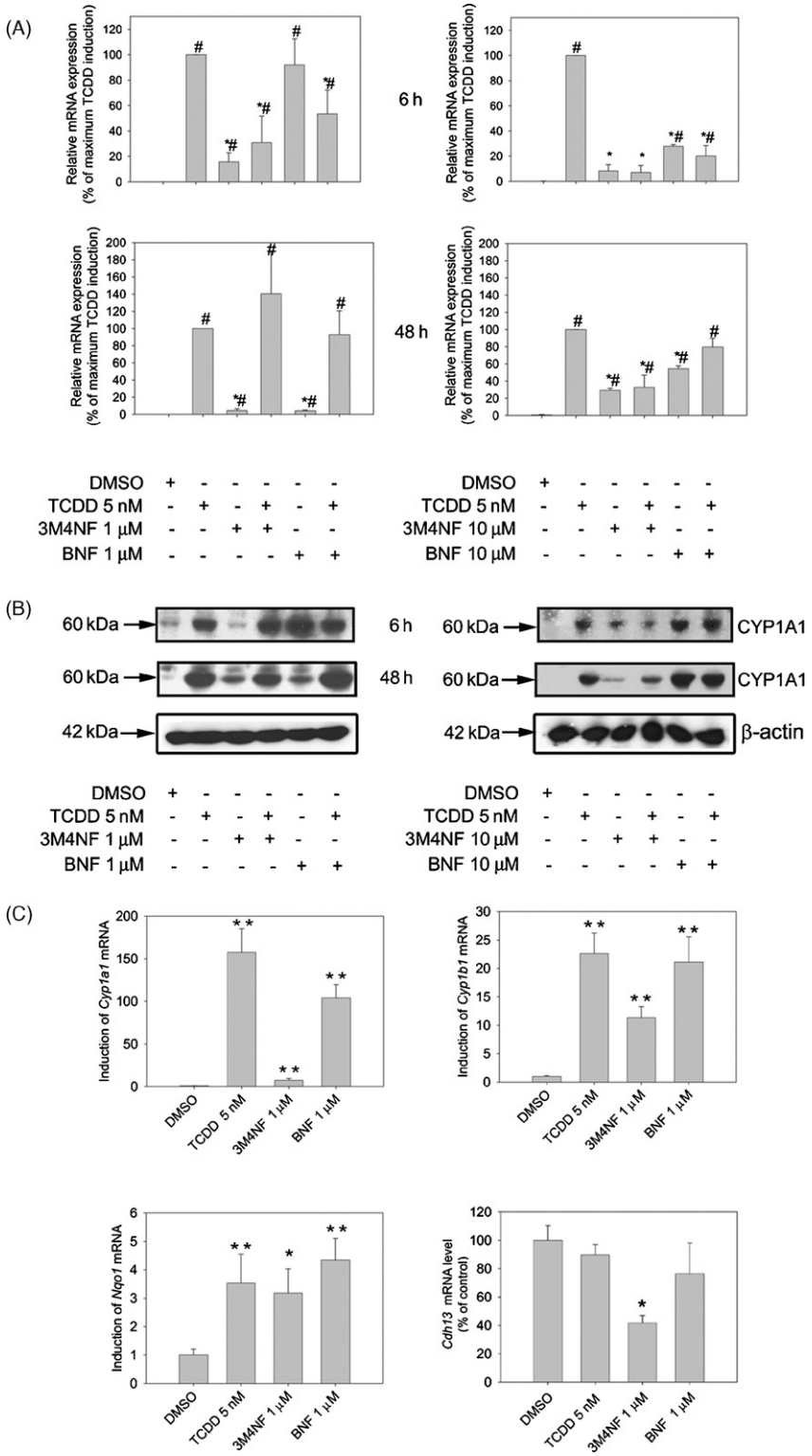

Fig. 4 – Differential effects of 3M4NF and BNF on expression of AhR target genes—*Cyp1a1*, *Cyp1b1*, *Nqo1* and *Cdh13* (T-cadherin). (A, B) WB-F344 cells were treated for 6 and 48 h with DMSO (0.1%), TCDD, 3M4NF and BNF, or with combination of flavones with TCDD. Total cell lysates were prepared as described in Section 2 and analyzed by real-time RT-PCR for CYP1A1 mRNA levels (A), and by western blotting for CYP1A1 and β-actin (loading control) proteins (B). RT-PCR data were analyzed using ANOVA followed by Tukey test. Symbol '#' denotes a significant difference between negative control (0.1% DMSO) and treated samples ($P < 0.05$). Symbol '*' denotes a significant difference between positive control (5 nM TCDD) and treated samples ($P < 0.05$). (C) Detection of CYP1A1, CYP1B1, NQO1 and T-cadherin mRNAs by RT-PCR after 24 h treatment of WB-F344 cells with DMSO (0.1%), TCDD, 3M4NF and BNF. The results represent means ± S.D. of three independent experiments. Symbol '*' and ''denote a significant difference between negative control (0.1% DMSO) and treated samples ($P < 0.05$ and $P < 0.01$, respectively).**

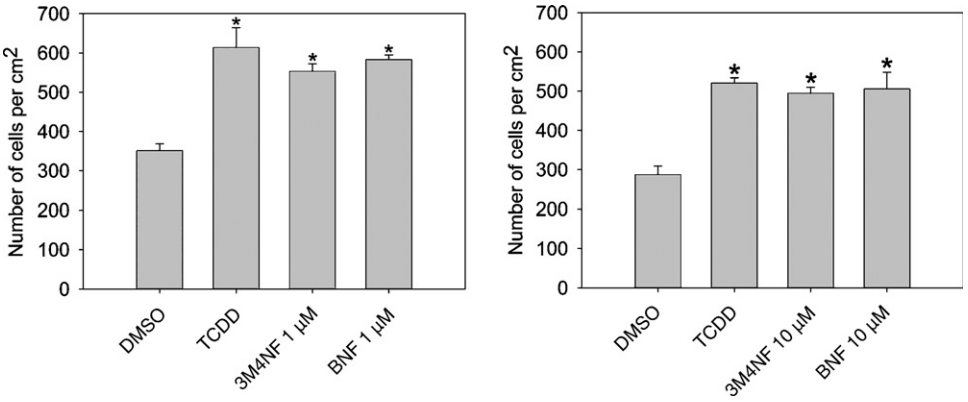

Fig. 5 – 3M4NF and BNF induced cell proliferation in contact-inhibited WB-F344 cells. Confluent cultures of WB-F344 cells were treated with DMSO (0.1%), TCDD, 3M4NF and BNF. After 72 h of treatment, cells were harvested by trypsinization and counted with Coulter Counter. The results represent means ± S.D. of three independent experiments. Data were analyzed using non-parametric Mann–Whitney *U*-test. Symbol '*' denotes a significant difference between negative control (0.1% DMSO) and treated samples ($P < 0.05$).

3.4. *Impact of flavones on proliferation of contact-inhibited WB-F344 cells*

