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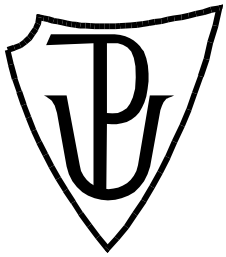

Construction of stably transfected human cell lines for toxicological and environmental applications

PhD. THESIS

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I hereby declare the presented thesis is based on my own research carried out at the Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Olomouc, in the period September 2009 – March 2013. Co-authors agree with the inclusion of published results.

Olomouc ..... ……..

Mgr. Aneta Novotná

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Novotna A., Doricakova A., Pavek P. and Dvorak Z. Construction and characterization of peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α over-expressing cell line derived from human hepatocyte carcinoma HepG2 cells). *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012*, [Epub ahead of print]. **[IF_{2011} 0,702]**

Novotna A., Pavek P. and Dvorak Z. Construction and characterization of a reporter gene cell line for assessment of human glucocorticoid receptor activation. *Eur J Pharm Sci. 2012*, **47** :842-7. **[IF_{2011} 3,212]**

SUMMARY

Cell-based *in vitro* models are invaluable tools in the development of new drugs, evaluation of drug-drug interactions with already known compounds, as well as for a better understanding of metabolism pathways of various toxicants and pollutants. Since the liver is the major site of metabolism for the majority of xenobiotics, most *in vitro* models are liver-derived systems including subcellular fractions, integral physiological cellular models such as primary human hepatocytes and engineered systems such as immortalized hepatocytes or liver-derived cell lines.

The present thesis focuses on the generation of stable transfected human cell lines for toxicological, pharmacological and environmental applications. In the first part of the thesis, AZ-AhR cell line for the assessment of transcriptional activity of the aryl hydrocarbon receptor (AhR) and AZ-GR cell line for the measurement of the glucocorticoid receptor (GR) transcriptional activity, were constructed and characterized. The AZ-AhR cell line was derived from HepG2 cells transfected with a reporter plasmid containing AhR-responsive sequences of the mouse *Cyp1a1* gene. The resulting AZ-AHR cells showed responsiveness to AhR ligands including TCDD (2,3,7,8-tetrachlorodibenzodioxin), 3-methylcholantrene, omeprazole, indirubine, SP600125 (1,9-pyrazoloanthrone) and resveratrol, yielding a dose- and time-dependent induction of luciferase activity. The assay was very sensitive allowing a high throughput format (96-well plate) and evaluation of luciferase activity immediately after 6 h. AZ-AHR cells remained fully functional over 15 passages and 30 days in culture, and the response of cryopreserved cells after thawing, to AhR ligands was not significantly different from that of fresh cells. The AZ-GR cell line was derived from HeLa cells transfected with reporter plasmid containing three copies of glucocorticoid response elements. A panel of 22 natural and synthetic steroids was tested in AZ-GR cells. The resulting cell line AZ-GR was highly specific and sensitive to glucocorticoids and there was no responsiveness to estrogens, gestagens or androgens was observed. Cells displayed very low responsiveness to mineralocorticoids, but the potency and efficacy of mineralocorticoids was much lower than those glucocorticoids. The sensitivity of the assay allowed a high throughput format of the analyses, using 96-well plates. Time-course analyses revealed that GR activators may be detected using AZ-GR cells soon after 14 hours of treatment. A functionality of the AZ-GR cells was not affected by the cryopreservation process and the cells fully maintained responsiveness to glucocorticoids for 32 days in the culture and over 16 passages without significant alterations.

The second part of the thesis is focused on a construction and characterization of hepatocyte-like cells with the aim to restore liver-specific cell functions, in particular, the biotransformation capacity of human hepatoma cells HepG2. Two transgenic human hepatoma cell lines derived from HepG2 cells, over-expressing transcriptional factors HNF4α or PGC-1α were developed. Stable over-expression of HNF4α in HepG2 cells improved some hepatospecific functions including secretion of the plasma proteins albumin, plasminogen and fibrinogen and increased expression of the xenoreceptors PXR and AhR. Dioxin-inducible expression of CYP1A1 was moderately higher than in parent HepG2 cells. Basal expression of CYP3A4 protein was increased, but rifampicin-inducible expression of CYP3A4 was lost in HepG2-HNF4α cells. Stable over-expression of PGC-1α in HepG2 cells increased secretion of fibrinogen but not albumin and α1-antitrypsin in all tested clones of HepG2-PGC-1α cells. We found, that over-expression of PGC-1α, and consequently increased expression of HNF4α protein, positively correlated with increased levels of PXR and AhR proteins in HepG2-PGC-1α. Dioxin-inducible expression of CYP1A1 mRNA and protein was not significantly altered compared to the parent HepG2 cells. Basal expression of CYP3A4 protein was increased in HepG2-PGC-1α cells, but rifampicin-inducible expression of CYP3A4 was consequently lost.

In conclusion, stable transfected luciferase reporter gene cell lines AZ-AHR and AZ-GR allow quick, sensitive and high throughput format of analysis of tested compounds and have potential use in pre-clinical research, drug discovery, food safety and environmental applications. HepG2 derived cell lines over-expressing transcriptional factors HNF4α and/or PGC-1α displayed some improved hepatospecific functions, such as the secretion of plasma proteins or increased expression of xenoreceptors PXR and AhR. However, xenobiotic-inducible expression of CYP1A1 and CYP3A4 was not substantially improved, implying that the approach of stable over-expression of hepatospecific transcription factors only partly addressed the issue of the generation of hepatocyte-like cell lines for pre-clinical testing. These cell lines have potential use in basic research of hepatocyte-specific cell functions.

SOUHRN

Buněčné *in vitro* modely jsou neocenitelným nástrojem využívaným při vývoji nových léčiv, hodnocení lékových interakcí již známých látek i pro lepší pochopení metabolismu toxických látek a polutantů. Jelikož jsou játra hlavním místem metabolismu xenobiotik, většina *in vitro* modelů jsou od jater odvozené systémy zahrnující subcelulární frakce, integrální fyziologické buněčné modely jako primární lidské hepatocyty a konstruované systémy zahrnující imortalizované hepatocyty nebo buněčné linie odvozené od jaterních buněk.

Předkládaná práce se zaměřuje na vytváření stabilně transfekovaných lidských buněčných linií pro toxikologické, farmaceutické a environmentální aplikace. V první části disertace byly připraveny a charakterizovány buněčná linie AZ-AHR pro hodnocení transkripční aktivity aryl uhlovodíkového receptoru (AhR) a buněčná linie AZ-GR umožňující měření transkripční aktivity glukokortikoidního receptoru (GR). Buněčná linie AZ-AHR byla odvozena od HepG2 buněk transfekcí s reportérovým plazmidem obsahujícím AhR-responzivní sekvence myšího genu *CYP1a1*. AhR ligandy zahrnující TCDD (2,3,7,8-tetrachlorodibenzodioxin), 3-metylcholantren, omeprazol, indirubin, SP600125 (1,9-pyrazoloantrone) a resveratrol indukovaly v AZ-AHR buňkách luciferázovou aktivitu v závislosti na koncentraci a čase. Linie byla vysoce senzitivní, což umožnilo „high throughput“ testování látek v 96-jamkových deskách a stanovení luciferázové aktivity již po 6 hodinách inkubace. Buňky zůstaly v kultuře plně funkční 30 dní, což odpovídá 15 pasážím. Odpověď kryoprezerovaných buněk na AhR ligandy po rozmrazení se od čerstvých buněk výrazně nelišila. Buněčná linie AZ-GR byla odvozena od HeLa buněk transfekcí s reportérovým plazmidem obsahujícím tři kopie glukokortikoidního responzivního elementu. Panel 22 přírodních a syntetických steroidů byl využit k charakterizaci připravené buněčné linie. AZ-GR linie byla vysoce specifická a senzitivní ke glukokortikoidům a nepozorovali jsme žádnou odpověď po inkubaci s estrogeny, gestageny a androgeny. Velmi nízkou luciferázovou aktivitu jsme zaznamenali po inkubaci s mineralokortikoidy, ale jejich síla a účinnost byla v porovnání s glukokortikoidy zanedbatelná. Buněčné linie AZ-GR je vysoce senzitivní a umožňuje „high throughput“ testování s použitím 96-jamkových desek. Analýzy v závislosti na čase ukázaly, že GR aktivátory mohou být detekovány již po 14 hodinách inkubace. Kryoprezervace nemá vliv na funkčnost buněk, které jsou plně responzivní na glukokortikoidy 32 dní v kultuře, což odpovídá 16 pasážím.

Druhá část disertace byla zaměřena na konstrukci a charakterizaci „hepatocyte-like“ buněčných linií s cílem obnovit specifické funkce jaterních buněk, především biotransformační kapacitu, v lidské hepatomové linii HepG2. Byly vyvinuty dvě

transgenní lidské hepatomové linie odvozené od HepG2 buněk over-exprimující transkripční faktory HNF4α nebo PGC-1α. Stabilní over-exprese HNF4α v HepG2 buňkách zlepšila některé hepatospecifické funkce včetně sekrece plasmatických proteinů albuminu, plasminogenu a fibrinogenu a zvýšené exprese xenoreceptorů PXR a AhR. Dioxin-inducibilní exprese CYP1A1 byla mírně vyšší než v parentních HepG2 buňkách. Bazální exprese CYP3A4 proteinu byla zvýšená, ale rifampicin-inducibilní exprese CYP3A4 nebyla v linii HepG2-HNF4α detekována. Stabilní over-exprese PGC-1α v HepG2 buňkách zvýšila sekreci fibrinogenu, ale ne albuminu a α1-anti-trypsinu ve všech testovaných klonech HepG2-PGC-1α. Zjistili jsme, že over-exprese PGC-1α, a v důsledku toho zvýšená exprese proteinu HNF4α, pozitivně koreluje se zvýšenou hladinou proteinů PXR a AhR v linii HepG2-PGC-1α. Dioxin-inducibilní exprese CYP1A1 mRNA a proteinu se výrazně nezměnila v porovnání s parentními HepG2 buňkami. Bazální exprese proteinu CYP3A4 byla zvýšená, ale rifampicin-inducibilní exprese CYP3A4 nebyla v buněčné linii HepG2-PGC-1α detekována.

Závěrem lze konstatovat, že stabilně transfekované reportérové buněčné linie AZ-AHR a AZ-GR umožňují rychlé a senzitivní analýzy testovaných látek s využitím „high throughput“ testování a mají potenciální využití v preklinickém výzkumu, vývoji léčiv, environmentálních aplikacích a sledování bezpečnosti potravin. Buněčné linie odvozené od HepG2 buněk over-exprimující transkripční faktory HNF4α nebo PGC-1α vykazují zlepšení některých hepatospecifických funkcí, jako je sekrece plasmatických proteinů nebo zvýšená exprese xenoreceptorů PXR a AhR. Nicméně, xenobiotiky indukovatelná exprese CYP1A1 a CYP3A4 nebyla výrazně zlepšena, což znamená, že stabilní exprese hepatospecifických transkripčních faktorů řeší pouze částečně otázkou přípravy „hepatocyte-like“ buněčných linií pro pre-klinické testování. Tyto linie mají potenciální využití v základním výzkumu jaterně specifických buněčných funkcí.

CONTENT

ABBREVIATIONS

3MC	3-methylcholanthrene
ADME	Absorption, distribution, metabolism, and excretion
AhR	Aryl hydrocarbon receptor
AHRR	Aryl hydrocarbon receptor repressor
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATF2	Activating transcription factor 2
C/EBPα	CCAAT enhancer binding protein α
CALUX	Chemical-activated luciferase expression
CAR	Constitutive androstane receptor
CBP/p300	(cAMP-response-element-binding protein)-binding protein
CREB	cAMP response element-binding protein
CYP P450	Cytochrome P450
DBD	DNA binding domain
DEX	Dexamethasone
DMSO	Dimethylsulfoxide
DR	Direct repeat
DRE	Dioxin response element
ED	Endocrine disruption
EDC	Endocrine disrupting chemical
EROD	Ethoxyresorufin-O-deethylase
FOXO-1	Forkhead box O1
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCs	Glucocorticoids
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GRE1/2s	Glucocorticoid response element half-sites
GRUs	Glucocorticoid responsive units
GST	Gluthathione S-transferase
HAHs	Halogenated aromatic hydrocarbons
HNF4α	Hepatocyte nuclear factor 4α
HPA	Hypothalamic–pituitary–adrenal
Hsp90	Heat-shock protein 90 kDa
IND	Indirubin

LBD	Ligand binding domain
MR	Mineralococrticoid receptor
NCoR	Nuclear receptor co-repressor
nGREs	Negative glucocorticoid response elements
NHR	Nuclear hormone receptor
NLS	Nuclear localization signal
NR	Nuclear receptor
OME	Omeprazole
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1α	Peroxisome proliferator-activated receptor-gamma co-activator 1 alpha
POPs	Persistent organic pollutants
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PRC	PGC-1-related co-activator
PXR	Pregnane X receptor
RE	Response element
RIF	Rifampicin
RVT	Resveratrol
RXR	Retinoid X receptor
SHP	Short heterodimer partner
SMRT	Silencing mediator of retinoid and thyroid receptors
SP600125	1,9-pyrazoloanthrone
SRC	Steroid receptor co-activator
TCDD	2,3,7,8- tetrachlorodibenzo-p-dioxin
TR	Thyroid receptor
UGTs	uridin-5´-diphosphate-glucuronosyltransferases
VDR	Vitamin D receptor
XAP2	Hepatitis B-virus X-associated protein 2

INTRODUCTION

Xenobiotics are compounds foreign to the living organism and include drugs, industrial chemicals, pesticides, pollutants, pyrolysis products in cooked food, alkaloids, secondary plant metabolites, toxins produced by moulds or plants and others. Some xenobiotics, such as persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dioxins and polychlorinated dibenzofurans are resistant to degradation. These compounds are introduced into the food chain as well as into the soil, water, air and accumulate there. Consequently, they can enter the human body and they may interact with diverse signaling pathways and produce a variety of biological effects, for instance immunological reactions, toxicity or cancer. With regard to the biological effects of xenobiotics, it is of topical interest to monitor their levels in the environment and to obtain as much information as possible about their toxic action in the body, including the dose-response effects, receptor interactions, mechanisms of toxicity as well as absorption, distribution, metabolism, and excretion (ADME).

Many *in vitro* models are used in the research of xenobiotic metabolism as well as in the study of hepatotoxicity and hepatocellular functions. The liver is the main site of xenobiotic metabolism therefore the most frequently used models are liver-derived *in vitro* systems. Primary human hepatocytes are valuable *in vitro* models for xenobiotic metabolism studies. They express the majority of cytochrome P450 (CYP) enzymes involved in the metabolism of exogenous compounds and are capable of generating a metabolic profile of tested compounds similar to that found *in vivo* (Ponsoda et al., 2001). Nevertheless their utilization is seriously complicated for several reasons including the restricted availability of fresh human liver, *in vitro* phenotypic instability and the limited life-span in the culture (Donato et al., 2008). On the other hand, the human liver-derived cell lines show a stable phenotype, an almost unlimited lifespan and continuous growth in the culture. The hepatic cell lines are easily available and culture conditions are simpler and easily standardized among laboratories. The major drawback of hepatoma cell lines is the low biotransformation capacity caused by a significantly lower expression of crucial CYP P450 genes in comparison t primary hepatocytes.

Reporter gene assays are frequently used as *in vitro* models for various environmental applications, pre-clinical research, screenings and drug discovery. These systems that are based on the transiently or stably transfected cells with the reporter plasmid containing a key regulatory sequence upstream the reporter gene and

allow the quick measurement of the expression of the genes involved in xenobiotic metabolism.

1. THEORETICAL PART

1.1 Nuclear receptors

Various mechanisms are involved in the regulation of drug-metabolizing enzymes. Besides the post-transcriptional regulation, post-translation regulation or proteasome - dependent protein degradation, the most common mechanism is a transcriptional regulation by receptors acting as sequence-specific ligand-activated transcriptional factors (Lekas et al., 2000; Raffalli-Mathieu et al., 1997; Zangar et al., 2002). The discovery of xenobiotic nuclear receptors (xenoreceptors) was an important milestone in the research of drug-drug interactions involving enzyme induction by xenobiotics.

The superfamily of nuclear receptors consists of a diverse array of transcription factors, which include nuclear hormone receptors (NHRs) and orphan nuclear receptors (Banner et al., 1993; Bertilsson et al., 1998; Forman et al., 1998). NHRs are receptors for which hormonal ligands have been identified, whereas orphan receptors are so named because their ligands are unknown, at least at the time the receptor is identified. Members of the nuclear receptor superfamily share a common structural organization (Fig. 1). The N-terminal region (A/B domain) is highly variable and contains at least one constitutionally active transactivation region (AF-1) and several autonomous transactivation domains (AD). The most conserved region is the DNA-binding domain (DBD; C domain), which is responsible for targeting the receptor at highly specific DNA sequences comprising a response element (RE). The largest domain is the moderately conserved ligand-binding domain (LBD; E domain) that controls many functions; mostly a ligand induced AF-2 transactivation, a strong dimerization interface and often a repression function. Between the DNA-binding and the ligand binding domain is a less conserved region (D domain) that behaves as a flexible hinge and contains the nuclear localization signals (NLSs). NLS are recognized by a group of nuclear translocation proteins that actively shuttle the receptor into the nucleus. Nuclear receptors may contain F domain, a final domain in the C-terminus of the E domain (Berg, 1989; Burbach et al., 1992).

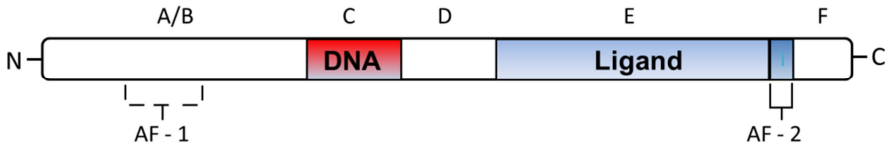

Figure 1: Nuclear receptor structure. [According to (Mangelsdorf et al., 1995)]. Members of the nuclear receptor superfamily consist of six different modular domains. The A/B domain in the N-terminal region containing transactivation region AF-1; the C domain represents the DNA-binding domain; the D domain contains a nuclear localization signal; the largest E domain with a ligand binding site; and the F domain.

So far, 48 nuclear receptors have been identified. In the transcriptionally active state, they form homodimers or heterodimers with each partner binding to specific RE sequences that exist as repetitive binding sites separated by variable length nucleotide spacers between direct or inverted binding site repeats. Several years ago, the classification of nuclear receptors was proposed by dividing them into four categories according to their transcription mechanism. Class 1 of NR is composed of steroid hormone receptors that control target gene transcription by binding as homodimers to RE palindromes. Representatives of class I are steroid receptors including ER (estrogen receptor), AR (androgen receptor), GR, MR (mineralocorticoid receptor) and PR (progesterone receptor). In class 2, the nuclear receptors heterodimerize with RXR (retinoid X receptor) and generally bind to direct repeat REs such as TR (thyroid hormone receptor), VDR (vitamin D receptor), FXR (farnesoid X receptor also called bile acid receptor), PPAR (peroxisome proliferator-activated receptor) and others. Class 3 consists of those orphan receptors that function as homodimers or heterodimers and bind to direct repeat REs, for instance PXR (pregnane X receptor), CAR (constitutive androstane receptor). Orphan receptors in group 4 function as monomers and bind to a single RE (Jacobs et al., 2003; Mangelsdorf et al., 1995).