Various AhR ligands have been previously shown to induce cell proliferation in contact-inhibited liver epithelial cells [25,37]. However, nothing is known about the impact of flavonoids, which may behave as full or partial antagonists, on cell proliferation in this model. The anti-proliferative effects of flavones in hepatoma cells have been suggested to be AhR-independent [3]. Therefore, the effects of model flavones on cell proliferation in confluent WB-F344 cells were investigated. It was found that, 3M4NF and BNF (Fig. 5), as well as ANF or combinations of flavones with TCDD, induced cell proliferation leading to almost 1.7-fold increase of cell number after 72 h. Effects of combination treatments were similar to effects of individual compounds (data not shown). These data corresponded with results of cell cycle analyses performed after 24 h treatment. Both 1 and 10 μM concentrations of 3M4NF and BNF induced an increase of percentage of cells in S-phase (Fig. 6). Consistent with these data, both 3M4NF- and BNF-treated cells expressed significantly higher levels of S-phase specific Cyclin A, with 3M4NF inducing even higher Cyclin A mRNA levels than TCDD (Fig. 7A). Both flavones also induced a sustained elevation of Cyclin A protein, with 3M4NF again being the most effective compound (Fig. 7B). The levels of E-cadherin and γ-catenin (plakoglobin) are upregulated in confluent cultures of nontransformed epithelial cells and have been suggested to participate in maintenance of contact inhibition [38]. The loss of contact inhibition and induction of cell proliferation in WB-F344 cells by TCDD is accompanied with changes in expression of proteins involved in cell–cell adhesion [39]. It was found that γ-catenin and E-cadherin expression are significantly reduced after 48 h treatment of cells with 3M4NF, but not by BNF (Fig. 8). Taken together, a model AhR antagonist, 3M4NF had strong proliferative effects in contact-inhibited WB-F344 cells.