1.1.1 AhR

The first xenoreceptor discovered was AhR that is often called a dioxin receptor according to its model activator dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD). AhR is not considered as a true nuclear receptor, but it is a member of the bHLH/PAS (basic helix-loop-helix/ PER ARNT Sim) family of transcriptional factors. Generally, constitutive/basal AHR levels are high in the liver but AhR is also abundant in diverse mammalian tissues such as the placenta, thymus, lung, kidney, small intestine, heart and pancreas (Dolwick et al., 1993; Mason & Okey, 1982). AhR regulates genes involved in drug metabolism such as CYP1A1, CYP1A2, CYP1B1, GSTs (gluthathione S-transferase), UGTs (uridin-5´-diphosphate-glucuronosyltransferases) and NADHP

reductase, but it also controls genes involved in various endogenous functions, for instance cell cycle and proliferation (TGFβ, IL-1β, p27), apoptosis (Bax, Jun), immune response or circadian rhythm (Matikainen et al., 2001; Richardson et al., 1998; Sutter et al., 1991; Zaher et al., 1998). It has been also demonstrated that AhR plays role in tumor-promotion in the absence of environmental toxic chemicals (Opitz et al., 2011).

In addition to the link between AhR and endogenous functions, evidence demonstrates the important role of AhR in normal physiology and development. AhR has been highly conserved throughout the evolution across vertebrate species from marine, terrestrial, and avian environments. Moreover, the up-regulated AhR target genes including orthologues of the *Cyp1* family of monooxygenases have been identified (Goldstone & Stegeman, 2006; Walker et al., 2000). Further, research on AhR mutant mice has shown developmental aberrations and pathological end points of experimental animals (Schmidt et al., 1996). Finally, many studies have reported the expression of DRE responsive genes *CYP1a1* and *CYP1b1* during embryogenesis in mice and in humans (Campbell et al., 2005; Choudhary et al., 2003; Omiecinski et al., 1990).

Besides the roles of AhR mentioned above, AhR mediates the chemical toxicity and carcinogenic effects of many exogenous compounds including planar hydrophobic halogenated aromatic hydrocarbons (HAHs) (polyhalogenated dibenzo-*p*-dioxins, dibenzofurans and biphenyls) and polycyclic aromatic hydrocarbons (PAHs) (such as 3-methylcholanthrene, benzo[*a*]pyrene). This wide ligand spectrum is represented abundantly in our ecosystems by cigarette smoke, combustible products, contaminated food (dioxins, PAH, PCBs) and natural food products (polyphenols, metabolites derivatives). As a consequence, human populations are exposed daily to AhR ligands.

Natural compounds (flavonoids, resveratrol) and drugs (omeprazole, lansoprazole, TSU-16) also belong to the group of AhR activators. Endogenous AhR ligands are indigo, indirubin, indole, bilirubin, biliverdin, tryptophan derivatives and arachidonic acid derivatives.

1.1.1.1 Mechanism of AhR action

In the resting state, AhR is sequestered in the cytosol in a multiprotein complex with two molecules of Hsp90 (heat-shock protein 90 kDa), the Hsp90-interacting protein p23 and the immunophilin-like protein ARA9 also termed XAP2 (hepatitis B-virus X-associated protein 2) (Carver & Bradfield, 1997; Denis et al., 1988; Nair et al., 1996). Upon the binding of a lipophilic ligand, AhR undergoes conformation changes and it is translocated to the nucleus, where it forms heterodimer with an aryl hydrocarbon receptor nuclear translocator (ARNT) (Pollenz et al., 1994). Heterodimer

AhR/ARNT binds the specific sequences called a dioxin responsive element (DRE) (consensus sequence 5´-T/GNGCGTGA/CG/CA-3´; core sequence 5´-GCGTG-3´) on the promoter of response genes and triggers the expression of CYP1A1, CYP1A2 and CYP1B1 (Fig. 2) (Lusska et al., 1992; Neuhold et al., 1989; Whitlock et al., 1989).

AhR transcriptional activity is modulated by interactions with other nuclear proteins including basal transcription factors, co-activators, co-repressors and components of other signaling pathways. The interactions between the AHR/ARNT and transcription factors (e.g. TFIIB, TFIIE), TATA binding protein and co-activators (e.g. SRC1, RIP140, p300, GRIP1, BRG-1) are thought to facilitate gene activation of the AHR/ARNT heterodimer (Kobayashi et al., 1997; Kumar & Perdew, 1999; Kumar et al., 1999; Rowlands et al., 1996; Swanson, 2002).

On the other hand, the interactions of AHR/ARNT heterodimer with the co-repressor proteins SMRT (silencing mediator of retinoid and thyroid receptors) and SHP (short heterodimer partner) inhibit AhR activity. In addition, the inhibition of AhR signaling is mediated by AhRR (AhR repressor) that competes with AhR for heterodimer formation with the ARNT and the subsequent binding to the DRE sequence (Baba et al., 2001; Watanabe et al., 2001). Another mechanism of negative regulation is the degradation of AhR by a 26S proteasome pathway upon ligand binding and nuclear transport (Davarinos & Pollenz, 1999).

An important regulating step in AhR signaling pathways is phosphorylation that controls transcriptional activity, subcellular distribution and the stability of AhR. Phosphorylation status of either the AHR or ARNT has an impact on AhR/ARNT heterodimer formation and on its binding to DNA. Phosphorylation of tyrosine residues plays a critical role in the ability of the AHR/ARNT heterodimer to bind to DNA. Phosphorylation at serine/threonine residues of either the AHR or ARNT regulates events that occur following DNA binding and the steps presumably involved in transcriptional activation (Li & Dougherty, 1997; Park et al., 2000; Swanson, 2002).

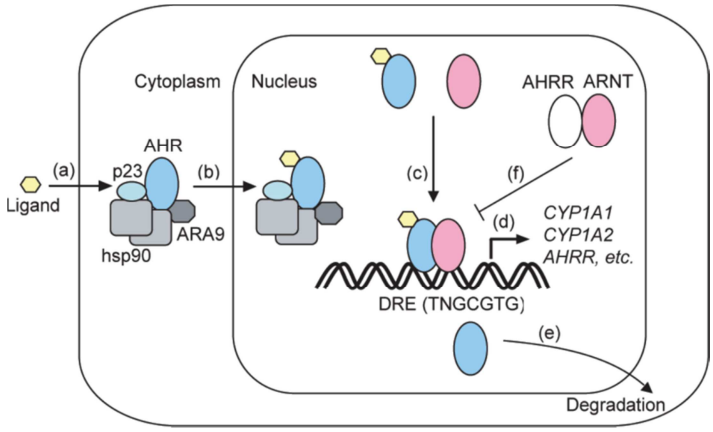

Figure 2: AhR activation. Adopted from (Nguyen & Bradfield, 2008). In resting state, AhR is sequestered in the cytosol in a multiprotein complex with chaperones. Upon ligand binding, AhR translocates to the nucleus and forms a heterodimer with the AhR-nuclear translocator (ARNT). The heterodimer AhR/ARNT binds to the promoter sequence called dioxin or xenobiotic responsive element (DRE/ XRE) and triggers the expression of the target genes (for instance CYP1A1 and CYP1A2).

1.1.1.2 AhR and endocrine disruption

Endocrine disruption (ED) refers to the ability of endocrine disrupting chemicals (EDCs) which interfere with hormonal systems that are needed for normal development and with the function of several important systems in the body, such as reproductive organs, the cardiovascular system, the immune system, brain and bones. EDCs actions contribute to wide range of metabolic diseases including type II diabetes and obesity, cardiovascular diseases or hormone related cancers (breast, testicular, uterus and prostate).

AhR ligands such as dioxin-like compounds and persistent organic pollutants including pesticides, can act as EDCs and induce dysregulation of several hormone signaling pathways, in particular estrogen, androgen, retinoid and thyroid hormones. Since the majority of these compounds show principally strong antiestrogenic effects, the examples of ED mediated via AhR presented in this part are related particularly to ER. AhR activators can affect estrogen receptor functions through interaction with AhR by several mechanisms.

(i) Ligand activated AhR modulates positively or negatively functions of estrogen and androgen receptors by a mechanism employing interference with ER/AR activity through direct protein degradation in proteasome (Ohtake et al., 2007; Ohtake et al., 2009).

(ii) There is a competition between the steroid hormone receptors and AhR for common available co-regulators. Activated AhR can recruit co-activators shared by

activated ER, including SRC-1 and SRC-2 (steroid receptor co-activator 1 and 2) and thereby inhibit ER transcriptional activity (Beischlag et al., 2002). It has also been reported that ARNT can act as an ER co-activator and competition between ER and AhR for ARNT is partly responsible for the anti-estrogenic properties of the TCDD (Brunnberg et al., 2003; Ruegg et al., 2008).

(iii) AhR may bind directly to sequences called inhibitory DRE (iDRE) on the target gene promoter of steroid receptors. This kind of interaction has been found between iDREs localized on the promoter of ER target genes such as c-fos, cathepsin D or hsp27 (Ahmed et al., 2009; Duan et al., 1999; Krishnan et al., 1995; Porter et al., 2001).

(iv) AhR ligands (i.e. EDCs) cause dysregulation of hormone metabolism. EDCs have been described as interfering with hormone biosynthesis, transport of the hormone to the target tissue and levels of hormone binding proteins. CYP P450 enzymes are involved in hormone catabolism, in particular CYP3A4, CYP1A1, CYP1A2 and CYP1B1 and therefore CYPs activation upon exposure to xenobiotics can lead to increased hormone catabolism, and consequently, compromise hormone signaling.

1.1.2 Glucocorticoid receptor

Glucocorticoid receptor (GR; NR3C1)) belongs to the subfamily of nuclear hormone receptors. It is a pivotal factor that mediates numerous cell-, tissue- and organ-specific biological functions as a response of organisms to glucocorticoid hormones (glucocorticoids; GCs), e.g. the development, growth, behaviour, apoptosis and the metabolism of glucose, proteins and lipids. Glucocorticoids, stress-induced steroid hormones, are synthesized in the adrenal cortex under the control of the hypothalamic–pituitary–adrenal (HPA) axis (Barnes, 1998; Sapolsky et al., 2000). Clinically, glucocorticoids represent one of the most commonly prescribed drugs worldwide, effectively used for their anti-inflammatory or immune-suppressive effects in asthma, dermatitis, rheumatoid arthritis, prevention of graft rejection, and autoimmune diseases (Coghlan et al., 2003; Kirkham et al., 1991; Zhou & Cidlowski, 2005). Synthetic (exogenous) glucocorticoids include dexamethasone, beclomethasone, betamethasone, triamcinolone etc. The most important physiological (endogenous) ligand of human GR is cortisol (hydrocortisone).

The human GR gene consists of 777 amino acids and contains 10 exons. Exon 1 is an untranslated region, exon 2 codes for the immunogenic domain (A/B), exon 3 and 4 are for the DNA-binding domain (C) and exons 5–9 are for the hinge region (D) and the ligand-binding domain (E). GR does not contain an F region, in contrast to the other steroid hormone receptors. The human GR gene contains two terminal exons 9

(exon 9α and 9β) of which alternative splicing generate two main GR isoforms, GRα and GRβ (Fig. 3) (Hollenberg et al., 1985). The transcriptionally active isoform that binds glucocorticoids is GRα, whereas GRβ does not bind the ligands and acts as a dominant-negative GRα inhibitor (Bamberger et al., 1995; Oakley et al., 1996). In addition to GRα and GRβ, there have been identified multiple isoforms of GR that are generated from the common hGR precursor RNA by the mechanism of alternative splicing. Isoforms GRγ, GR-A and GR-P play a role, at least partly, in differential glucocorticoid-induced responsiveness, particularly as it relates to human diseases such as acute lymphoblastic leukaemia or multiple myeloma (Beger et al., 2003; Moalli et al., 1993). GR is expressed in all tissues and regulates gene expression in a cell-type-specific manner (Gross & Cidlowski, 2008).

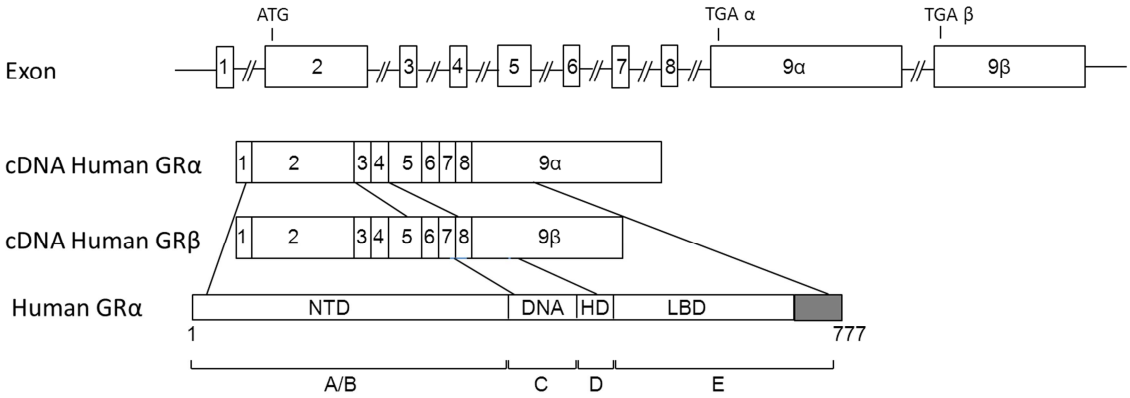

Figure 3: Genomic DNA, protein structure and functional organization of GR. Taken from (Kino & Chrousos, 2011). Human GR consists of 9 exons. The alternative splicing of exon 9 generates two main isoforms GRα and GRβ. Locations of several functional domains are indicated. AF-1 and -2 - activation function 1 and 2; DBD - DNA-binding domain; HD - hinge region; LBD - ligand-binding domain; NTD - N-terminal region, NL1 and 2 - nuclear localization 1 and 2.

1.1.2.1 Mechanism of GR action

The inactive form of GR is sequestered in the cytosol in complex with two molecules of Hsp90. Following binding of GC, the receptor undergoes a series of conformational changes, leading to its dissociation from the cytoplasmic chaperones and the exposure of its nuclear localization signals. These signals are then recognized by nuclear translocation proteins, which actively shuttle the receptor into the nucleus. In the nucleus, GR forms homodimer that binds to the regulating sequences of responsive genes and alters the gene expression.

Four types of GR binding sites have been distinguished. Glucocorticoid response elements (GREs), glucocorticoid response element half-sites (GRE1/2s) and glucocorticoid responsive units (GRUs) are involved in the activation of gene

expression. A consensus GRE has been defined as the pentadecameric imperfect palindrome GGTACAnnnTGTTCT. The 5´ half, however, shows a high degree of flexibility. The flexibility does not necessarily mean a reduced response to GCs, but the positions -3, -2, +2, +3 and +5 in the GRE (nucleotide numbering relative to the dyad axis of symmetry) are of critical importance to proper GR transcriptional activity (Cairns et al., 1991; Nordeen et al., 1990). Although these GREs exhibit a good match to the consensus GRE, glucocorticoid induction is only moderate.

The transcriptional activity of GR does not solely depend on GR binding to GRE, but additionally requires the binding of other transcription factors to adjacent binding sites to result in stronger glucocorticoid-mediated induction. These response elements referred to as GRUs that are both spatially and functionally clustered were found in promoters of several genes, for instance phosphoenolpyruvate carboxykinase (PEPCK), 6-phosphofructo-2-kinase and phenylethanolamine N-methyltransferase (Scott et al., 1998; Schoneveld et al., 2004; Tai et al., 2002; Zimmermann et al., 1997). When looking at a subset of hepatic genes involved in gluconeogenesis, regulatory regions contain, besides the presence of GREs, also binding sites for the liver-enriched transcription factors C/EBPα (CCAAT enhancer binding protein α) and FoxA (McGrane et al., 1990). Other studies of human CYP genes have shown that CYP2C9 is transactivated by dexamethasone in the presence of the liver-enriched transcriptional factor HNF4α (hepatocyte nuclear factor α). It has been reported that CYP2C9 in placental cells lacking HNF4α was transactivated by dexamethasone only after co-transfection with HNF4α expression vector (Pavek et al., 2007). Likely, these transcription factors fulfil specific regulatory tasks within this class of genes.

GR can also bind DNA as a monomer to a GRE half site (GRE1/2), however, GRE1/2s alone are not responsible for glucocorticoid response and require additional elements or they may act without accessory elements by making use of multiple GRE1/2s. Two GRE1/2s (TGTTCT), separated by 160bp in the promoter of the human CYP3A5 gene that are responsible for glucocorticoid - dependent gene regulation have been found (Schoneveld et al., 2004; Schuetz et al., 1996).

Direct GR binding to negative response elements (nGREs) in the promoter region of the target gene represses gene expression. The sequence of nGREs shows the similarity with GREs although the consensus sequence of an nGRE is more variable (ATYACnnTnTGATCn) (Truss & Beato, 1993).

Otherwise, GR can act as a ligand-inducible co-regulator that employs protein–protein interactions to exert its effect. In this case, GR does not bind directly to the DNA but recruits DNA-bound transcription cofactors in a regulatory complex, for instance the p160 family co-activators SRC-1 and SRC-2, PGC1-α (peroxisome proliferator-

activated receptor-gamma co-activator 1 alpha), NCoR (nuclear receptor co-repressor), SMRT and many others (Lu & Cidlowski, 2006). This kind of GR interaction (also termed as trans-regulation) can regulate gene expression both positively and negatively. An example of this phenomenon is the increased expression of CYP2A6 by dexamethasone despite the fact that the CYP2A6 promoter does not contain the functional GRE. Increased expression of CYP2A6 is mediated by GR via interactions of HNF4α with the HNF4-response element (Onica et al., 2008). No consensus of GRE has been identified in the CYP3A4 promoter although CYP3A4 activation by glucocorticoids has been reported. The reason seems to be that GR interactions with HNF3/CEBPα in the promoter of CYP3A4 since the mutation of binding sites of these liver-enriched transcriptional factors decreased glucocorticoid-mediated CYP3A4 reporter activity (El-Sankary et al., 2002).

1.1.2.2 Crosstalk between GR and AhR

Several lines of evidence indicate that there is an interaction between AhR and GR in terms of cellular signaling and regulation of drug metabolizing enzymes. The first study performed on the cell line PLHC-1 derived from fish demonstrated that dexamethasone enhances TCDD inducible expression of the CYP1A protein and catalytic activity (Celander et al., 1996). Another research with C57BL/6N mice has shown the synergistic interactions between TCDD and hydrocortisone for the induction of cleft palate (Abbott et al., 1994). It has also been reported that dexamethasone modulated TCDD-induced transcriptional activity of AhR, and TCDD modulated dexamethasone-induced transcriptional activity of GR. Further, both dexamethasone and TCDD modulates expression of AhR and GR mRNAs in HepG2 cells (Dvorak et al., 2008). In another study it was shown that dexamethasone controls AhR-mediated CYP1A1 and CYP1A2 expression and activity in primary human hepatocytes. Additionally, dexamethasone reduced both basal and inducible CYP1A1/2 ethoxyresorufin-O-deethylase (EROD) activities but the GR antagonist RU486 abolished this effect. This suggested the involvement of GR in the process (Vrzal et al., 2009). It has been also reported that dexamethasone decreased AhR mRNA level in HeLa cells (derived from human cervix carcinoma), although basal and TCDD-inducible AhR transcriptional activity was augmented by dexamethasone (Vrzal et al., 2007).

1.1.2.3 GR interactions with PXR and CAR

An induction of CYP3A by dexamethasone has been extensively documented. It has been found that, in contrast to the other xenobiotic inducers of CYP3A4, glucocorticoids play a dual role in CYP3A4 expression in primary human hepatocytes. Dexamethasone in nanomolar concentrations induced PXR, CAR and RXRα mRNAs

and proteins and enhanced CYP3A4 induction by rifampicin. Supramicromolar concentrations of dexamethasone activated PXR and caused CYP3A4 induction independent of GR (Pascussi et al., 2000a; Pascussi et al., 2001). Another study reported that submicromolar concentrations of dexamethasone enhance phenobarbital-mediated induction of CYP3A4, CYP2B6, and CYP2C8 mRNAs in cultured human hepatocytes, as the consequence of increased CAR mRNA a protein (Pascussi et al., 2000b). Based on these results, the transcriptional analysis of the CAR promoter has been performed and a glucocorticoid responsive unit located between -4477 bp and -4410 bp upstream of the transcription start was identified. Indeed, the GRE that was characterized at -4447/-4432 has a classical GRE structure (Pascussi et al., 2003).

1.2 Reporter gene cell lines

Experimental *in vitro* assays - reporter gene assays - have been extensively developed for various environmental applications, screenings and also for drug discovery. These systems are based on stable transfected cell lines with reporter plasmid containing key regulatory sequences upstream the reporter gene and allow the quick, sensitive and selective evaluation of transcriptional activity of nuclear receptors involved in drug metabolism.

Construction of stably transfected cell lines is a time and material consuming process, but experimentation with stably transfected cells has several advantages: (i) there is no need to transfect cells before each experiment, which saves time and materials; (ii) there is no need to normalize data per β-Gal due to the variable transfection efficiency; hence, the data are more homogeneous (iii) there is a much higher sensitivity of the assay, because all the cells are transfected; (iv) taking together points i-iii, stable cell lines usually allow high through-put measurements in a 96-well plate format.

Basically, two approaches are used for the generation of stable reporter gene cell lines:

(i) The first strategy uses parent cell lines, that do not contain the receptor of interest and they are usually rapid growing. Then, the process requires two transfection steps; firstly, with the reporter vector containing regulatory sequences and subsequently the transfection with the expression plasmid encoding the nuclear receptor of interest. This approach is lengthy and requires selection with two different antibiotics but the resulting cell line is usually very highly selective for the desired group of compounds (Sedlak et al., 2011).

(ii) The second approach is based on the use of a parent cell line expressing a fully functional endogenous receptor. The cells are transfected only with reporter

plasmid containing regulatory sequences for receptor binding and only one selection agent is necessary (Mori et al. 2008). The resulting cell line has usually a lower selectivity as compared to the first approach. On the other hand, the receptor of interest and other transcriptional factors involved in the receptor functions are expressed in natural stoichiometry and the data obtained reflect a nearly physiological state.

Several stably transfected gene reporter cell lines have been developed for the assessment of the transcriptional activity of nuclear receptors. An *in vitro* bioassay called CALUX (chemical-activated luciferase expression) has been invented. A rat hepatoma cell line (H4IIE) was stably transfected with a construct containing a DRE sequence and the luciferase reporter gene (Murk et al., 1996). The assay was developed for the detection of AhR active compounds in sediments and pore water, but it has been modified for many other applications. A stable cell line called DR-EcoScreen was derived from mouse hepatoma cells Hepa1c1c7 transfected with a reporter plasmid containing seven copies of DRE. This line was used for the sensitive, rapid and simple identification of AhR agonists among a large number of environmental chemicals (Takeuchi et al., 2008).

Since there are substantial differences between nuclear receptor signaling in rodent and human cells, it has been of great interest to establish a cell line based on human cells. The first attempt to develop a human stable reporter system for assessment of AhR transcriptional activity was performed by Postlind et al. who transfected reporter plasmid pLUC1A1 containing CYP1A1 promoter to HepG2 cells. The resulting cell line referred to as 101 L, was used to establish dose response relationships for TCDD and polycyclic aromatic hydrocarbons (Postlind et al., 1993). Cell line 101 L was also used as a high-volume screening system for identifying CYP1A inducers, such as flavonoids, resverarol, apigenin, curcumin, kaempferol and quercetin (Allen et al., 2001).

Other stable reporter cell lines derived from human tumor cells have been established for the evaluation of transcriptional activity of steroid receptors. Wilkinson et al. have developed a panel of steroid hormone receptor cellular assays by stably engineering the expression of Gal4-DBD/NR-LBD chimeras in a parental HEK293 cell line containing the beta-lactamase reporter gene under transcriptional control of an upstream activation sequence (UAS-bla) (Wilkinson et al., 2008). These cell lines are commercially available as GeneBLAzer™ nuclear receptor cells, allowing assessment of AR, ERα and ERβ, GR, MR and PR transcription activity.

1.3 Human hepatocytes

The liver is the major site of drug metabolism and contains the highest concentration of drug-metabolizing enzymes in comparison to other organs. For this reason, human liver-derived experimental *in vitro* models are one of the most extensively used systems for the evaluation of human-specific drug properties (e.g. interactions, toxicity, metabolism, transport). The models involve subcellular fractions (liver microsomes, liver slices) and metabolically competent cellular models such as primary human hepatocytes, immortalized hepatocyte and liver-derived cell lines.

Liver microsomes are the simplest model for monitoring the metabolic stability of compounds, enzyme inhibition and drug-drug interactions (Rodrigues, 1999). Since CYP enzymes are localized in the inner membrane of smooth endoplasmic reticulum, liver microsomes are easily prepared from endoplasmic reticulum of liver tissue and contain mostly enzymes of phase I metabolism. The major limitation is absence enzymes of phase II, except UGTs.

Primary human hepatocytes are considered as the best *in vitro* model of fully competent metabolic cells. They express majority of cytochrome CYP P450 enzymes involved in metabolism of exogenous compounds and are capable of generating a metabolic profile of drugs similar to those that are found *in vivo* (Ponsoda et al., 2001). Nevertheless their utilization is seriously complicated because of the restricted availability of fresh human livers for cell harvesting purposes, the high batch-to-batch functional variability of hepatocytes preparations obtained from different human liver donors, *in vitro* phenotypic instability and the limited life-span in a culture (Donato et al., 2008).

Not only in toxicological research, but also in the treatment of liver disease by hepatocyte transplantation, immortalized human hepatocytes are broadly used, for instance Fa2N-4 or HC-04 cell lines. However, not all immortalized hepatocytes express the full profile of liver-selective transporters and drug-metabolizing enzymes which is their major weakness. At this time, many cryopreservation procedures are known (Alexandre et al., 2002; Loretz et al., 1989; Mitry et al., 2010; Saliem et al., 2012; Steinberg et al., 1999). After thawing, cells can be easily maintained in the culture and they allow a high-throughput analysis and screening. However, cryopreserving the cells declines the viability and diminishes cytosolic enzymes due to cell membrane damage.

1.4 Human liver-derived cell lines

The human liver-derived cell lines have been widely used as an *in vitro* model in the hepatotoxicity research as well as in the study of hepatocellular functions. They show several advantages as compared to primary hepatocytes, such as a stable

phenotype, an almost unlimited lifespan and continuous growth in the culture. The hepatic cell lines are easily available and culture conditions are simple and easily standardized among laboratories. The major drawback of hepatoma cell lines is the low biotransformation capacity that is the consequence of a very low expression of the crucial CYP P450 genes. One possible explanation of this phenomenon can be a decrease or lack of liver specific transcriptional factors controlling P450 genes expression. Very poor expression of liver specific transcriptional factors HNF1, -3, 4 (hepatocyte nuclear factor) and C/EBPα have been identified by a comparative analysis between HepG2 cells and human hepatocytes (Castell et al., 2006). Alternatively, this phenomenon of low biotransformation capacity can be explained by the decreased expression of transcriptional co-activators, for instance PGC-1α or p160 SRC co-activators and the increased expression of transcriptional repressors SMRT, SHP or NCoR (Castell et al., 2006). Another explanations can be the alteration in the isoform of pattern transcriptional factors and co-activators or chromatin compaction.

Several strategies were suggested to overcome low biotransformation activity and increase expression levels of key P450 enzymes in hepatoma cell lines. The first promising way was the restoration of the lacking liver transcriptional factors. It has been shown that the overexpression of C/EBPα or HNF3 causes a significant increase in several CYPs of the CYP2 and CYP3 family (Bort et al., 2004; Castell et al., 2006; Jover et al., 1998). Another way to improve the biotransformation properties of hepatoma cell lines is by the reactivation of non-functional transcriptional factors or the blocking of transcriptional repressors (Martinez-Jimenez et al., 2006a).

The human hepatoma cell line HepG2 is one of the most widely used cell lines in pharmacological and toxicological research. HepG2 cells show many liver-specific functions. They express conjugating enzymes, but lack the functional expression of almost all the relevant drug-metabolizing P450 enzymes. Several hypotheses to explain the low biotransformation activity of HepG2 cells were proposed. This could be due to a malfunction of HNF4α including an imbalanced expression of HNF4α splicing variants (α1, α2, α3, α7) or an increased expression of co-repressors SHP, NCoR or SMRT. However, the most important reason for the malfunction of HNF4α in HepG2 cells is a very low expression of transcriptional co-activators PGC-1α and SRC-1.

1.4.1 HNF4α

The differentiation of human hepatocytes and the functions of the adult human liver require transcriptional factors that belong to five families, including homeodomain homolog HNF1, the forkhead homolog HNF3, homeodomain homolog HNF6, basic leucin zipper protein C/EBPα and zinc finger protein HNF4. The subfamily HNF4 is

comprised of more than 150 highly conserved proteins that include many receptors and factors, such as HNF4α, HNF4β, HNF4γ and their splice variants (Mangelsdorf et al., 1995). HNF4α (NR2A1) is crucial for the development of fetal liver and it is linked to several diseases including diabetes, haemophilia, atherosclerosis, and hepatitis (Bogan et al., 2000; Gupta et al., 2005; Chen et al., 1994). It is highly expressed in the liver, kidney, small intestine and pancreatic β-cells (Sladek et al., 1990; Yamagata et al., 1996). Initially, HNF4α was considered as an orphan receptor, but it was "deorphanized" with the identification of fatty acyl-CoA thioesters as an endogenous ligands of HNF4α (Hertz et al., 1998).

In the liver, HNF4α is localized exclusively in the nucleus and it forms homodimer that binds to direct repeats (AGGTAC) in DNA and recruits transcriptional co-activators and other accessory proteins. HNF4α regulates the constitutive expression of a large number of target gene encoding enzymes and transporters involved in the metabolism of lipids, glucose, bile acids and xenobiotics (Guan et al.; Hirota et al., 2008; Chiang, 2009). HNF4α also controls the constitutive expression of the genes encoding the coagulation factors FXII and XIIIB and genes involved in amino acid metabolism such as proline oxidase (Inoue et al., 2006; Kamiya et al., 2004). HNF4α regulates the expression of HNF1 which is involved in the expression of hepatospecific markers albumin, fibrinogen and plasminogen (Maire et al., 1989; Tronche et al., 1997; Yamagata et al., 1996).

In addition to the direct regulation of target genes, HNF4α modulates the expression of other genes controlled by nuclear receptors. The importance of HNF4α for the expression of drug-metabolizing hepatic P450s was demonstrated in mice and in human hepatocytes (Jover et al., 2001; Wiwi et al., 2004; Wiwi & Waxman, 2005). It has been reported that the mouse PXR promoter contains an HNF4α binding site and that HNF4α is required for the expression of PXR in fetal hepatocytes (Kamiya et al., 2003; Tirona et al., 2003). It has also been shown that HNF4α binds to the DR1 element located at －88/－76 of the hPXR (human PXR) promoter and increases hPXR transcriptional activity (Iwazaki et al., 2008). These findings suggest that HNF4α may be involved in a positive activation of the CYP3A4 gene by PXR.

HNF4α is involved in gene activation in concert with transcription co-activators and co-repressors through its AF domains. It was shown that HNF4α interacts with the p160 family co-activators SRC-1, -2 and -3, CBP [CREB-binding protein]/p300 or PGC-1α (Dell & Hadzopoulou-Cladaras, 1999; Eeckhoute et al., 2001; Wang et al., 1998). The negative regulator of HNF4α is SHP, which inhibits activity of HNF4α by competition with co-activators and by direct transcriptional repression (Lee et al., 2000).

The expression of HNF4α target genes is seriously impaired in HepG2 cells, however, HNF4α is present at the same high levels as in hepatocytes while the expression level of transcriptional co-activators is decreased (Castell et al., 2006). These findings suggest that the low biotransformation activity of HepG2 cells is caused by the poor expression of key co-activators, particularly PGC-1α and SRC-1 that are needed for the stimulation of HNF4-mediated CYP transcription.

1.4.2 PGC-1α

PGC-1α was identified as the first member of PGC-1 family of co-activators (Puigserver et al., 1998). Its homologs PGC-1β and PGC-1-related co-activator (PRC) display high similarity in the C-terminus, the RNA recognition motif, the conserved L1 motif and the nuclear receptor interaction domain (L2) in the N-terminus (Andersson & Scarpulla, 2001; Lin et al., 2002). PGC-1 co-activators form a complex with several HAT-containing proteins (CBP/p300, SRC-1) at their N-terminal regions or dock the protein mediator complex called TRAP/DRIP on the C-terminal region of PGC-1α (Puigserver et al., 1999; Wallberg et al., 2003). PGC-1α transcriptional activator displaces repressor proteins and consequently increases the gene transcription (Borgius et al., 2002). PGC-1α plays an important role in regulation of a wide range of nuclear receptors such as HNF4α, glucocorticoid receptor, PPARα, and PPARβ and others (Rhee et al., 2003; Shin et al., 2003; Yoon et al., 2001).

The expression of PGC-1α is controlled by nutritional and hormonal signals as well as by circadian pacemakers (Spiegelman & Heinrich, 2004). The highest expression of PGC-1α was found in tissues with a high density of mitochondria, i.e. heart, kidney, skeletal muscles, brown adipose tissue, liver and brain (Esterbauer et al., 1999; Knutti et al., 2000; Puigserver et al., 1998). PGC-1α has an important role in adaptive thermogenesis and it is involved in glucose metabolism and the maintenance of lipid and energy homeostasis.

Depending on the cell type, PGC-1α is regulated in different ways. The major mechanism of PGC-1α induction is the activation of the cAMP signaling pathways through cAMP binding to the CREB binding site in the proximal promoter of PGC-1α (Herzig et al., 2001). Another mechanism of PGC-1α induction involves the stimulation of ATF2 (activating transcription factor 2) in brown adipose tissue by cold, or calcium signaling in skeletal muscles and the heart during physical exercise (Cao et al., 2004; Handschin et al., 2003). Fasting induces PGC-1α expression in an adult liver, which leads to an activation of gluconeogenic enzymes PEPCK and glucose-6-phosphatase through interactions with the transcription factor HNF4α and forkhead box O1 (FOXO-1)(Boustead et al., 2003; Puigserver et al., 2003; Rhee et al., 2003).

As mentioned above, PGC-1α serves as the transcriptional co-activator of HNF4α and it stimulates HNF4-mediated CYP transcription. The transactivation effect of PGC-1α on P450 genes mediated by HNF4α was corroborated by transfection experiments using MZ-Hep-1 cells that have a low level of HNF4α. Co-transfection of PGC-1α and HNF4α increased the levels of CYP2C9, CYP1A1 and CYP1A2, whereas transfection of PGC-1α or HNF4α alone had no significant effect on P450s expression (Martinez-Jimenez et al., 2006a). Compared to primary human hepatocytes, the expression of PGC-1α in HepG2 is strongly decreased, whereas the expression of HNF4α is maintained at the same level (Martinez-Jimenez et al., 2006b). The transient transfection of HepG2 cells with PGC-1α significantly increased the expression of CYP2C9, CYP1A1 and CYP1A2 genes and to a lesser extent the expression of CYP3A4, CYP3A5 and CYP2D6 (Martinez-Jimenez et al., 2006a; Martinez-Jimenez et al., 2006b).

2. OBJECTIVES

The aim of the present work was the development of stable transfected human cell lines for toxicological, pharmacological and environmental applications. The main achievements planned were:

1. Construction and characterization of stable gene reporter human cell lines, generated from HepG2 and HeLa cells, allowing the assessment of transcriptional activities of the human glucocorticoid receptor and the human aryl hydrocarbon receptor.

2. Construction and characterization of hepatocyte-like human cell lines, generated from the commercial human cancer cell line HepG2 stably transfected with PGC-1α and HNF4α vectors.

3. EXPERIMENTAL PART

3.1 Biological material

3.1.1 HepG2 cell line

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

3.1.2 HeLa cell line

Human Negroid cervix epitheloid carcinoma cells HeLa (ECACC No. 93021013) were cultured in Dulbecco`s modified Eagle`s medium (DMEM) supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 µg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

3.2 Reporter plasmids

3.2.1 pGL-4.27-DRE

The reporter plasmid pGL-4.27-DRE was constructed as follows: Two copies of F site sequences, one copy of B site and D site sequences of mice *Cyp1a1* gene 13 were synthesized and inserted using KpnI-XhoI enzymes into the multiple cloning region of pGL4.27[luc2P/minP/Hygro]vector (Cat. Nuber E8451, Promega, UK).

F site sequence: 5'-GATCGCCGGG**T**T**TGCGT**GCGATA-3'

B site sequence: 5´-GATCTACCTGT**G**T**GCGTG**CCAAGG-3´

D site sequence: 5´-ATCTCTT**C**T**CACGC**A**A**CTCCGA-'3

3.2.2 pGL-4.27-GRE

The reporter plasmid pGL-4.27-GRE was constructed as follows: Three copies of tandem glucocorticoid response element (GRE) were synthesized and inserted using KpnI-XhoI enzymes into pGL4.27 [luc2P/minP/Hygro] vector (Cat. Number E8451, Promega, UK).

GRE sequences: **GGTACA**TTT**TGTTCT**

3.3 Expression plasmids

3.3.1 pcDNA3-neo-HNF4α

The expressing vector pcDNA3-neo-HNF4α contains full length sequence of human liver HNF4α (Chartier et al., 1994) inserted into expressing vector pcDNA3.1 (Cat. Number V790-20 Invitrogen, USA).