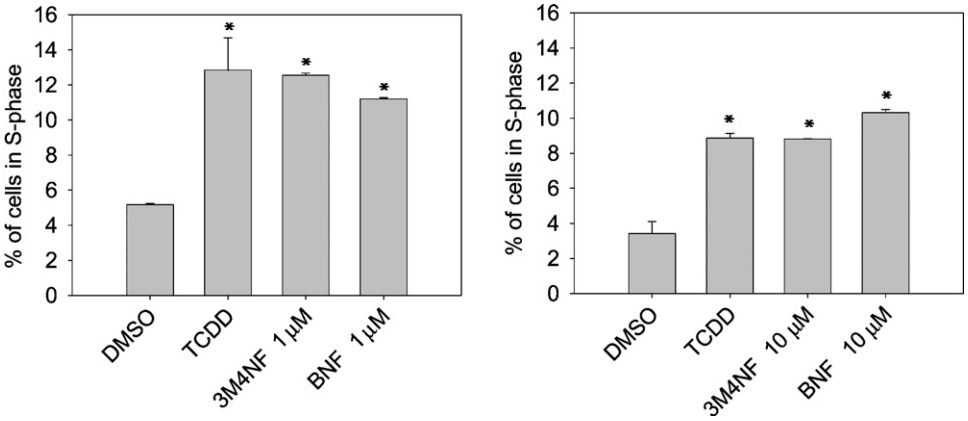

Fig. 6 – 3M4NF and BNF stimulated cell cycle progression. Confluent cultures of WB-F344 cells were exposed to DMSO (0.1%), TCDD, 3M4NF and BNF. After 24 h, cells were trypsinized, fixed in 70% ethanol and DNA content was determined by flow cytometry as described in Section 2. The percentage of cells in S-phase is indicated and represents the mean ± S.D. of three independent experiments. Symbol '*' denotes a significant difference between negative control (0.1% DMSO) and treated samples ($P < 0.05$), as obtained in non-parametric Mann–Whitney *U*-test.