HNF4α full length sequence (X76930)

```
   1 ctccaaaacc ctcgtcgaca tggac**ATG**gc cgactacagt gctgcactgg acccagccta
  61 caccaccctg gaatttgaga atgtgcaggt gttgacgatg ggcaatgaca cgtccccatc
 121 agaaggcacc aacctcaacg cgcccaacag cctgggtgtc agcgccctgt gtgccatctg
 181 cggggaccgg gccacgggca aacactacgg tgcctcgagc tgtgacggct gcaagggctt
 241 cttccggagg agcgtgcgga agaaccacat gtactcctgc agatttagcc ggcagtgcgt
 301 ggtggacaaa gacaagagga accagtgccg ctactgcagg ctcaagaaat gcttccgggc
 361 tggcatgaag aaggaagccg tccagaatga gcgggaccgg atcagcactc gaaggtcaag
 421 ctatgaggac agcagcctgc cctccatcaa tgcgctcctg caggcggagg tcctgtcccg
 481 acagatcacc tcccccgtct ccgggatcaa cggcgacatt cgggcgaaga agattgccag
 541 catcgcagat gtgtgtgagt ccatgaagga gcagctgctg gttctcgttg agtgggccaa
 601 gtacatccca gctttctgcg agctcccccct ggacgaccag gtggccctgc tcagagccca
 661 tgctggcgag cacctgctgc tcggagccac caagagatcc atggtgttca aggacgtgct
 721 gctcctaggc aatgactaca ttgtccctcg gcactgcccg gagctggcgg agatgagccg
 781 ggtgtccata cgcatccttg acgagctggt gctgcccttc caggagctgc agatcgatga
 841 caatgagtat gcctacctca aagccatcat cttctttgac ccagatgcca aggggctgag
 901 cgatccaggg aagatcaagc ggctgcgttc ccaggtgcag gtgagcttgg aggactacat
 961 caacgaccgc cagtatgact cgcgtggccg ctttggagag ctgctgctgc tgctgcccac
1021 cttgcagagc atcacctggc agatgatcga gcagatccag ttcatcaagc tcttcggcat
1081 ggccaagatt gacaacctgt tgcaggagat gctgctggga gggtccccca gcgatgcacc
1141 ccatgcccac cacccctgc accctcacct gatgcaggaa catatgggaa ccaacgtcat
1201 cgttgccaac acaatgccca ctcacctcag caacggacag atgtgtgagt ggccccgacc
1261 caggggacag gcagccaccc ctgagacccc acagccctca ccgccaggtg cgtcagggtc
1321 tgagccctat aagctcctgc cgggagccgt cgccacaatc gtcaagcccc tctctgccat
1381 cccccagccg accatcacca agcaggaagt tatc**TAG**caa gccgctgggg cttgggggct
```

3.3.2 pcDNA3-neo-PGC-1α

The expressing vector pcDNA3-neo-PGC-1α was derived from the plasmid pBS/HA-hPGC-1, which carries full length sequence of human liver PGC1α downstream of the hemagglutinin (HA) epitope-encoding sequence. The full-length HA-hPGC-1 construct was then subcloned as BamHI-NotI fragments into the pcDNA3 vector (Invitrogen) (Knutti et al., 2000).

PGC1-α full length sequence (NG_028250.1)

```
   1 ATGgcgtggg acatgtgcaa ccaggactct gagtctgtat ggagtgacat cgagtgtgct
  61 gctctggttg gtgaagacca gcctctttgc ccagatcttc ctgaacttga tctttctgaa
 121 ctagatgtga acgacttgga tacagacagc tttctgggtg gactcaagtg gtgcagtgac
 181 caatcagaaa taatatccaa tcagtacaac aatgagcctt caaacatatt tgagaagata
 241 gatgaagaga atgaggcaaa cttgctagca gtcctcacag agacactaga cagtctccct
 301 gtggatgaag acggattgcc ctcatttgat gcgctgacag atggagacgt gaccactgac
 361 aatgaggcta gtccttcctc catgcctgac ggcacccctc caccccagga ggcagaagag
 421 ccgtctctac ttaagaagct cttactggca ccagccaaca ctcagctaag ttataatgaa
 481 tgcagtggtc tcagtaccca gaaccatgca aatcacaatc acaggatcag aacaaaccct
 541 gcaattgtta agactgagaa ttcatggagc aataaagcga agagtatttg tcaacagcaa
 601 aagccacaaa gacgtccctg ctcggagctt ctcaaatatc tgaccacaaa cgatgaccct
 661 cctcacacca aacccacaga gaacagaaac agcagcagag acaaatgcac ctccaaaaag
 721 aagtcccaca cacagtcgca gtcacaacac ttacaagcca aaccaacaac tttatctctt
 781 cctctgaccc cagagtcacc aaatgacccc aagggttccc catttgagaa caagactatt
 841 gaacgcacct taagtgtgga actctctgga actgcaggcc taactccacc caccactcct
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1561 ctgagctacc cttgggatgg cacgcaatcc tattcattgt tcaatgtgtc tccttcttgt
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2221 tcaaacgaaa ctgactttga gctgtacttt tgtggacgca agcaattttt caagtctaac
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2341 gactctctgg attttgatag tttactgaaa gaagctcaga gaagcttgcg caggTAA
```

3.4 Reagents

Dulbecco`s modified Eagle`s medium (DMEM), penicillin, streptomycin, L-glutamine were purchased from Sigma-Aldrich (Czech Republic). The fetal bovine serum was from GE Healthcare (France). Oligonucleotide primers used in RT-PCR reactions were from Invitrogen (USA). LightCycler FastStart DNA MasterPLUS SYBR Green I and the FuGENE HD transfection reagent were from Roche Diagnostic Corporation (Czech Republic). 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) was from Ultra Scientific (USA). Geneticin (G418) was purchased from Invitrogen (USA). DMSO (dimethylsulfoxide), rifampicin, hygromycin B, dexamethasone, beclometasone, betamethasone, cortisol, corticosterone, prednisolone, methylprednisolone, testosterone, estradiol, diethylstilbestrol, 4-hydroxytamoxifen, spironolactone, aldosterone, 17α-hydroxy- progesterone and progesterone were purchased from Sigma-Aldrich (Czech Republic). Raloxifene hydrochloride, tamoxifen citrate salt, genistein, cyproterone acetate, danazol, triamcinolone and mifepristone were purchased from Santa Cruz Biotechnology (USA). Reporter lysis buffer was from Promega (UK). Primary antibodies against albumin (mouse monoclonal; sc-70340, 1.B.730), fibrinogen (mouse monoclonal; sc-65966, GMA-035), plasminogen (mouse monoclonal; sc-65967, GMA-039), α1-antitrypsin (mouse monoclonal; sc-73431, 13702), HNF4α (rabbit polyclonal; sc-8987, H-171), PGC-1α (goat polyclonal; sc-8987, H-171), PXR (mouse monoclonal; sc-48340, H-11), AhR (rabbit polyclonal; sc-5579, H-211), CYP1A1 (goat polyclonal; sc-9828, G-18), CYP3A4 (mouse monoclonal; sc-53850, HL3) and actin (goat polyclonal; sc-1616, I-19), all purchased from Santa Cruz Biotechnology (USA). Secondary horseradish peroxidase conjugated antibody, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (USA). Hyperfilm^{TM} ECL was from Amersham-Pharmacia Biotech (UK). All other chemicals were of the highest quality commercially available.

3.5 Methods

3.5.1 Cytotoxicity assay

HepG2 and HeLa cells were seeded at a density of 25×10^4 on the 96-well plate. Following 16 h of incubation, HepG2 cells were treated with increasing concentrations of G418 (400-1400 µg/ml) and hygromycin B (50-1000 µg/ml) for 1, 2, 3, 6 and 7 days. HeLa cells were treated with increasing concentrations of hygromycin B (50-800 µg/ml). In parallel, the cells were treated with vehicle (DMSO; 0.1%, v/v) and Triton X-100 (2%, v/v) to assess the minimal and maximal cell damage, respectively. After the treatment, the medium was removed and the cells were washed with 1xPBS.

Subsequently, 100 µl of MTT (0.3 mg/ml) was added to each well and incubated at 37˚C for 1 h. Following the incubation, the MTT solution was removed and 100 µl of DMSO was added to dissolve the purple formazan. The absorbance was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments LLC). The values of IC_{50} were calculated using data acquired from three independent cell passages.

3.5.2 Transfection and selection process

3.5.2.1 Stably transfected human reporter gene cell lines

HepG2 cells were transfected with reporter pGL-4.27-DRE (10 µg) using the Lipofectamin 2000 transfection reagent in ratio DNA (µg):transfection reagent (µl) = 1:2.5 and seeded at the density of 1.3×10^6 cells in 60 mm culture dishes in 5 ml of the DMEM culture medium. HeLa cells were transfected with pGL-4.27-GRE (2 µg) reporter plasmid using the Fugene HD reagent in ratio DNA (µg):transfection reagent (µl) = 1:3. The cells were seeded at the density of 8×10^5 cells in 60 mm culture dishes in 5 ml of the DMEM culture medium. Following 36 h of incubation, the culture medium was replaced by the selection medium supplemented with hygromycin B (0.6 mg/ml in the case of HepG2 cells; 0,2 mg/ml in the case of HeLa cells). The medium was changed every 3-4 days for the period of 9 weeks (HepG2) or 3 weeks (HeLa), until a polyclonal population was selected. Thereafter, the cells were transferred to 10 cm culture dishes at the density of 500-1000 cells and cultured for an additional 4 weeks (HepG2) and 2 weeks (HeLa) under hygromycin resistance, until small colonies were visible. Subsequently, twelve colonies derived from HepG2 cells (termed AZ-AHR cells) and seventeen colonies derived from HeLa cells (termed AZ-GR) were subcloned into a 48-well tissue culture plate to obtain monoclonal populations.

3.5.2.2 Stably transfected hepatocytes-like cells

For the establishment of HepG2 cells stably overexpressing HNF4α or PGC-1α, the cells were transfected with pcDNA3-neo-HNF4α expressing vector (2 µg) or pcDNA3-neo-PGC-1α expressing vector (2 µg) using the Fugene HD transfection reagent. Simultaneously, HepG2 cells were transfected with an empty vector pcDNA3-neo. The ratio of DNA (µg):transfection reagent (µl) = 1:3. The cells were seeded at the density of 2×10^6 cells in 60 mm culture dishes in 5 ml of DMEM culture medium. Following the 36 h of incubation, the culture medium was replaced by selection medium supplemented with G418 (1.1 mg/ml). The medium was renewed every 3-4 days for the period of 8 weeks for HepG2-HNF4α or 12 weeks for HepG2-PGC-1α, until polyclonal populations were selected. Thereafter, cells were transferred in 10 cm culture dishes at a density of 500-1000 cells and cultured for additional 3 weeks (in the

case of HepG2-HNF4α) or 5 weeks (in the case of HepG2-PGC-1α) under neomycin resistance, until small colonies were visible. After that, nine colonies of HepG2-HNF4α and six colonies of HepG2-PGC-1α were subcloned into 48-well tissue culture plate to obtain monoclonal populations.

3.5.3 RNA isolation and qRT-PCR

Total RNA was isolated using TRIZOL Reagent (Invitrogen). Cells (1.5×10^6 cells) were washed by PBS buffer pH 7.4 ± 0.2 (137 mM NaCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4 and 2.7 mM KCl) and lysed by adding 1 ml of TRIZOL Reagent per well. Further, 0.2 ml of chloroform per 1 ml of TRIZOL reagent was added; the samples were shaken for 60 s and incubated at room temperature for 8 min. The samples were centrifuged at 13 000 RPM/ 15 min/ 4°C. Following centrifugation the aqueous phase was transferred into a new tube. The RNA was precipitated by adding 0.5 ml isopropyl alcohol. Samples were incubated at room temperature for 10 min and centrifuged at 13 000 RPM/ 15 min/ 4°C. The pellet was washed twice with 1 ml of 75% ethanol. The RNA pellet was briefly dried and dissolved in RNase-free water. cDNA was synthesized from 1000 ng of total RNA using M-MLV Reverse Transcriptase (Invitrogen) at 37°C for 1h in the presence of random hexamers (Amersham Biosciences). One tenth was used for qRT-PCR amplification using the Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The following program was used: an activation step at 95°C for 10 min was followed by cycles of PCR (denaturation at 95°C for 10 seconds; annealing of individual primers for 7 seconds, elongation at 72°C for 17 s). The levels of CYP1A1, CYP3A4, AhR, PXR, HNF4α, PGC-1α, *neo* gene and GAPDH mRNAs were determined using primers and conditions as described in Table 1. The measurements were performed in duplicates. Gene expression was normalized per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by delta-delta method.

Table 1: Oligonucleotides used for quantitative RT-PCR

Oligonucleotid name		Oligonucleotid sequence	Tm [°C]	Number of cycles
GAPDH	Forward	5′-TTCAGCAAGAAGAACAAGGACAA-3′	68	30
GAPDH	Reverse	5′-GGTTGAAGAAGTCCTCCTAAGC-3′		
CYP3A4	Forward	5′-TCCGGGACATCACAGACAGC-3′	65	40
CYP3A4	Reverse	5′-ACCCTGGGGTTCATCACCAA-3′		
CYP1A1	Forward	5′-CAAAGTTGTCATGGATGACC-3′	65	35
CYP1A1	Reverse	5′-GGTCGGAGTCAACGGATTTGGTCG-3′		
PXR	Forward	5′-TCCGGAAAGATCTGTGCTCT-3′	65	45
PXR	Reverse	5′-AGGGAGATCTGGTCCTCGAT-3′		
AhR	Forward	5′-CATCCCCCACAGCACAACAA-3′	60	40
AhR	Reverse	5′-TCCCACTTGGCCAGGACTTC-3′		
HNF4α	Forward	5′-GCCTACCTCAAAGCCATCAT-3′	61	40
HNF4α	Reverse	5′-GACCCTCCCAGCAGCATCTC-3′		
PGC-1α	Forward	5´-AAT GTG TCT CCT TCT TGT TCT T-3´	60	40
PGC-1α	Reverse	5´-GGT GTC TGT AGT GGC TTG A-3´		
neo gene	Forward	5′-GGTCTTGTCGATCAGGATG-3′	58	40
neo gene	Reverse	5′-ACGAGGAAGCGGTCAG		

3.5.4 Protein detection and Western blotting

HepG2 cells, G418-resistant HepG2-HNF4α cells, HepG2-PGC1-α cells and HepG2-pcDNA3 cells were seeded at density 5×10^5 on 6-well plates using culture media enriched with fetal bovine serum (10% v/v). Following 16 h of incubation, the medium was changed for a serum-free one (in case of detection of hepatospecific markers - albumin, fibrinogen, plasminogen and α1-antitrypsin) and the cells were maintained in serum - free conditions for 48 h. For assessment of the expression level of CYP1A1 and CYP3A4 proteins, the cells were treated for 48 h with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) or rifampicin (RIF; 10 μM), and in parallel with DMSO as a vehicle for control. Expression levels of xenoreceptors AhR and PXR proteins and transcriptional factors HNF4α and PGC1-α proteins were analysed in cell lysates after 48 h of incubation. Total protein extracts were prepared as follows: the cells were washed twice with ice-cold PBS and scraped into 1 ml of PBS. The suspension was centrifuged at 5000 RPM/ 5 min/ 4˚C. The pellet was re-suspended in 150 μL of ice-cold lysis buffer (10 mM Hepes pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; anti-protease cocktail, 0.2% (v/v) sodium dodecylsulfate), vortex for 10 minutes and centrifuged at 13 000 RPM/ 15 min/ 4°C. A supernatant was collected and the protein content was determined by the Bradford methods. SDS–PAGE gels (8%) were run on a Mini protean tetra cell apparatus according to the general procedure.

Protein transfer onto the PVDF membrane was carried out. The membrane was saturated with 5% non-fat dried milk for 1 h at room temperature. Blots were probed with primary antibodies against albumin (mouse monoclonal; dilution 1/2000), fibrinogen (mouse monoclonal; dilution 1/200), plasminogen (mouse monoclonal; dilution 1/200), α1-antitrypsin (mouse monoclonal; dilution 1/500), HNF4α (rabbit polyclonal; dilution 1/500), PGC-1α (goat polyclonal; dilution 1/500), PXR (mouse monoclonal; dilution 1/500), AhR (rabbit polyclonal; dilution 1/500), CYP1A1 (goat polyclonal; dilution 1/500), CYP3A4 (mouse monoclonal; dilution 1/500) and actin (goat polyclonal; dilution 1/2000). A chemiluminescence detection was performed using a horseradish peroxidase-conjugated secondary antibody and a western blotting luminol reagent (Santa Cruz, USA).

3.5.5 Reporter gene assay

The cells were seeded on 96-well plates in density of 25 000 cells per well. Following 16 h of incubation, cells were treated with tested compounds. After the treatments, cells were lysed with reporter lysis buffer (Promega) and luciferase activity was measured in 96-well plate format, using Tecan Infinite M2000 plate luminometer.

4. Results

4.1 Construction and characterization of human stable transfected reporter gene cell lines

4.1.1 Construction and characterization of the stably transfected reporter human hepatoma cell line for the assessment of aryl hydrocarbon receptor transcriptional activity - AZ-AHR cells

4.1.1.1 Response of AZ-AHR cells to TCDD

Human hepatocellular carcinoma cells HepG2 were transfected with pGL-4.27-DRE luciferase reporter plasmid responsive to the transcriptionally active aryl hydrocarbon receptor AhR. We isolated twelve clones of AZ-AHR hygromycin-resistant cells. We tested the response of the AZ-AHR clones to the model AhR ligand TCDD. For this purpose, parent HepG2 cells and AZ-AHR cells were seeded in 96-well plates and following 16 h of stabilization, the cells were treated with 5 nM TCDD and vehicle (DMSO; 0.1% v/v) for 24 h, and luciferase activity was measured. There was no induction of luciferase activity in the parent HepG2 cells. Out of twelve clones examined, significant induction of luciferase activity was attained in eight clones (1, 2, 3, 5, 7, 8, 9, 11), with fold induction ranging from 4× to 1 262×. For further analyses, the most responsive clones (1, 2, 8, 9, 11), yielding both high relative luciferase units (RLU) and a high fold induction over the vehicle-treated cells, were used (Fig. 4).

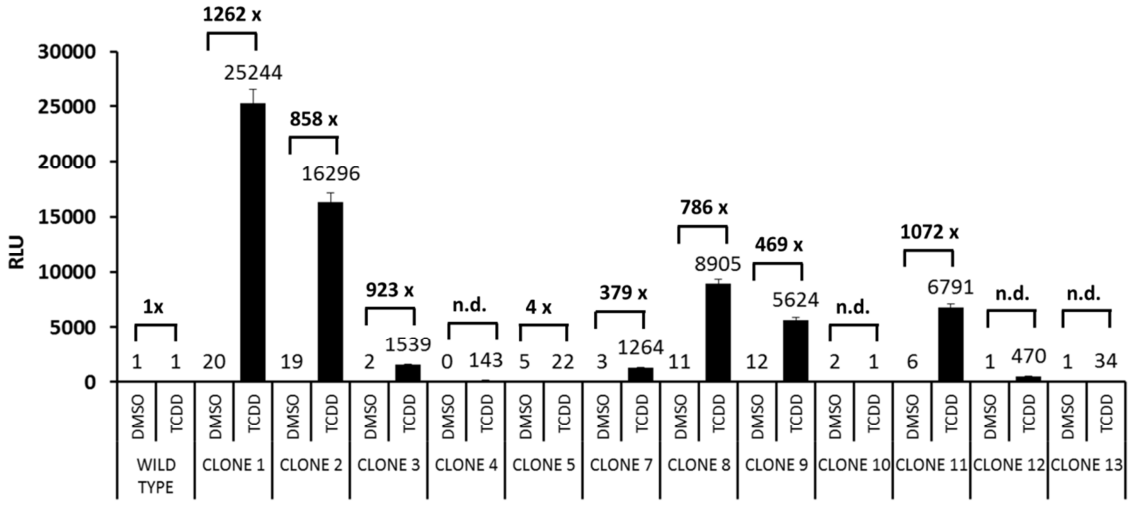

Figure 4: Response of AZ-AHR cells to TCDD. Parent HepG2 cells and clones of AZ-AHR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated with 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% v/v) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are mean from triplicate measurements and are expressed as relative luciferase units (RLU).

4.1.1.2 Dose-response analyses of AZ-AHR cells treated with AhR activators

In the first series of functional tests, we performed dose-response experiments in five clones (1, 2, 8, 9, 11) of the AZ-AHR cell line. Cells were treated in 96-well plates with increasing concentrations of TCDD, resveratrol (RVT), 3-methylcholanthrene (3MC), 1,9-pyrazoloanthrone (SP600125), omeprazole (OME), indirubin (IND) and vehicle (DMSO; 0.1% v/v) for 24 h, and luciferase activity was measured in cell lysates.

Treatment of AZ-AHR cells with TCDD (10 pM – 100 nM), the most potent AhR ligand, yielded typical sigmoid logarithmic dose-response curves for each tested clone (Fig. 1A). With the exception of clone 1 (EC_{50} = 3.93 nM), the values of EC_{50} were homogenous between the clones, ranging from 1.64 nM to 2.31 nM (Tab. 2). Sigmoid-shaped curves were also obtained in all clones of AZ-AHR cells treated with polyaromatic hydrocarbon 3MC (10 nM – 50 µM), with EC_{50} values ranging from 1.46 µM to 2.26 µM. As in the case of TCDD, clone 1 yielded a higher EC_{50} (9.17 µM) when compared to other clones (Tab. 2). Interestingly, we have systematically observed a local minimum in the 10 µM concentration of 3MC (18 independent dose-response experiments), indicating that more than one mechanism of 3MC action is involved in signal transduction in high concentrations of 3MC (Fig. 5B).

Indirubin, an endogenous ligand of AhR, induced luciferase activity in a dose dependent manner in all tested clones with EC_{50} ranging from 0.95 µM to 2.42 µM (Tab. 2). In concentrations of indirubin 10 µM and higher, the activity of luciferase descended, suggesting the inhibition of luciferase catalytic activity or feed-back mechanisms in supra-physiological concentrations (Fig. 5C). The efficacy of TCDD, 3MC and indirubin were of the same order of magnitude (Fig. 5A-5C).

Omeprazole, a drug inhibiting proton pump, is an activator of AhR and inducer of CYP1A genes, but it does not bind to AhR *in vitro* and the mechanism of AhR activation by omeprazole is unknown (Gerbal-Chaloin et al., 2006). In gene reporter assays with 5 clones of AZ-AHR cells, omeprazole displayed an atypical dose-response profile of the U-shaped curve, with the maximum induction of luciferase activity (efficacy comparable to 3MC) at 100 µM concentration of omeprazole (Fig. 5D). We have also tested the effects of SP600125 (specific inhibitor of JNK) and resveratrol (natural antioxidant) on luciferase expression in AZ-AHR cells, since both compounds were described as partial agonists AhR (Dvorak et al., 2008). Both resveratrol (Fig. 5E) and SP600125 (Fig. 5F) caused dose-dependent induction of luciferase activity in all tested clones, with maximal luciferase activity at 50 µM and 100 µM, respectively. A further increase in SP600125 and resveratrol concentrations caused a decrease of

luciferase activity, probably due to the interactions with other cell targets and inhibition of luciferase activity.

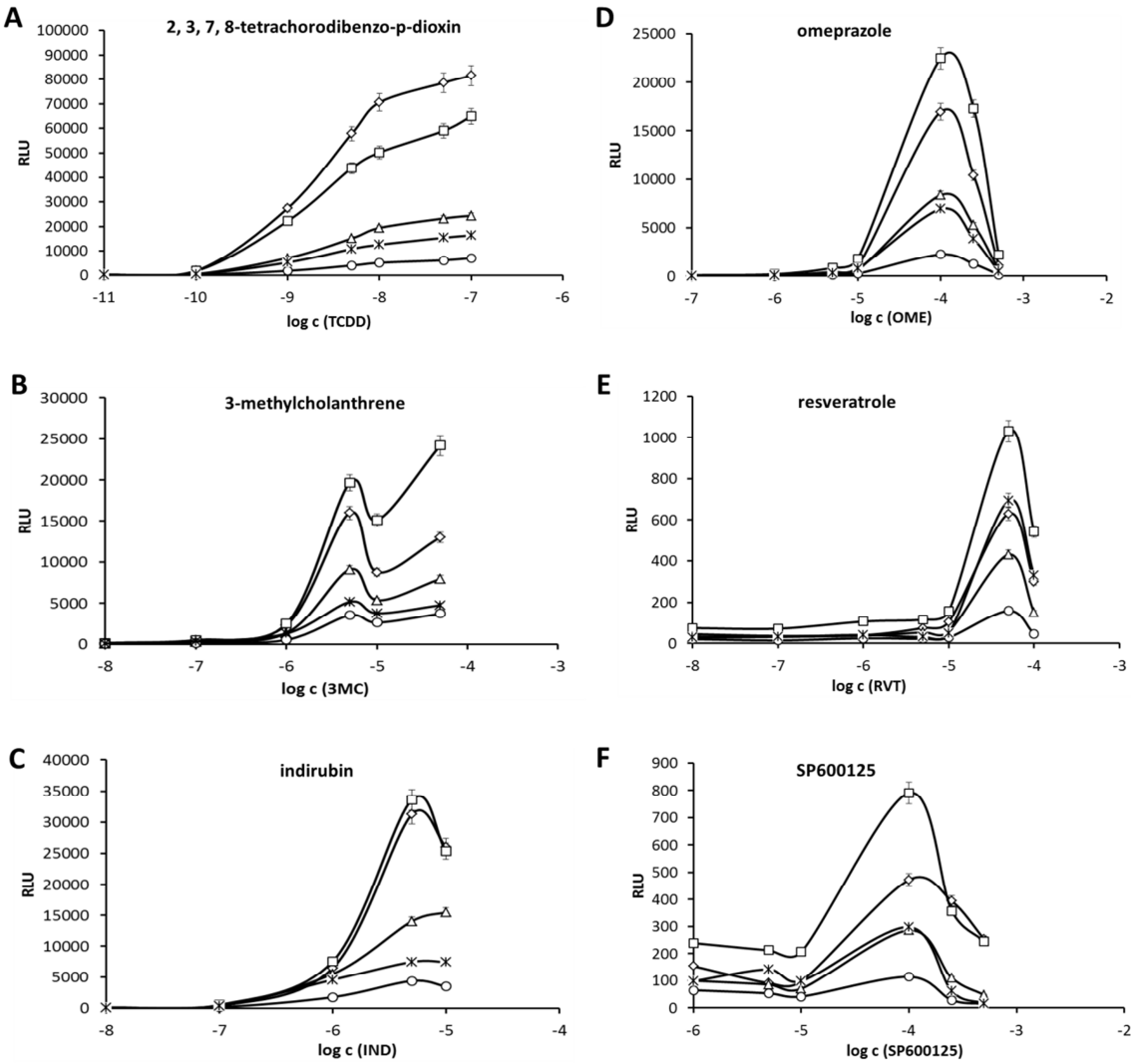

Figure 5: Dose-response analyses of AZ-AHR cells treated with AhR activators. Clones 1, 2, 8, 9 and 11 of AZ-AHR cells were treated with AhR ligands for 24 h. **Panel A:** 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 0.01 nM – 100 nM); **Panel B:** 3-methylcholanthrene (3MC; 0.01M – 50 µM); **Panel C:** Indirubin (IND; 0.01 – 10 µM); **Panel D:** Omeprazole (OME; 0.1 – 500 µM); **Panel E:** Resveratrol (RVT; 0.01– 100 µM); **Panel F:** 1,9-pyrazoloanthrone (SP600125; 1 – 500 µM). After the treatments, cells were lysed and luciferase activity was measured. The data are the mean from triplicate measurements and are expressed as relative luciferase units (RLU). Legend to plots: Clone 1 (◊); Clone 2 (□); Clone 8 (Δ); Clone 9 (o); Clone 11 (x).

Table 2: EC_{50} values of tested compounds on the AZ-AHR cell line

	EC_{50}		
AZ-AHR cells	**TCDD [nM]**	**3MC [µM]**	**IND [µM]**
Clone 1	3.93 ± 2.75	9.17 ± 2.60	2.37
Clone 2	1.64 ± 1.08	2.26 ± 0.80	2.04 ± 0.09
Clone 8	2.04 ± 0.77	1.90 ± 0.27	2.42 ± 0.25
Clone 9	2.31 ± 1.19	1.46 ± 0.25	0.95 ± 0.19
Clone 11	1.87 ± 0.81	2.15 ± 0.42	1.97 ± 1.12

4.1.1.3 Time-course analyses of AZ-AHR cells treated with TCDD

In the next series of functional tests, we examined time effects of TCDD on luciferase expression in AZ-AHR cells. Clones 1, 2, 8, 9 and 11 were seeded in 96-well plates and following 16 h of stabilization, cells were treated for 2 h, 4 h, 6 h, 8 h and 24 h with TCDD (5 nM) and DMSO (0.1% v/v). In all clones tested, TCDD caused a time-dependent increase of luciferase activity. The assay was very sensitive and a significant induction of luciferase activity was attained after 6 h of treatment with TCDD, with a fold induction ranging from 8 fold to 24 fold (Fig. 6). This implies the potential use of the AZ-AHR cell line in testing the cytotoxic compounds which may kill cells in a longer time periods. There were not substantial differences between individual clones, with the exception of clone 1 that displayed a much higher fold induction of luciferase after 24 h as compared to the other clones (Fig. 6).

In clone 2, we performed more detailed analysis, where the induction of luciferase was measured every 2 h in the period of 24 h. The maximal luciferase activity by TCDD was attained between 12 and 18 h of the treatment (Fig. 6, bottom right plot).

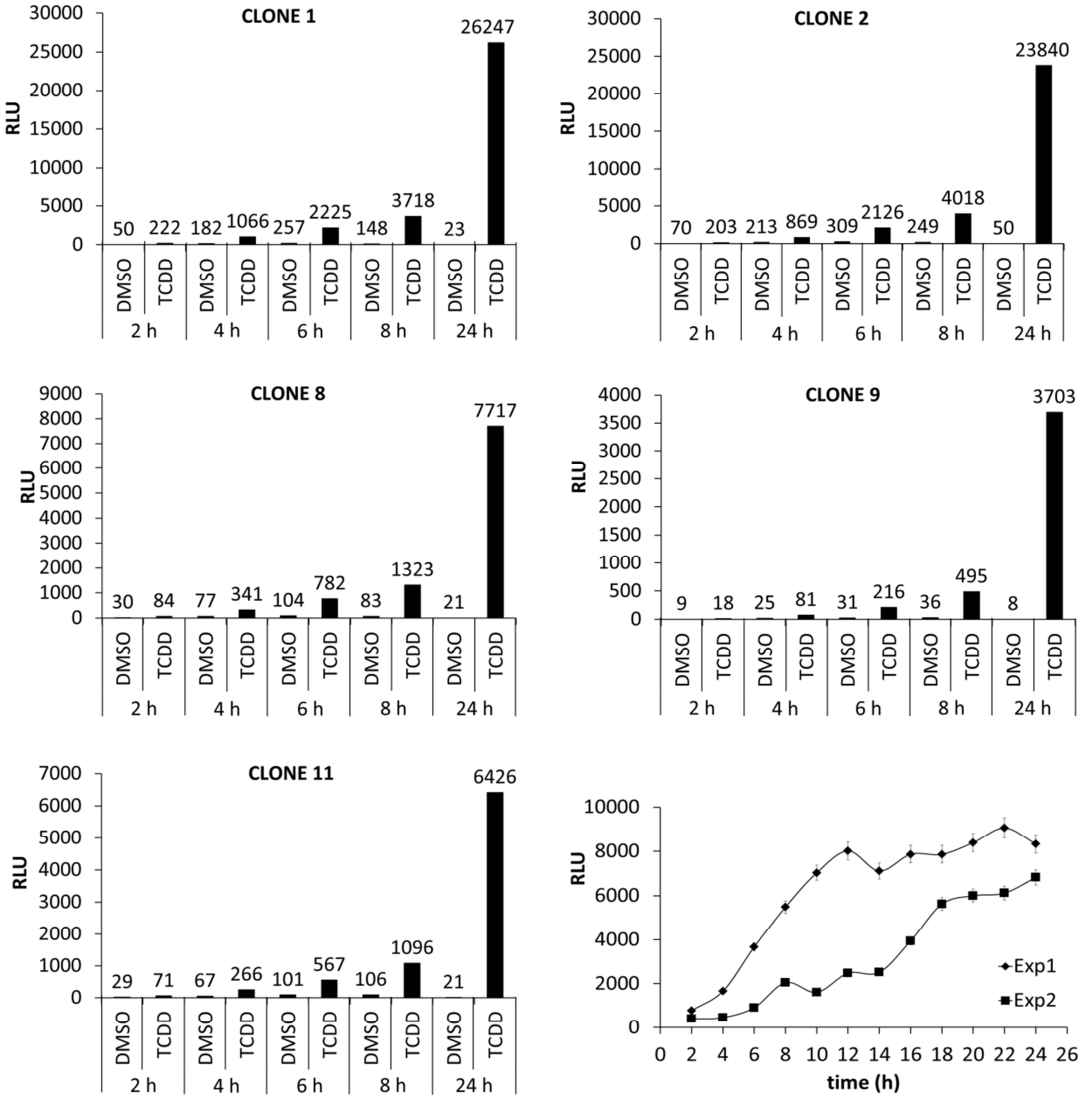

Figure 6: Time-course analyses of AZ-AHR cells treated with TCDD. Bar graphs: Clones 1, 2, 8, 9 and 11 of AZ-AHR cells were treated for 2 h, 4 h, 6 h, 8 h and 24 h with TCDD (5 nM) and vehicle (DMSO; 0.1% v/v). The data are the mean from triplicate measurements and are expressed as relative luciferase units (RLU in bar graphs). **Bottom right plot:** Clone 2 was treated for 2 h, 4, 6, 8, 10, 12, 14, 18, 20, 22, and 24 h with TCDD and/or DMSO. Showed are data from two independent experiments. The data are mean from triplicate measurements and are expressed as relative luciferase units (RLU) over DMSO-treated cells.

4.1.1.4 Response of AZ-AHR cells to AhR ligands after freeze-thaw cycle

We tested the functionality of AZ-AHR cells (clone 1) after cryopreservation. We treated fresh cells (8th passage = fresh 1 and 12th passage = fresh 2) with TCDD (0.01 nM – 100 nM), 3MC (1 µM), SP600125 (10 µM), RVT (10 µM), IND (1 µM) and DMSO for 24 h. Thereafter, cells were frozen according to a general procedure, using fetal bovine serum and DMSO as a cryoprotectant. Cells were stored in liquid nitrogen for

three weeks. After thawing, the treatments (*vide supra*) were repeated on the thawed cells.

Dose-response curves and EC_{50} values for TCDD were similar for fresh cells and the cells after a freeze-thaw cycle (Fig. 7A). There were not substantial differences between the fold induction of luciferase activity by AhR ligands (3MC, IND, RVT, SP600125) in fresh and cryopreserved cells (Fig. 7B). Collectively, the novel reporter AZ-AHR cell line is fully functional and responsive to AhR ligands after the cryopreservation process.

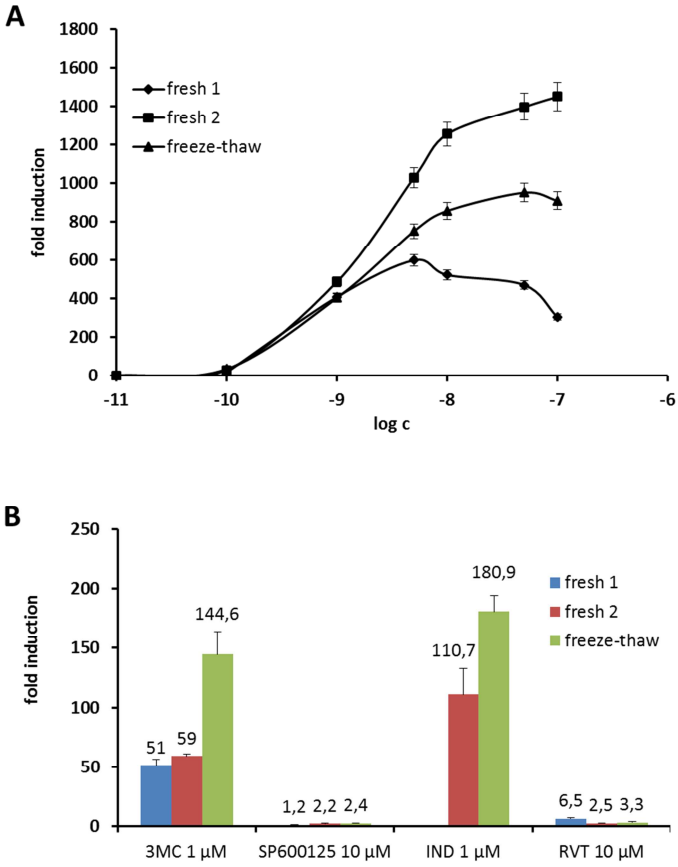

Figure 7: Response of AZ-AHR cells to AhR ligands after a freeze-thaw cycle. Experiments were performed in clone 1 of AZ-AHR fresh cells (8th passage = fresh 1 and 12th passage = fresh 2), and in clone 1 of AZ-AHR cells (freeze-thaw) after cryopreservation. Cells were treated with TCDD (0.01 nM – 100 nM), 3MC (1 µM), SP600125 (10 µM), RVT (10 µM), IND (1 µM) and DMSO (0.1% v/v) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are the mean from triplicate measurements and are expressed as fold inductions over DMSO-treated cells. **Panel A:** Dose-response curves for fresh and cryopreserved AZ-AHR cells treated with TCDD. **Panel B:** Effects of 3MC, SP600125, RVT, IND on luciferase activity in fresh and cryopreserved AZ-AHR cells.

4.1.1.5 Maintenance of luciferase inducibility by TCDD in AZ-AHR cells

Finally, we tested the ability of five clones (1, 2, 8, 9, 11) of the AZ-AHR cell line for response to TCDD over long term period. We checked the response of the cells to TCDD (5 nM; 24 h) after each passage of the cells. The induction of luciferase activity by TCDD was stable during the 30 days of the AZ-AHR cells in culture, which corresponds to 15 passages (Tab. 3).

Table.3: Maintenance of luciferase inducibility by 5 nM TCDD

Passage	Days in	Clone 1		Clone 2		Clone 8		Clone 9		Clone 11	
number	culture	RLU	Fold	RLU	fold	RLU	fold	RLU	fold	RLU	fold
3	1	16565	1273	16088	567	11570	788	9578	522	6307	787
5	7	25244	1262	16296	858	8905	786	5624	469	6791	1072
7	12	40016	599	5401	149	3608	1503	11750	746	9065	339
8	14	19815	417	21605	483	12709	643	4693	335	13803	746
9	16	nd	nd	36338	689	18931	653	5567	445	7629	254
12	22	57912	1059	43785	431	15131	496	3975	370	10827	274
13	26	27544	599	16428	345	7423	244	2817	352	8359	237
14	28	28698	2870	17583	440	6920	706	2368	592	9084	966
15	30	26247	944	23840	475	7717	371	3703	411	6426	300

4.1.2 Construction and characterization of the stable reporter gene cell line for the assessment of human glucocorticoid receptor activation - AZ-GR cells

4.1.2.1 Selection process and response of AZ-GR clones to dexametasone

HeLa cells were transfected with pGL-4.27-GRE reporter plasmid and stably transfected clones were selected under the pressure of hygromycin. We found slight morphological differences between the parent HeLa cells and stably transfected AZ-GR cells. HeLa cells have an epithelial like morphology and elongated shape, while transfected cells were rather oval (Fig. 8).

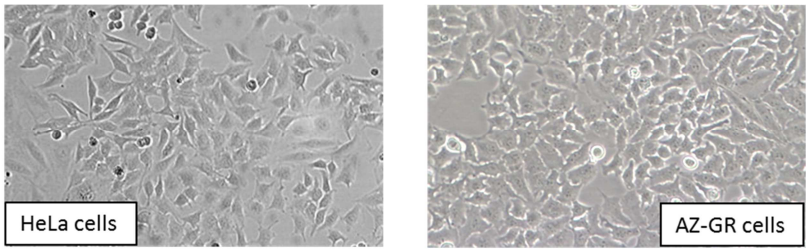

Figure 8: Morphology of AZ-GR cells and HeLa cells. Phase contrast micrographs of parent HeLa cells (at 7th passage) and AZ-GR cells (5th passage, clone 2).