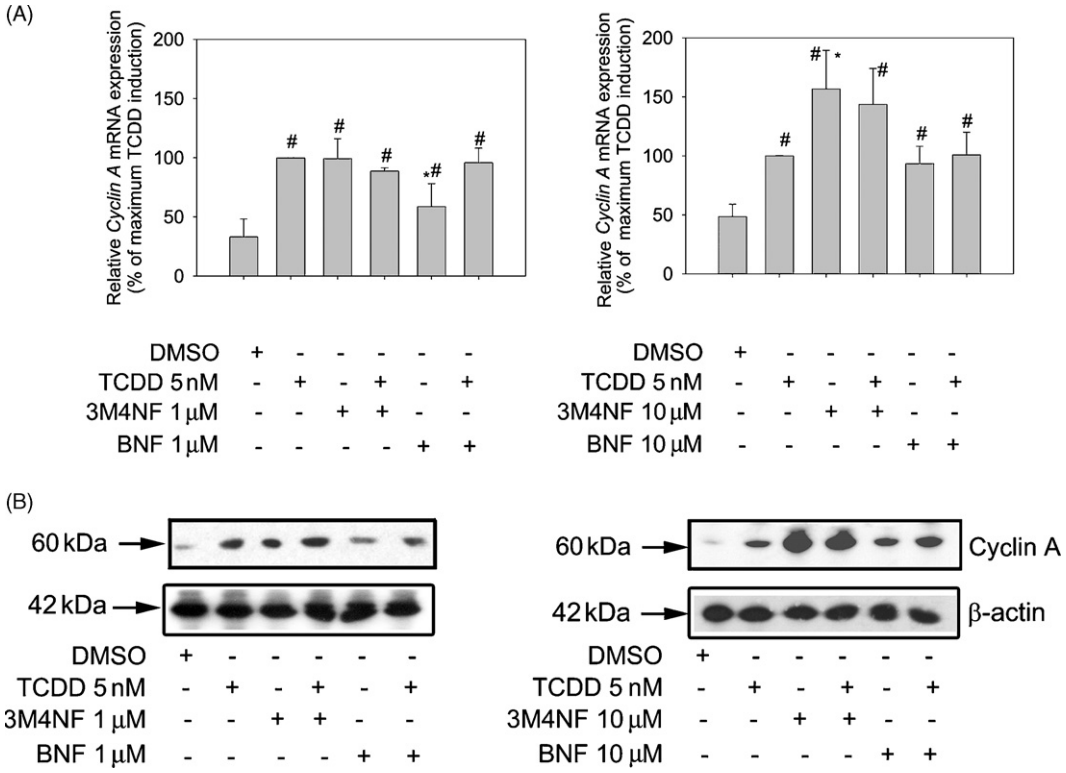

Fig. 7 – Both 3M4NF and BNF increased Cyclin A mRNA and protein levels. WB-F344 cells were exposed for 48 h to DMSO (0.1%), TCDD, 3M4NF and BNF, or the combination of flavones with TCDD. (A) Relative Cyclin A mRNA levels were measured by quantitative RT-PCR and data were analyzed using Mann–Whitney *U*-test. The results represent means ± S.D. of three independent experiments. Symbol '#' denotes a significant difference between negative control (0.1% DMSO) and treated samples ($P < 0.05$). Symbol '*' denotes a significant difference between positive control (5 nM TCDD) and treated samples ($P < 0.05$). (B) Cyclin A and β-actin (loading control) protein levels were determined by Western blotting in total cell lysates. The results are representative of three independent experiments.

A study of Weiss et al. (submitted) has documented that effects of TCDD on cell proliferation in contact-inhibited rat liver epithelial cells are AhR-dependent. Therefore, we used siRNA targeted against AhR, in order to confirm the role of AhR in 3M4NF action on cell proliferation. Cells transfected with AhR-specific or control siRNA (against DsRed protein), as well as untreated control WB-F344 cells, were exposed to DMSO and 3M4NF for 48h and they were futher analyzed for effects of test compounds on cell numbers and Cyclin A, AhR and CYP1A1 protein levels. The transfection with anti-AhR siRNA fully prevented the 3M4NF-induced increase in cell numbers (Fig. 9A). The downregulation of AhR also reduced the 3M4NF-induced Cyclin A and CYP1A1 protein levels (Fig. 9B). These data suggest that effects of 3M4NF in WB-F344 cells are mediated by AhR, which are in agreement with the above results suggesting that 3M4NF acts as AhR agonist, when end-points other than *Cyp1a1* expression are considered.

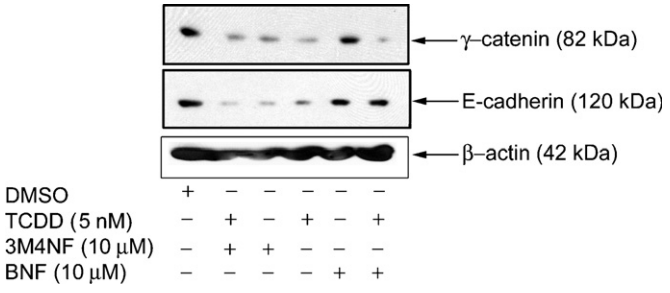

Fig. 8 – 3M4NF decreased γ-catenin and E-cadherin protein levels. Confluent cultures of WB-F344 cells were exposed to DMSO (0.1%), TCDD, 3M4NF and BNF. After 48 h, total cell lysates were prepared and analyzed by Western blotting for γ-catenin (plakoglobin), E-cadherin and β-actin (loading control). The results are representative of three independent experiments.