Hygromycin-resistant clones were tested for responsiveness to synthetic glucocorticoid dexamethasone (DEX). For this purpose, stably transfected clones cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated with 100 nM DEX and vehicle (DMSO; 0.1% v/v) for 24 h. From seventeen hygromycin resistant clones, five showed strong induction of luciferase activity by dexamethasone. The fold induction of clones 1, 2, 3, 11 and 15 by dexamethasone ranged from 32× to 101×. There was no induction of luciferase activity in parent HeLa cells (Fig 9). The most responsive clones 2 and 15 of AZ-GR cells, yielding both high relative luciferase units (RLU) and high fold induction over the vehicle-treated cells, were used for further detailed analyses.

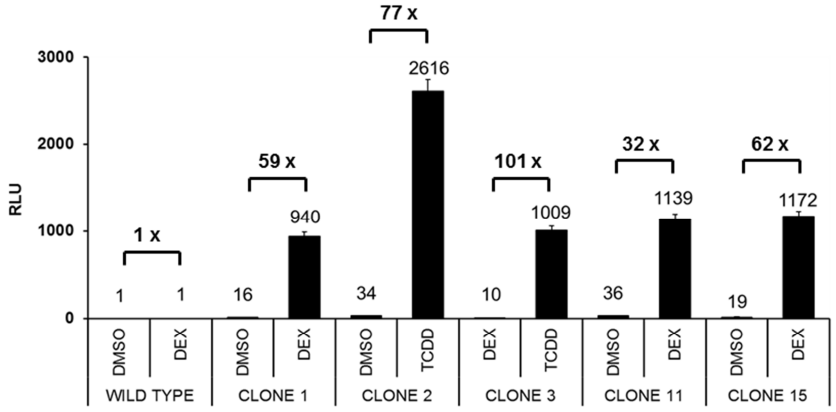

Figure 9: Response of stably transfected GR-responsive clones to dexamethasone. Clones 1, 2, 3, 11 and 15 of AZ-GR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated with dexamethasone (DEX; 100 nM) and vehicle (DMSO; 0.1% v/v) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are the mean from triplicate measurements and are expressed as relative luciferase units (RLU).

4.1.2.2 Dose-response analyses of AZ-GR cells treated with steroids

We performed dose-response analyses with 22 well-established natural and synthetic ligands for steroid receptors, including glucocorticoids, mineralocorticoids, estrogens, androgens and gestagens. AZ-GR cells were treated in 96-well plates with increasing concentrations of tested compounds and vehicle (DMSO; 0.1% v/v) for 24 h, and luciferase activity was measured in cell lysates. The values of E_{MAX}, $LogEC_{50}$ and efficacies were calculated from dose-response curves (Fig. 10; Tab. 4).

The treatment of AZ-GR cells with glucocorticoids, including dexamethasone, beclometasone, betamethasone, cortisol, prednisolone, methylprednisolone and triamcinolone (0.01 nM – 10 µM) yielded typical sigmoid logarithmic dose response curves for both clones (Fig. 10). Sigmoid-shaped curves were also obtained for mineralocorticoids corticosterone (0.01 nM – 10 µM) and aldosterone (1 nM – 100 µM). However, the potency and efficacy of mineralocorticoids were substantially lower than those for glucocorticoids (Fig. 10; Tab. 4). Aldosterone can bind to GR, which is probably the reason for slightly increased luciferase activity in AZ-GR cells after treatment with mineralocorticoids (Fuller et al., 2000).

Mifepristone, the synthetic antagonist (partial agonist) of the glucocorticoid receptor and progesterone receptor, induced luciferase activity in a dose dependent manner. While the potency of mifepristone was much higher ($LogEC_{50}$ -10.65) as

compared to the potent glucocorticoid agonist dexamethasone ($LogEC_{50}$ -8.02), the efficacy of mifepristone was negligible (Fig. 10; Tab. 4).

Estrogens, androgens and gestagens did not induce luciferase activity in AZ-GR cells (Tab. 4). Collectively, these results reveal a very high selectivity of the novel cell line AZ-GR for glucocorticoids.

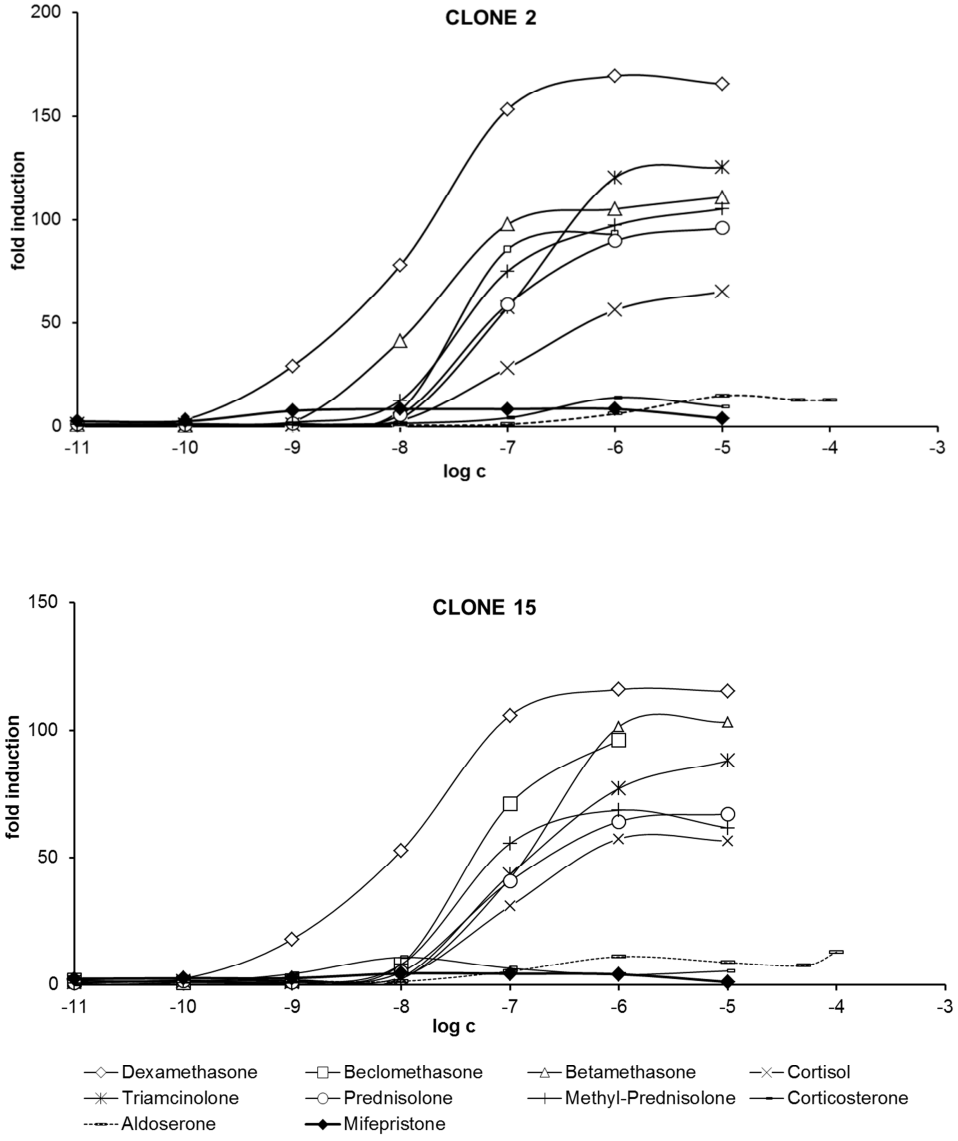

Figure 10: Dose-response analyses of AZ-GR cells treated with ligands for steroid receptors. AZ-GR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated for 24 h with: dexamethasone, beclometasone, betamethasone, cortisol, prednisolone, methylprednisolone, triamcinolone, mifepristone, corticosterone (0,01 nM – 10 µM) and aldosterone (1 nM – 100 µM). **Upper Panel:** Clone 2; **Bottom Panel**: Clone 15. After the treatments, cells were lysed and luciferase activity was measured. The data are the mean from triplicate measurements for each clone and are expressed as fold induction over DMSO-treated cells. Differences in RLU units within the triplicates were lower than 5%.

Table 4: Characteristics of AZ-GR cells in comparison with published data

	CLONE 2				Literature Log EC_{50}	
COMPOUND	**Log EC_{50}**	**E_{MAX} - fold**	**Efficacy - %**	**Log EC_{50} cryo**	**Sedlak et al.**	**Wilkinson et al.**
Dexametasone	-8.02 ± 0.01	170	262	-8.16	-8.78 ± 0.28	-9.81
Beclometasone	-7.52 ± 0.06	99	152	-7.42	-8.98 ± 0.14	nd
Betametasone	-7.81 ± 0.00	111	171	-7.85	-8.95 ± 0.15	nd
Cortisol	-7.18 ± 0.07	65	100	-7.17	-7.34 ± 0.35	-8.01
Triamcinolone	-7.27 ± 0.04	125	192	-7.25	-8.33 ± 0.12	nd
Prednisolon	-7.35 ± 0.01	96	148	-7.39	nd	nd
M-Prednisolon	-7.52 ± 0.04	105	162	-7.59	nd	-9.37
MIfepristone	-10.65 ± 0.16	8	12	-9.85	-8.23 ± 0.34	-8.16
Corticosterone	-6.62 ± 0.13	14	21	-7.21	-7.52 ± 0.15	-6.36
Aldosteron	-5.95 ± 0.11	13	20	-6.42	-8.61 ± 0.72	> -2.00
17-α-OH-progesteron	> -5.00	1.0	1.5	> -5.00	> -5.00	Nd
Progesteron	> -5.00	1.0	1.5	> -5.00	-6.87 ± 0.18	> -2.00
Testosterone	> -5.00	1.3	2.0	> -5.00	-6.89 ± 0.10	> -2.00
Danazol	> -5.00	1.1	1.7	> -5.00	> -5.00	> -2.00
Cyproterone acetate	> -5.00	1.6	2.4	> -5.00	> -5.00	> -2.00
Spironolactone	> -5.00	0.7	1.1	> -5.00	> -5.30	> -2.00
17-β-estradiol	> -5.00	0.2	0.3	> -5.00	> -5.00	> -2.00
Genistein	> -5.00	1.8	2.8	> -5.00	> -5.00	nd
Diethylstilbestrol	> -5.00	1.3	2.0	> -5.00	> -5.00	nd
4-OH-tamoxifen	> -5.00	0.9	1.4	> -5.00	> -5.00	> -2.00
Raloxifen hydrochlorid	> -5.00	1.1	1.7	> -5.00	nd	> -2.00
Tamoxifen citrate	> -5.00	2.2	3.4	> -5.00	> -5.00	> -2.00

Cryo - cells after freeze-thaw cycle; **EC_{50}** - half maximal effective concentration; **E_{MAX}** - maximal effective concentration - expressed as maximal fold activation; **Efficacy** - percentage of E_{MAX} attained by cortisol.

4.1.2.3 Time-course analyses of AZ-GR cells treated with dexamethasone

In the next series of experiments, we examined the time course of luciferase induction by dexamethasone in AZ-GR cells. The cells were treated with 100 nM dexamethasone and luciferase activity was measured after 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h and 24 h of incubation. In both clones, dexamethasone caused a time-dependent induction of luciferase activity. The significant induction was attained after 14 h of the treatment with dexamethasone, with fold induction ranging from 7 fold to 10 fold (Fig. 11).

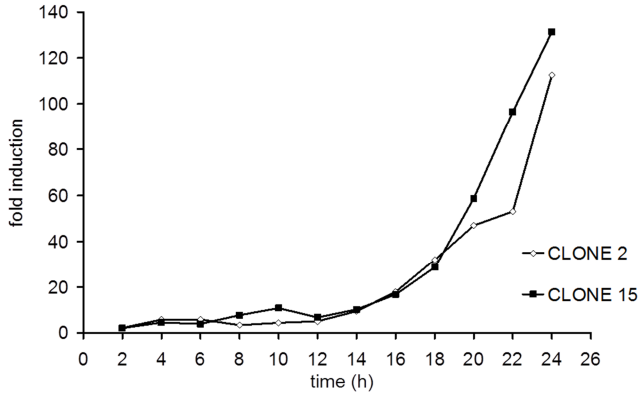

Figure 11: Time-course analyses of AZ-GR cells treated with dexamethasone. Clones 2 and 15 of AZ-GR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated for 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h and 24 h with DEX (100 nM) and vehicle (DMSO; 0.1% v/v). After the treatments, cells were lysed and the luciferase activity was measured. The data are the mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Differences in RLU units within the triplicates were lower than 5%.

4.1.2.4 Response of AZ-GR cells to steroid compounds after cryopreservation

AZ-GR cells were frozen according to a general procedure, using fetal bovine serum and DMSO as cryoprotectant. Cells were stored in liquid nitrogen for two weeks. After the thawing, we tested functionality of the AZ-GR cell line. The cells were seeded in 96-well plates and following 16 h of stabilization, treated with increasing concentrations of steroids and vehicle (DMSO; 0.1% v/v) for 24 h, and the luciferase activity was measured in cell lysates. The values of log EC_{50} were calculated from dose response curves for individual compounds. The fold inductions and the values of Log EC_{50} obtained in cryopreserved cells were not significantly different from those obtained in fresh cells (Fig, 12; Tab. 4). Taking together, the novel reporter AZ-GR cell line is fully functional and responsive to glucocorticoids after the cryopreservation process.

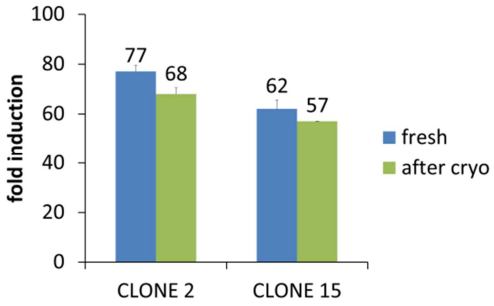

Figure 12: Response of AZ-GR cells to dexamethasone after cryopreservation. Experiments were performed in clones 2 and 15 of AZ-GR fresh cells (9th passage) and in clones 2 and 15 of AZ-GR cells after cryopreservation (freeze-thaw cycle). Cells were treated with DEX (100 nM) and DMSO (0.1 % v/v) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are the mean from triplicate measurement and are expressed as fold induction over DMSO-treated cells.

4.1.2.5 Maintenance of luciferase inducibility in AZ-GR cells by dexamethasone

Finally, we tested the ability of AZ-GR cell lines to respond to dexamethasone over the long term period. We checked the response of the cells to dexamethasone (100 nM; 24 h) after each passage of the cells. The induction of luciferase activity by dexamethasone was stable over the 32 days of the AZ-GR cells in culture, which corresponds to 16 passages (Table 5).

Table 5: Maintenance of luciferase inducibility by 100 nM DEX

Passage number	Days in culture	Clone 2		Clone 15	
		RLU	Fold	RLU	Fold
3	6	2612	72	2896	80
5	10	1371	67	1399	78
6	12	2202	110	2576	161
10	19	1139	68	961	57
11	21	637	31	685	55
13	26	736	35	647	51
14	28	1247	68	1291	88
16	32	971	47	890	68

4.2 Construction and characterization of hepatocyte-like cells

4.2.1 Construction and characterization of the HNF4α1 over-expressing cell line derived from human hepatoma HepG2 cells

4.2.1.1 Cytotoxicity of geneticin

We determined the cytotoxicity of G418 antibiotic in HepG2 cells for the periods of 1, 2, 3, 6 and 7 days. Employing a standard MTT assay, the values of IC_{50} for each time period were determined (data not shown). Based on these results, we have chosen G418 at a concentration of 1.1 mg/ml for the selection process.

4.2.1.2 HNF4α expression in the stable HepG2 transfectants

HepG2 cells were transfected with the pcDNA3-HNF4α and the control empty plasmid pcDNA3 carrying a *neo* selection marker conferring resistance to the antibiotic G418 (*neo* gene). We selected nine G418-resistant HepG2-HNF4α clones and one HepG2-pcDNA3 control clone. We measured the expression of HNF4α mRNA and the protein in HepG2-HNF4α, HepG2-pcDNA3 and in parent HepG2 cells. Surprisingly, there was no significant difference in the expression levels of HNF4α mRNA in HepG2-HNF4α cells as compared to HepG2-pcDNA3 and parent HepG2 cells. On the other hand, strongly increased protein levels of HNF4α were observed in all analysed HepG2-HNF4α clones (except for clone 4) as compared to HepG2-pcDNA3 and parent HepG2 cells (Fig. 13A). To verify the stable integration of the expressing vector, we determined mRNA expression level of *neo* gene in HepG2-HNF4α, HepG2-pcDNA3 and parent HepG2 cells. We found high levels of *neo* gene mRNA in HepG2-HNF4α cells and HepG2-pcDNA3 cells as compared to non-transfected HepG2 cells (Fig. 13B).

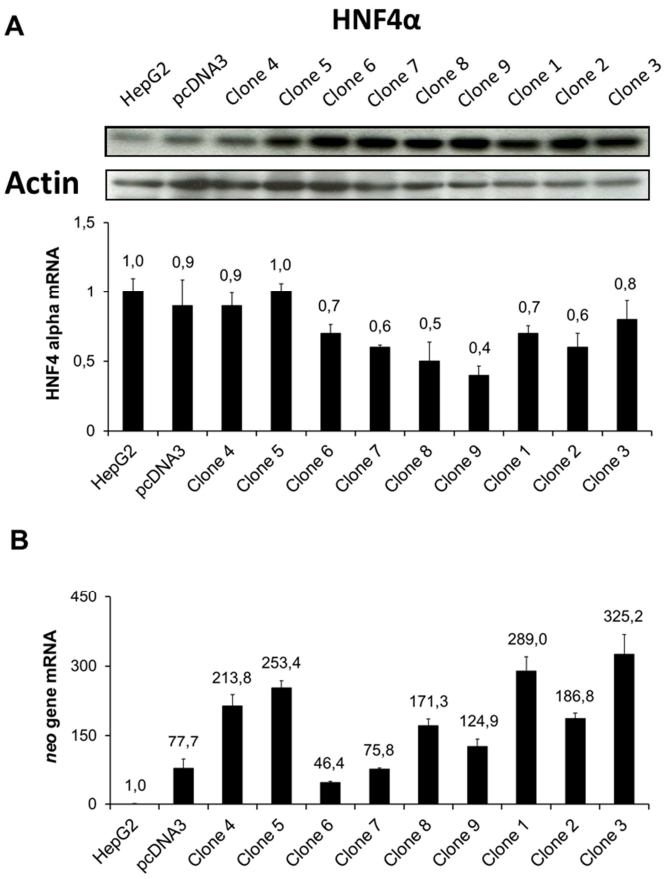

Figure 13: HNF4α expression in the stable G418-resistant HepG2-HNF4α cells. The expression of HNF4α and *neo* gene mRNAs and HNF4α protein was measured in 9 clones of HepG2-HNF4α cells, in HepG2-pcDNA3 cells and in parent HepG2 cells. **Panel A:** Representative RT-PCR analysis of HNF4α mRNA (bar graph) and western blot analysis of HNF4α protein (insert in graph) are shown. **Panel B:** Representative RT-PCR analysis of *neo* resistance gene mRNA in samples corresponding to Panel A. RT-PCR data are the mean ± SD from duplicate measurements and are expressed as fold induction over parent HepG2 cells. The data were normalized per GAPDH mRNA levels.

4.2.1.3 Secretion of hepatospecific markers

We analysed HepG2-HNF4α clones for the secretion of hepatospecific markers including α1-antitrypsin, albumin, fibrinogen and plasminogen in the medium. The cells were maintained in serum-free medium for 48 h. Secretion proteins in extracellular medium were analysed by Western blotting. Parent HepG2 and HepG2-pcDNA3 secreted α1-antitrypsin, albumin and fibrinogen, but the secretion of plasminogen was not detectable (Fig. 14). The secretion of fibrinogen was clearly increased in all clones (except for clone 4), which corresponds with the protein level of HNF4α (Fig. 13A). We found no change (clones 4, 5), slight increase (clones 2, 3, 6, 7, 8) or drastic decrease (clones 1, 9) of albumin secretion in HepG2-HNF4α cells. Plasminogen was detected in

clones 6, 7 and massively in clone 9. The protein level of α1-antitrypsin was not significantly altered in any clone as compared to HepG2-pcDNA3 and parent HepG2 cells.

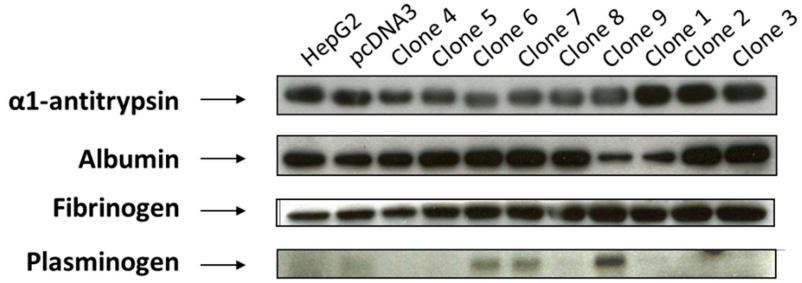

Figure 14: Secretion of hepatospecific markers in G418-resistant HepG2-HNF4α cells. Representative Western blotting analysis. The secretion of hepatospecific markers α1-antitrypsin, albumin, fibrinogen and plasminogen was monitored in culture media from G418-resistant HepG2-HNF4α cells, HepG2-pcDNA3 cells and parent HepG2 cells cultured in the absence of serum for 48 h.

4.2.1.4 Expression of xenoreceptors PXR and AhR

In the next series of experiments, we analysed the expression of xenoreceptors AhR and PXR in HepG2-HNF4α clones. The level of AhR mRNAs in HepG2-pcDNA3 control cells was decreased by approx. 50%; the levels in clones 1-9 varied between 30% – 90% of the AhR level in parent HepG2 cells (Fig. 15). In contrast, the level of AhR protein in HepG2-pcDNA3 control cells was increased as compared to parent HepG2 cells. The levels of AhR protein in clones 1-9 varied, and they were indirectly proportional to AhR mRNA levels; the highest expression of the AhR protein was in clones 2, 3, 6, 7, 8 and 9 (Fig. 15). The level of PXR mRNAs in HepG2-pcDNA3 control cells was slightly decreased (by approx. 10%); PXR mRNA levels in clones 1-9 varied between 40% – 100% of the PXR level in parent HepG2 cells (Fig. 15). The levels of PXR protein were elevated in all clones with exception of clone 4; the strongest expression of the PXR protein was in clones 6, 7, and 8 (Fig. 15).

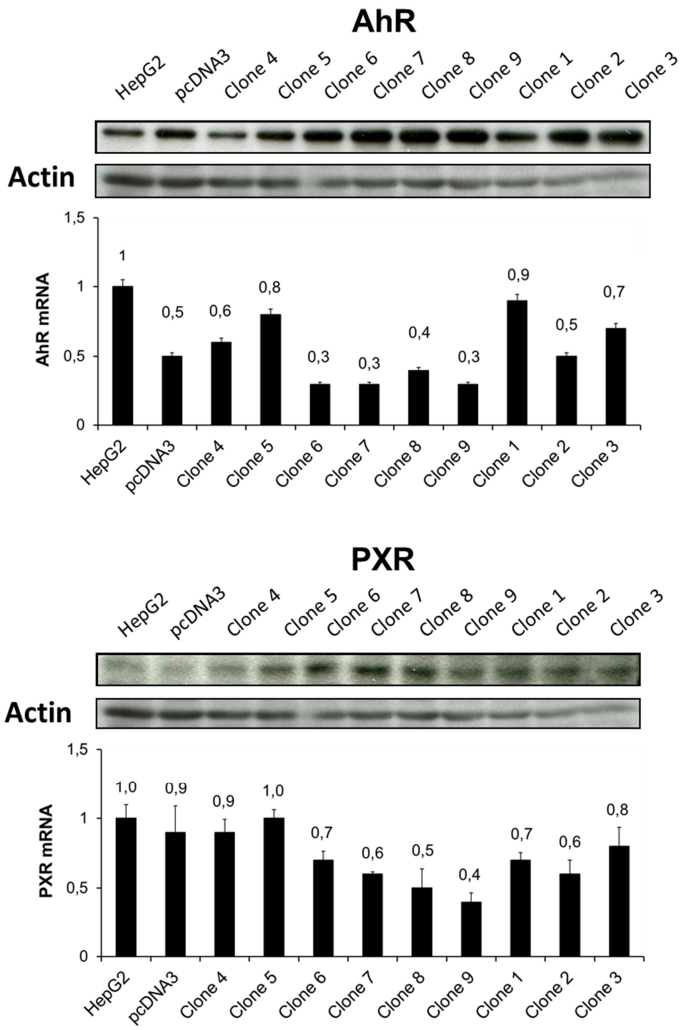

Figure 15: Expression of xenoreceptors AhR and PXR in G418-resistant HepG2-HNF4α cells. Representative RT-PCR analyses of AhR and PXR mRNAs (bar graphs) and western blots of AhR and PXR proteins (inserts in graphs) in G418-resistant HepG2-HNF4α cells, HepG2-pcDNA3 cells and parent HepG2 cells are showed. RT-PCR data are the mean ± SD from duplicate measurements and are expressed as fold induction over the value of HepG2 parent cells. The data were normalized per GAPDH mRNA levels.

4.2.1.5 Induction of drug-metabolizing enzymes CYP1A1 and CYP3A4

As a measure of the functionality of the hepatocyte-like HepG2-HNF4α cell line, we investigated ligand-inducible expression (induction) of CYP1A1 and CYP3A4 proteins and mRNAs. Cells were treated with rifampicin (RIF; 10 μM), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% v/v) for 24 h (mRNA analyses) and 48 h (protein analyses).

Treatment with TCDD resulted in strong induction of CYP1A1 mRNA (110 fold) and protein in parent HepG2 cells. Induction of CYP1A1 was much weaker in HepG2-pcDNA3 cells, at both mRNA (22 fold) and protein level. The magnitude of CYP1A1 mRNA induction in HepG2-HNF4α cells varied between 35 fold to 463 fold in individual clones. The expression at mRNA level was well correlated with CYP1A1 protein levels (Fig. 16). It is worth noting that CYP1A1 induction was significantly stronger in all tested clones of HepG2-HNF4α cells as compared to HepG2-pcDNA3 cells stably transfected with an empty resistance vector. When compared to parent HepG2 cells, the induction of CYP1A1 mRNA and protein was the highest in clone 1 (Fig. 16).

Induction of CYP3A4 mRNA by RIF in parent HepG2 cells was negligible (1.8 fold). The level of CYP3A4 mRNA in HepG2-pcDNA3 cells treated by RIF was even slightly decreased as compared to vehicle-treated cells; i.e. no induction. Similarly, the induction of CYP3A4 mRNA by RIF in HepG2-HNF4α cells varied in individual clones between 0.8 fold to 2.4 fold, and these values were not significantly different from CYP3A4 mRNA induction in parent HepG2 cells (Fig. 16). The basal level of CYP3A4 protein in parent HepG2 was not hardly detectable, but treatment with RIF resulted in a clear induction of CYP3A4 protein. The levels of both basal and RIF-induced CYP3A4 protein in HepG2-pcDNA3 cells were higher as compared with HepG2 cells, which was inconsistent with the expression of CYP3A4 mRNA. While basal expression of CYP3A4 protein was detected in all clones of HepG2-HNF4α cells, the RIF-induced levels of CYP3A4 protein were in all clones lower than in parent HepG2 and HepG2-pcDNA3 cells (Fig. 16).

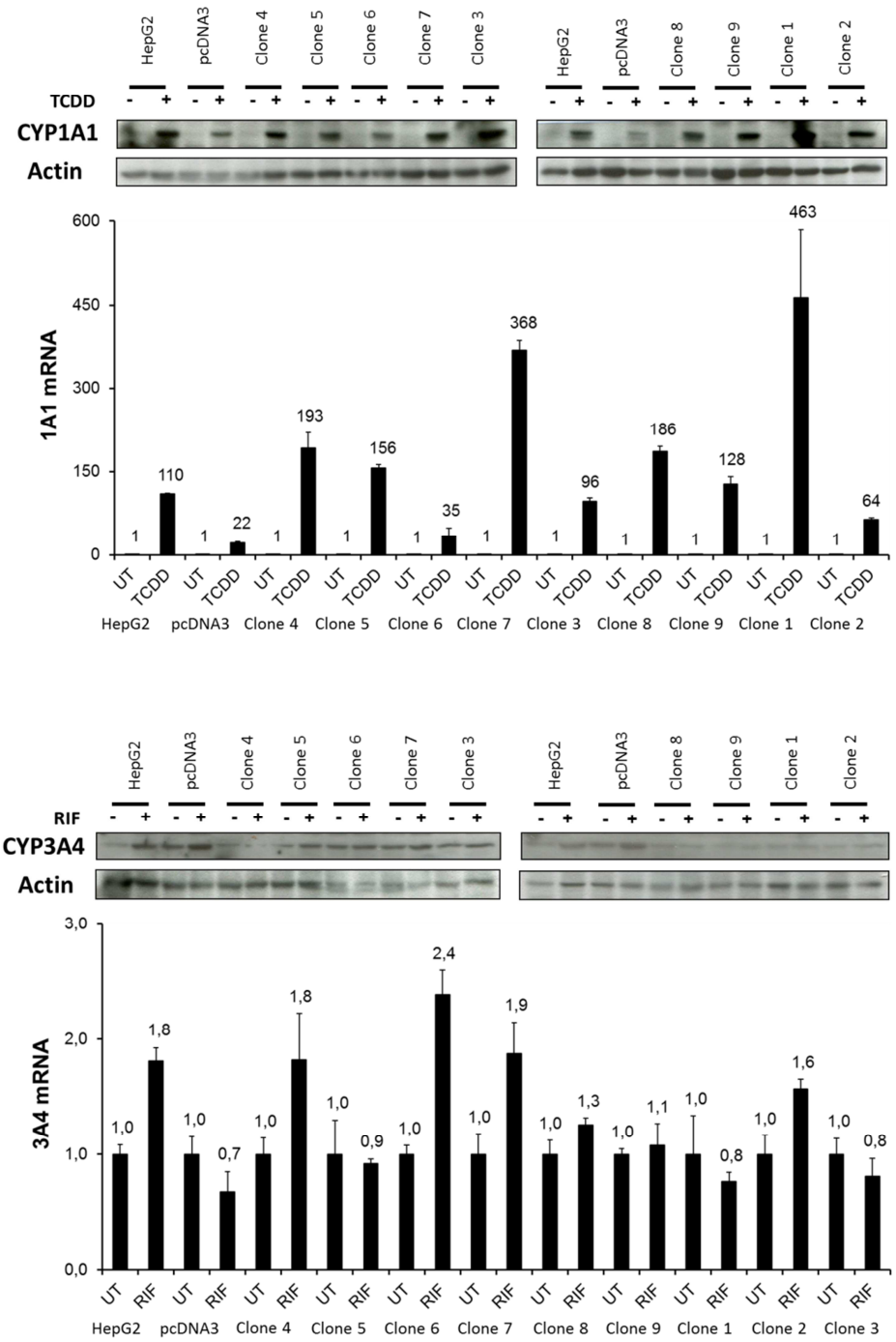

Figure 16: Induction of CYP1A1 and CYP3A4 in G418-resistant HepG2-HNF4α cells. G418-resistant HepG2-HNF4α cells, HepG2-pcDNA3 cells and parent HepG2 cells were treated with rifampicin (RIF; 10 µM), 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% v/v) for 24 h (mRNA analyses) and 48 h (protein analyses). Representative RT-PCR analyses of CYP1A1 and CYP3A4 mRNAs (bar graphs) and western blots of CYP1A1 and CYP3A4 proteins (inserts in graphs) are showed. RT-PCR data are the mean ± SD from duplicate measurements and are expressed as fold induction over vehicle-treated cells in each cell type or clone. The data were normalized per GAPDH mRNA levels.

4.2.2 PGC-1α over-expressing cell line derived from human hepatocyte carcinoma HepG2 cells

4.2.2.1 PGC-1α expression in the stable HepG2 transfectants

HepG2 cells were transfected with the pcDNA3-PGC-1α and the control empty plasmid pcDNA3 carrying a *neo* selection marker conferring resistance to the antibiotic G418 (*neo* gene). We selected six G418-resistant HepG2-PGC-1α clones and one HepG2-pcDNA3 control clone. We measured the expression of PGC-1α mRNA and protein in HepG2-PGC-1α, HepG2-pcDNA3 and in parent HepG2 cells. We found no significant difference in the expression levels of PGC-1α mRNA in HepG2-PGC-1α cells as compared to HepG2-pcDNA3 and parent HepG2 cells. On the other hand, we observed a largely increased PGC-1α protein level in clones 3, 4, 5 and 6 as compared to the control HepG2-PGC-1α and parent HepG2 cells (Fig. 17A). We determined the mRNA expression the level of *neo* gene in HepG2-PGC-1α, HepG2-pcDNA3 and parent HepG2 cells to verify the stable integration of the expressing vector (Fig. 17B). The morphology of HepG2-PGC-1α cells was slightly different from parent HepG2 cells (Fig. 18).

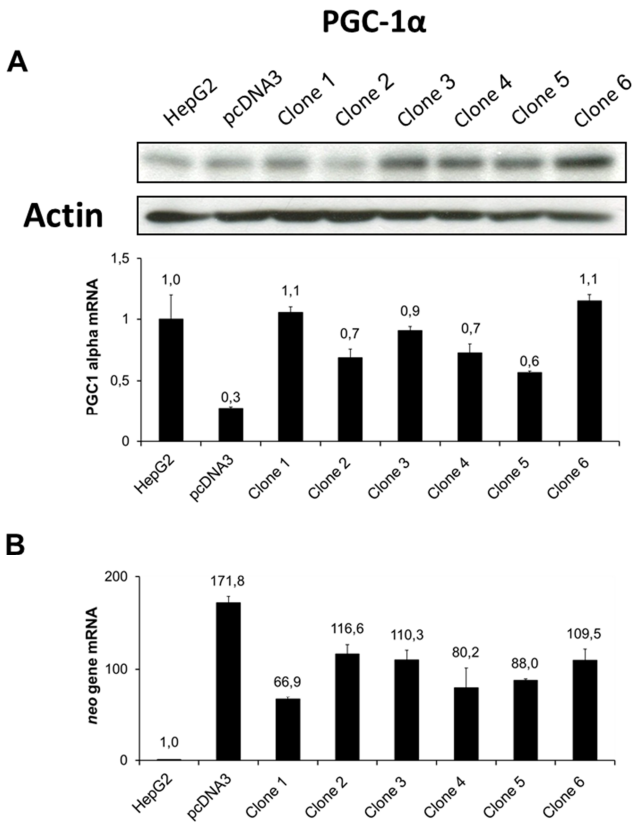

Figure 17: PGC-1α expression in the stable G-418 resistant HepG2-PGC-1α cells. The expression of PGC-1α and *neo* gene mRNAs and PGC-1α protein was measured in 6 clones of HepG2-PGC-1α cells, in HepG2-pcDNA3 cells and in parent HepG2 cells. **Panel A:** Representative RT-PCR of PGC-1α mRNA (bar graph) and western blot analyses of PGC-1α protein (insert in graph). **Panel B:** RT-PCR analyses of *neo* resistance gene mRNA in samples corresponding to Panel A. RT-PCR data are the mean ± SD from duplicate measurements and are expressed as fold induction over the value from parent HepG2 cells. The data were normalized per GAPDH mRNA levels.

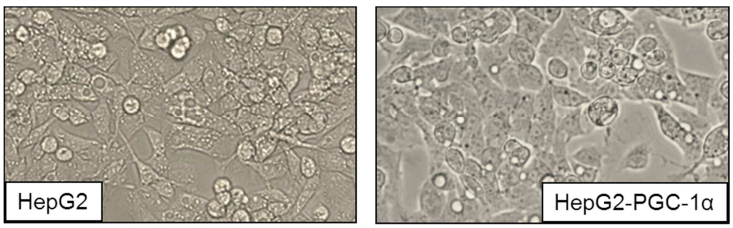

Figure 18: Morphology of parent HepG2 and HepG2-PGC-1α stable cell line. The picture shows HepG2 at passage 8 and HepG2-PGC-1α stable cell line at passage 21.

4.2.2.2 Secretion of hepatospecific markers

We analysed HepG2-PGC-1α clones for the secretion of hepatospecific markers including α1-antitrypsin, albumin and fibrinogen. The cells were maintained in serum-free medium for 48 h. Secretion proteins in an extracellular medium were analysed by Western blotting. The secretion of fibrinogen was increased in all clones (Fig. 19). The greatest increase was observed in clones 3, 4 and 6, which correlates with the protein level of PGC-1α and HNF4α (Fig. 17A and Fig. 20). We found no increase in the secretion of albumin and α1-antitrypsin in any clone, as compared to HepG2-pcDNA3 and parent HepG2.

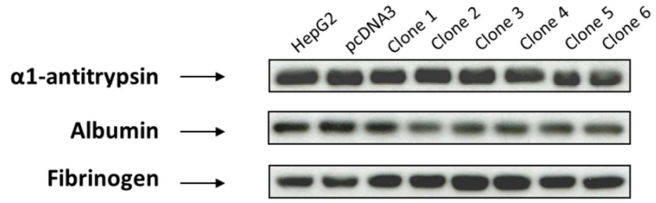

Figure 19: Secretion of hepatospecific markers α1-antitrypsin, albumin and fibrinogen in G418-resistant HepG2-PGC-1α cells. The secretion of hepatospecific markers α1-antitrypsin, albumin and fibrinogen was monitored in culture media from G418-resistant HepG2-PGC-1α cells, HepG2-pcDNA3 cells and parent HepG2 cells cultured in the absence of serum for 48 h.

4.2.2.3 Expression of transcription factor HNF4α

Next, we analysed the expression of the transcription factor HNF4α, the most important regulator of hepatic drug-metabolizing P450s. We found an increased expression of HNF4α protein in all clones (except for clone 2) and in the control HepG2-pcDNA3 cells as compared to parent HepG2 cells (Fig. 20). The greatest increase was detected in clones 3, 4, 5 and 6 and this correlates with PGC-1α protein level. The expression level of HNF4α mRNA was moderately decreased in HepG2-pcDNA3 control cells in comparison with parent HepG2 cells. HNF4α mRNAs level in tested HepG2-PGC-1α cells ranged between 0.7 fold to 1.3 fold compared to the parent HepG2.