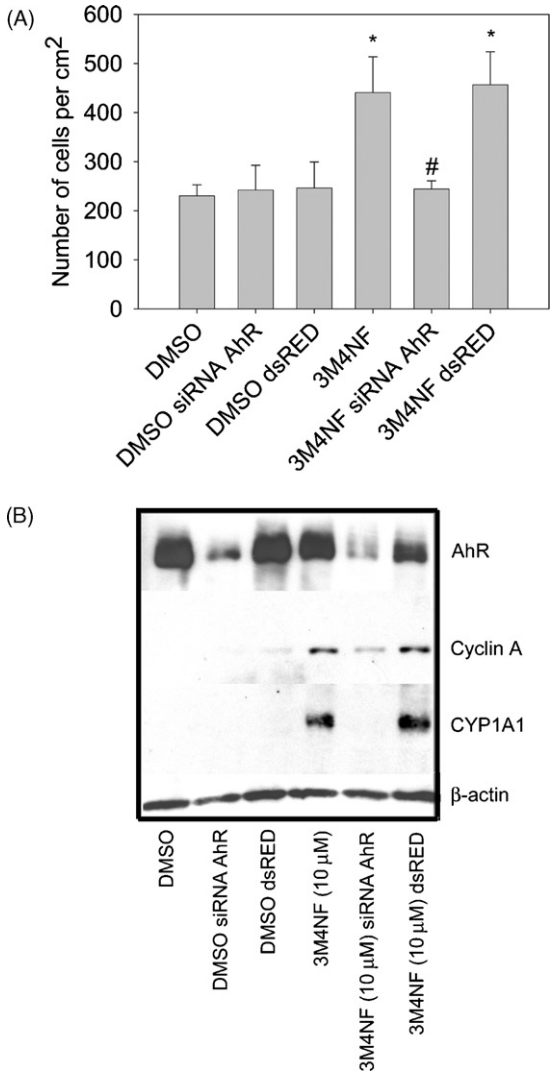

Fig. 9 – siRNA-mediated downregulation of AhR protein suppresses both the 3M4NF-induced expression of CYP1A1 and cell proliferation. WB-F344 cells were transfected either with AhR-specific or non-specific (DsRed) siRNAs as described in Section 2. Transfection mix was removed 24 h later, and cells were cultivated for another 24 h in medium with antibiotics, followed by exposure to 3M4NF (10 μM) or DMSO (0.1%) for 48 h. (A) Cells were harvested by trypsinization and counted with Coulter Counter. The results represent means ± S.D. of three independent experiments. Data were analyzed using non-parametric Mann–Whitney *U*-test. Symbol '*' denotes a significant difference between negative control (0.1% DMSO) and treated samples ($P < 0.05$). Symbol '#' denotes a significant difference between effect of 3M4NF in non-transfected cells and in cells transfected with siRNA ($P < 0.05$). (B) Western blotting was performed on SDS-PAGE fractionated lysates for AhR, Cyclin A, CYP1A1 and β-actin (loading control). The results are representative of three independent experiments.

4. Discussion

Many flavonoids, representing a large group of dietary AhR ligands, appear to act both as AhR agonists and antagonists in a concentration-dependent manner [9]. Several synthetic flavones are therefore being employed in experimental studies as inhibitors of the AhR-mediated activity, with ANF being the most investigated AhR antagonist. Another synthetic flavone, 3M4NF, was shown be an efficient AhR antagonist in MCF-7 cells [8,20], and it was consequently used as AhR inhibitor in a number of studies [5–7,17,21]. However, its antagonistic effects on the AhR have recently become questioned, since 3M4NF has been shown to enhance *Cyp1a1* transcription in mouse hepatoma cells [21]. It was suggested that the agonist/antagonist activity of 3M4NF may depend on species, concentration and the promoter context of a particular AhR-target gene [17,21]. Since the antagonistic effects of flavones, such as 3M4NF or ANF, have been studied almost exclusively within the context of the AhR-dependent regulation of *Cyp1a1* transcription, little is known about their impact on other AhR targets. Therefore, in the present study, rat liver epithelial WB-F344 cells were used as an in vitro model of liver progenitor cells, in order to investigate impact of synthetic flavones on expression of diverse AhR target genes and disruption of cell proliferation control.

3M4NF molecule has been suggested to impair both nuclear translocation of AhR and its transcriptional activation [6,7]. However, in the present study we observed that 3M4NF induces a partial AhR cytosol-nucleus translocation in WB-F344 cells, which was followed by only a limited degradation of AhR. 3M4NF also partially inhibited the TCDD-induced nuclear translocation and subsequent degradation of AhR. 3M4NF was shown to compete with TCDD for AhR receptor and thus preventing TCDD-induced AhR nuclear uptake and AhR degradation [5,7,40]. As the proteasomal degradation of AhR may depend on proportion of AhR translocation to the nucleus and AhR transformation to its active form capable to bind enhancer/promoter regions of AhR target genes [30], the lack of AhR degradation after 3M4NF treatment might be attributed to inefficient transformation to its active conformation. Taken together, effects of 3M4NF on AhR subcellular localization and degradation suggested that it might act both as a weak AhR agonist and antagonist within liver epithelial cells.

Induction of *Cyp1a1* transcription is widely used as a measure of the transcriptional activity of AhR [31], and a majority of information on agonist/antagonist properties of flavonoids is based on studies of their effects on *Cyp1a1* regulation. It was found that a minor part of AhR, which translocated into the nucleus was sufficient to initiate *Cyp1a1* transcription, similar to previously reported data from the mouse hepatoma Hepa1c1c7 cells [21]. Although we did not observe 3M4NF-induced binding of AhR/ARNT to synthetic DRE oligonucleotide, this may be due to a limited sensitivity of the method as the DRE binding induced by 3M4NF is very low [7], or due to the ability of 3M4NF-transformed AhR to bind enhancer/promoter regions only within the chromatin context. In agreement with its effects of AhR translocation and degradation, 3M4NF inhibited the TCDD-induced DRE binding and induction of CYP1A1 mRNA expression. It was also found that high concentrations of BNF transiently inhibited the

induction of CYP1A1 mRNA by TCDD in WB-F344 cells, suggesting that, within the context of *Cyp1a1* regulation, it may behave as a partial antagonist. Our results also suggest that even presumed flavonoid AhR agonists, like BNF, are able to inhibit the effects of TCDD on CYP1A1 mRNA levels in a concentration-dependent manner.

The effects of 3M4NF on AhR-dependent gene expression were suggested to be promoter context-specific [21]; however, this was demonstrated only for luciferase reporter constructs as compared to *Cyp1a1* gene expression. Since nothing is currently known about the impact of 3M4NF on other endogenous AhR gene targets, we selected three AhR-regulated genes, *Cyp1b1*, *Nqo1* and *Cdh13*, which we anticipated to respond differently to flavonoids. It was found that T-cadherin mRNA levels were significantly decreased after 3M4NF treatment, while TCDD only marginally decreased its expression in WB-F344 cells. T-cadherin belongs among non-classic types of cadherins with unclear functions. Evidence that 3M4NF is able to efficiently suppress T-cadherin expression in WB-F344 cells might support the hypothesis that AhR ligands can repress expression of this target gene. However, ANF did not change expression of T-cadherin in vascular smooth muscle cells [36], suggesting that additional regulatory mechanism might be involved in control of T-cadherin expression. Although 3M4NF was a relatively weak inducer of CYP1A1 expression, mRNA of two other AhR-regulated enzymes (NQO1 and CYP1B1) was strongly induced in response to 3M4NF exposure. Thus, agonist/antagonist properties of 3M4NF seem to be gene context-specific and 3M4NF can be an effective inducer of AhR-dependent gene expression. The same may apply to other widely used AhR antagonists, such as ANF.

AhR function is not limited only to the regulation of expression of xenobiotic-metabolizing enzymes, but this receptor plays a role in cell cycle control, regulation of apoptosis and cell proliferation [41–43]. Exposure of hepatoma cells to TCDD results in inhibition of cell proliferation and G1 cell cycle arrest [13,15]. The inhibition of cell proliferation has been attributed both to AhR-dependent transcriptional activation of genes such as $p27^{Kip1}$ cdk inhibitor and to co-repression of E2F activity through association of AhR with pRb protein, resulting in prevention of p300/CBP access to E2F [12–14]. Synthetic flavonoids have been reported to induce a similar effect in rat hepatoma cells, which was, however, AhR-independent [3]. In a marked contrast, various types of AhR ligands induce an increased cell proliferation in quiescent contact-inhibited rat liver epithelial cells [25,26,44]. This effect is associated with an increased Cyclin A expression and increased activity of Cyclin A/cdk2 complex [25], and it is AhR-dependent (Weiss et al., submitted). In the present study, we found that 3M4NF induced a release of non-proliferating WB-F344 cells from contact inhibition, similar to agonist AhR ligands, BNF and TCDD, or to another AhR partial agonist/antagonist ANF. Accordingly, 3M4NF induced a marked increase of Cyclin A expression, even higher than TCDD. Nevertheless, 3M4NF was effective only at much higher concentrations than TCDD.

The loss of contact inhibition is accompanied with deregulation of cell adhesive proteins and proteins mediating communication between cells. It has been reported that both E-cadherin and γ-catenin are highly expressed in contact-inhibited epithelial cells [25,26,44]. We observed a decrease of E-cadherin and γ-catenin expression after 3M4NF treatment, similar to the previously reported effects of TCDD on γ-catenin expression [39]. Transfection with siRNA targeted against AhR prevented both induction of CYP1A1 and induction of cell proliferation, as well as accumulation of Cyclin A, suggesting that proliferative effect of 3M4NF is AhR-dependent. These results suggested that activation of Ah receptor is responsible for the effects of 3M4NF on deregulation of cell cycle in contact-inhibited cells, which is in contrast with the AhR-independent cell cycle arrest induced by high concentrations of synthetic flavones in hepatoma cells [3].

Taken together, our data suggest that 3M4NF has many properties of AhR agonists, including induction of expression of AhR target genes and disruption of cell cycle control in the AhR-dependent manner. Therefore, synthetic flavones should be used with care when studying effects of AhR ligands as the present study stresses the need to study different types of AhR ligands in cell- and gene-specific context. On the other hand, 3M4NF properties seem to make it a suitable tool for elucidation of functions of AhR affected by endogenous or dietary agonists/antagonists, which may be different from those of toxic AhR ligands, such as TCDD. It may thus provide more insight into the possible physiological role of AhR in regulation of cell cycle and cell-to-cell communication.

Acknowledgements

Authors thank Dagmar Faust and Cornelia Dietrich (Institute of Toxicology, Johannes Gutenberg University, Mainz, Germany) for the help with establishing siRNA transfection protocol, and Thomas A. Gasiewicz (University of Rochester School of Medicine and Dentistry, NY) and Josef Abel (Heinrich Heine University, Düsseldorf, Germany) for providing 3M4NF. This study was supported by grant no. KJB6004407 from the Grant Agency of the Academy of Sciences of the Czech Republic and by grant no. 524/06/0517 from the Czech Science Foundation. Institutional support was provided by the Research Plan of the Academy of Sciences of the Czech Republic (no. AVOZ50040507), and by the Czech Ministry of Agriculture (no. MZE0002716201).

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