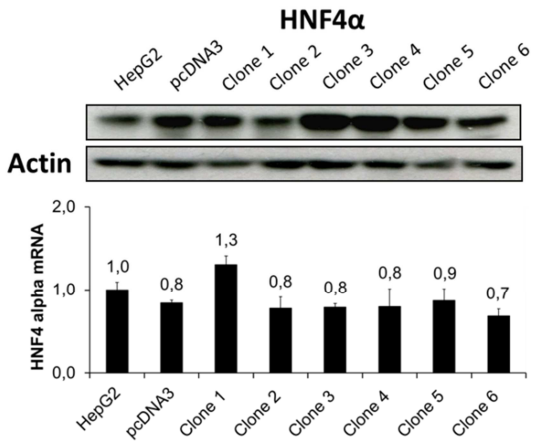

Figure 20: Expression of HNF4α in the stable G-418 resistant HepG2-PGC-1α cells. Representative RT-PCR analysis of HNF4α mRNA (bar graph) and western blot of HNF4α protein (insert in graph) in G418-resistant HepG2-PGC-1α cells, HepG2-pcDNA3 cells and parent HepG2 cells. RT-PCR data are the mean ± SD from duplicate measurements and are expressed as fold induction over the value of HepG2 parent cells. The data were normalized per GAPDH mRNA levels.

4.2.2.4 Expression of xenoreceptors

In the next series of experiments, we examined the expression of the xenoreceptors AhR and PXR. The level of AhR mRNA in the control HepG2-pcDNA3 cells was slightly increased (by approx. 40%) and in clones 1-6 varied between 90% - 160% of the AhR level in parent HepG2 cells. The AhR protein level in HepG2-pcDNA3 was not altered in comparison with parent HepG2 cells. In contrast, we found major increase in AhR protein levels in clones 3, 4 and 6 and moderate increase in clone 5 (Fig. 21), which correlates with PGC-1α protein level (Fig. 17A). PXR mRNA in HepG2-pcDNA3 control cells was decreased by approx. 30% and the levels in clones 1-6 varied between 20% and 110% of the PXR level in parent HepG2 cells. On the other hand, the protein level of PXR was increased in all clones as well in the control HepG2-pcDNA3 cells compared to parent HepG2 cells (Fig. 21).

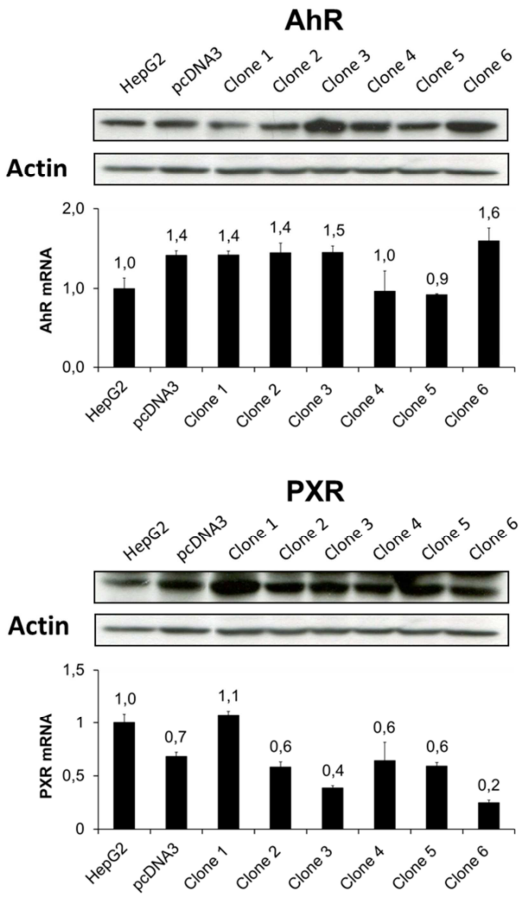

Figure 21: Expression of AhR and PXR in the stable G-418 resistant HepG2-PGC-1α cells. Representative RT-PCR analyses of AhR and PXR mRNAs (bar graphs) and western blots of AhR and PXR proteins (inserts in graphs) in G418-resistant HepG2-PGC-1α cells, HepG2-pcDNA3 cells and parent HepG2 cells. RT-PCR data are the mean ± SD from duplicate measurements and are expressed as fold induction over the value of HepG2 parent cells. The data were normalized per GAPDH mRNA levels.

4.2.2.5 Expression of drug-metabolizing enzymes CYP1A1 and CYP3A4

In the last series of experiments, we measured the basal and ligand inducible expression of CYP1A1 and CYP3A4, the most prominent drug-metabolizing cytochromes P450. HepG2-PGC-1α cells were treated with rifampicin (RIF; 10 µM), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% v/v) for 24 h (mRNA analyses) and 48 h (protein analyses).

The basal expression of CYP3A4 protein in HepG2-PGC-1α cells was markedly increased in all clones, as compared to the parent HepG2 and the control HepG2-pcDNA3 cells. Rifampicin strongly induced CYP3A4 protein in HepG2 and HepG2-pcDNA3 cells. However, no induction of CYP3A4 protein by rifampicin in HepG2-PGC-1α cells was found, and the levels of CYP3A4 protein were nearly identical in DMSO- and RIF-treated cells. An apparent loss of CYP3A4 protein induction by RIF was due to the elevated basal levels of CYP3A4 protein in HepG2-PGC-1α cells (Fig. 22). Induction of CYP3A4 mRNA by RIF ranged from 1.5 to 1.7 fold in parent HepG2 and HepG2-pcDNA3 cells. The basal and RIF-inducible expression of CYP3A4 mRNAs in HepG2-PGC-1α did not significantly differ from CYP3A4 mRNA expression in parent HepG2 cells (Fig. 22).

The typical AhR agonist TCDD, induced CYP1A1 mRNA with similar potency in all examined cell lines, i.e., in HepG2 cells (approx. 163 fold), in HepG2-pcDNA3 cells (approx. 150 fold) cells, and in HepG2-PGC-1α cell clones (from 65 fold to 198 fold in individual clones) (Fig. 22). No basal level of CYP1A1 protein was found in either cell line under examination. TCDD-inducible expression of CYP1A1 protein was diminished in control cells HepG2-pcDNA3 compared to parent HepG2 cells. The level of CYP1A1 inducible protein was in all clones of HepG2-PGC-1α cells higher than in the control cells HepG2-pcDNA3, but similar to the levels in parent HepG2 cells. In clone 1 we found a stronger induction of CYP1A1 protein than in parent HepG2 but this did not correlate with CYP1A1 mRNA level (Fig. 22).

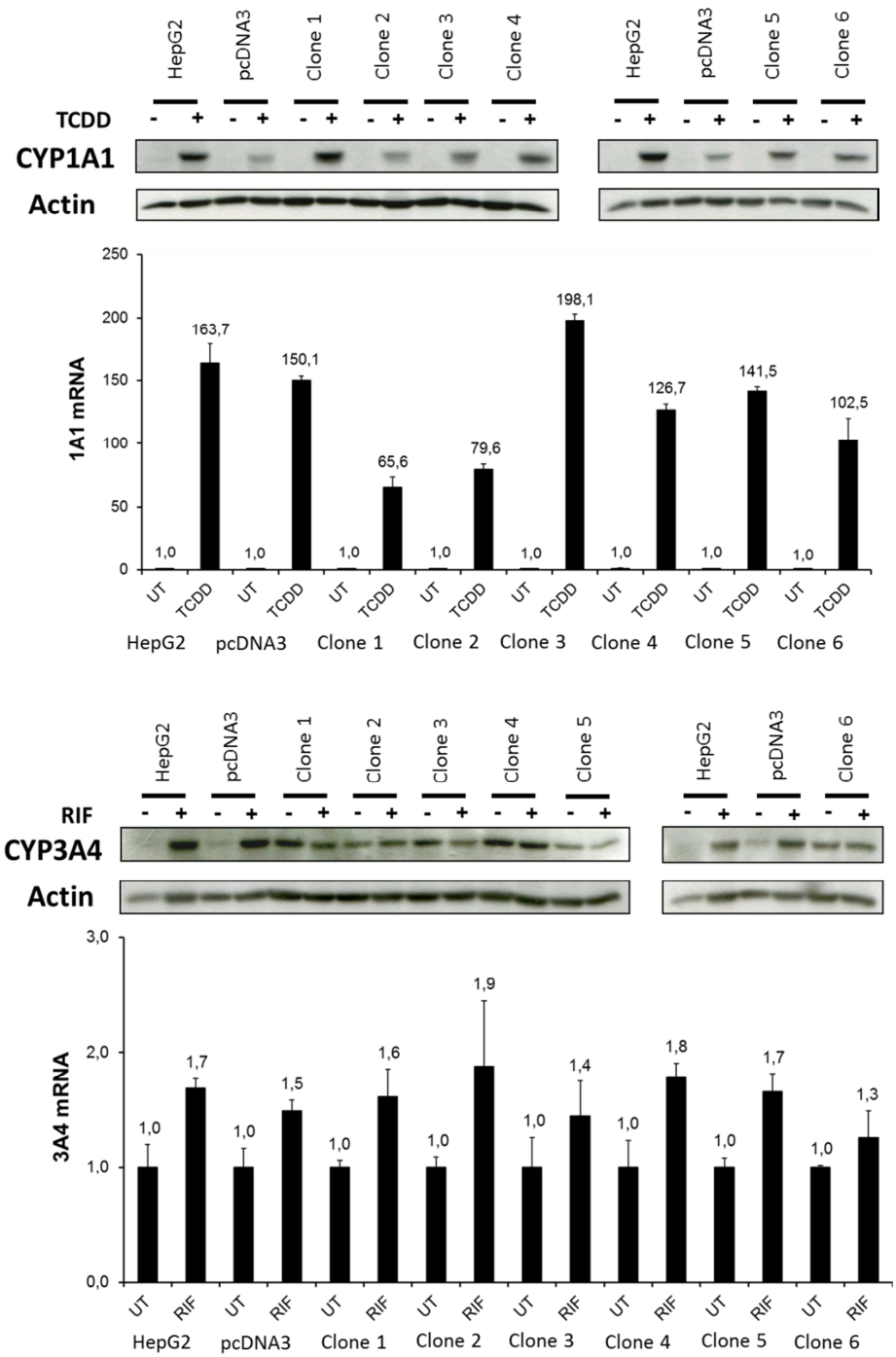

Figure 22: Expression of CYP1A1 and CYP3A4 in the stable G-418 resistant HepG2-PGC-1α cells. G418-resistant HepG2-PGC-1α cells, HepG2-pcDNA3 cells and parent HepG2 cells were treated with rifampicin (RIF; 10 µM), 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% v/v) for 24 h (mRNA analyses) and 48 h (protein analyses). Representative RT-PCR analyses of CYP1A1 and CYP3A4 mRNAs (bar graphs) and western blots of CYP1A1 and CYP3A4 proteins (inserts in graphs). RT-PCR data are the mean ± SD from duplicate measurements and are expressed as fold induction over vehicle-treated cells in the each cell type or clone. The data were normalized per GAPDH mRNA levels.

5. DISCUSSION

Various *in vitro* assays are used for environmental applications, screenings and for drug discovery. Reporter gene assays are experimental systems mainly used for large scale detection of various activators and profiling of a wide range of compounds affecting transcriptional factors and nuclear receptors, based on the plasmid transfected. While generation of a stable cell line is a time consuming process (weeks to months), they have several advantages, such as good reproducibility of results due to the homogenous transfection, high sensitivity because 100% of cells are transfected etc. Fundamentally, two common approaches are used for generation of stable reporter gene cell lines:

(i) Cell line is derived from cells that do not contain the receptor of interest and they are usually rapid growing, low-differentiated cell lines such as CHO, CV-1 etc. The transgenic cells are then constructed in two steps; the first step is transfection with reporter vector containing regulatory sequences and in the second step the transfection with expression plasmid encoding corresponding steroid receptor follows. Disadvantage of this approach is the length of this process, which is twice longer than for single transfection process and requires selection with two different antibiotics (Sedlak et al., 2011). In addition, resulting cell line may be far away from physiological situation, in term of expression of transcriptional co-activators etc. Advantage of this approach is usually very high selectivity of the cell line for desired group of compounds (e.g. glucocorticoids).

(ii) Starting parent cell line expresses fully functional endogenous receptor, which is the case of HeLa cells expressing functional GR or HepG2 expressing AhR. The cells are transfected only with reporter plasmid and only one selection agent is necessary (Mori et al. 2008).

Several stably transfected gene reporter cells lines have been developed for an assessment of transcriptional activity of AhR. Probably the most known system is an *in vitro* bioassay CALUX (chemical activated luciferase expression), which is a rat hepatoma H4IIE cell line stably transfected with a construct containing DRE sequence fused to the luciferase reporter gene (Murk et al., 1996). The assay was used for environmental applications, but it was modified for other purposes (Bovee et al., 1998; Seidel et al., 2000). Rat cells H4IIE were also used by others (Cui et al., 2002). The examples of stable cell lines derived from mouse hepatoma cells Hepa1c1c7 are DR-EcoScreen cells and third generation of CALUX (He et al., 2011). Since there are substantial differences between AhR signaling in rodent and human cells, for example the different effects of glucocorticoids on AhR signaling, this is of great interest to

establish cell line derived from human cells, responding to AhR activation (Sonneveld et al., 2007). The examples of reporter cell lines derived from human hepatoma HepG2 cells are 101L cells that allow simultaneous assessment of the transcriptional activation of CYP1A1 and CYP1A2 genes (Sato et al., 2010).

Several stably transfected reporter gene cell lines have been developed for assessment of GR transcriptional activity. Sedlak et al. constructed two panels of potent and selective luciferase reporter cell lines derived from U2OS cells (Sedlak et al., 2011). Wilkinson et al. have established complete panel of cell-based steroid hormone receptor assays for the androgen receptor, estrogen receptor α and β, glucocorticoid receptor, mineralocorticoid receptor, and progesterone receptor by stably engineering a Gal4 DNA-binding domain/nuclear receptor ligand-binding domain fusion protein into an upstream activation sequence beta-lactamase reporter cell line (Wilkinson et al., 2008). Stable reporter gene cell line MDA-kb2, derived from breast cancer cells MDA-MB-453, has been originally developed for screening AR agonist and antagonists and to characterize its specificity and sensitivity to EDCs (Wilson et al., 2002).

I have developed stably transfected gene reporter human hepatoma cell lines AZ-AHR and AZ-GR allowing assessment of aryl hydrocarbon receptor and glucocorticoid receptor transcriptional activities, respectively. The AZ-AHR cell line was prepared from HepG2 transfected with reporter plasmid pGL-4.27-DRE, containing two copies of F site sequences, one copy of B site and one copy of D site sequences. Resulting cells yielded dose- and time- dependent induction of luciferase activity in response to treatment with various AhR ligands, including TCDD, 3MC, IND, RVT, OME and SP600125. The assay was very sensitive allowing high throughput format (96-well plate) and evaluation of luciferase activity as soon as after 6 h, which allows testing of highly toxic compounds. AZ-AHR cells remained fully functional over 15 passages and 30 days in culture, and the response of cryopreserved cells after thawing to AhR ligands did not decline. AZ-GR cell line was derived from HeLa cells transfected with reporter plasmid containing three copies of glucocorticoid response elements. A panel of 22 natural and synthetic steroids was tested in cell line AZ-GR and the values of E_{MAX}, EC_{50} and efficacies were calculated, and they were comparable with the data from literature (Table 1) (Wilkinson et al. 2008). AZ-GR cells were highly specific and sensitive to glucocorticoids, and no responsiveness to estrogens, gestagens or androgens was observed. Cells displayed very low responsiveness to mineralocorticoids, and the potency and efficacy of mineralocorticoids was much lower than those for glucocorticoids. The sensitivity of the assay allowed high throughput format of the analyses, using 96-well plates. Time-course analyses revealed that GR

activators may be detected using AZ-GR cells soon after 14 h of the treatment. A functionality of AZ-GR cells was not affected by cryopreservation process and the cells fully maintained responsiveness to glucocorticoids for 32 days in the culture and over 16 passages without significant alterations.

Collectively, stably transfected luciferase reporter gene cell lines AZ-AHR and AZ-GR allowing high throughput, rapid and sensitive detection of AhR and GR activators were constructed and characterized. They have potential use in pre-clinical research, in environmental applications and screenings and in drug discovery.

The second part of the present thesis was focused on the construction of hepatocyte-like human cell lines. Liver-derived systems are of great importance and they involve liver microsomes, liver slices, primary human hepatocytes, immortalized hepatocyte and liver-derived cancer cell lines. Primary human hepatocytes are considered as a gold *in vitro* model for drug metabolism and interactions studies. However, their use is limited due to the low availability of liver tissue, *in vitro* phenotypic instability, limited life-span, insufficient quality of cryopreserved cells, inter-individual variability etc. Human liver-derived cancer cell lines have some advantages over human hepatocytes, such as availability, unlimited life-span, proliferation in culture and easy cryopreservation. However their phenotype is altered and they do not exert major hepatospecific functions, including drug-metabolizing capacity. It was demonstrated that the deficient expression of hepatic genes is at least partly caused by a lower level of transcriptional factors and co-activators (PGC-1α, SRC-1, SRC-2, C/EBPα, HNF4α) in human hepatoma cells.

In the present thesis, hepatocyte-like cells were constructed with the aim to restore the biotransformation capacity of human hepatoma cells HepG2. It has been demonstrated, that HepG2 cells lack a functional expression of almost all drug-metabolizing P450s, probably due to the malfunction of HNF4α that is caused by a low level of transcriptional co-activators PGC-1α, SRC-1 and SRC-2. PGC-1α activates P450 genes through co-activation of the transcription factor HNF4α which is a key factor for the expression of hepatic drug-metabolizing P450s and for the maintaining of hepatospecific functions (Jover et al., 2001). The over-expression of PGC-1α and SRC1 led to up-regulation of HNF4α - dependent genes (ApoCIII, ApoAV, PEPCK, AldoB, OTC, CYP7A1) and forced HepG2 cells toward a more differentiated phenotype (Martinez-Jimenez et al., 2006b). The transient transfection of PGC-1α to HepG2 cells resulted in an increased expression of CYP2C9, CYP1A1, CYP1A2 and CYP7A1 genes, and a moderate activating effect on CYP3A4, CYP3A5, and CYP2D6 (Martinez-Jimenez et al., 2006a). The up-regulation of HNF4α dependent genes mediated by PGC-1α was confirmed in co-transfection experiments with MZ-Hep1 HNF4α lacking

hepatoma cells. A significant increase in the expression of the cytochromes P450 was observed only with a transient co-transfection of PGC-1α together with HNF4α. Separate transfections of PGC-1α and HNF4α had no effects (Martinez-Jimenez et al., 2006a; Martinez-Jimenez et al., 2006b). Consistently, silencing of HNF4α by si-RNA in human hepatocytes resulted in down-regulation of CYP3A4, CYP3A5, CYP2A6, CYP2B6, CYP2C9 and CYP2D6 (Jover et al., 2001). Another study reported that PGC- 1α activates the transcription of CYP2A5 gene through the co-activation of HNF4α (Arpiainen et al., 2008). It was reported elsewhere, that the interactions of PXR with HNF4α, SRC-1 and PGC-1α are important for the induction of CYP3A4 (Gass et al., 2006; Matsumura et al., 2004). The importance of HNF4α in the expression of other drug metabolizing enzymes was also demonstrated, e.g. for CYP2A6 (Onica et al., 2008), CYP2B6 (Jover et al., 2001) and CYP2C subfamily (Chen et al., 2005; Kawashima et al., 2006; Pavek et al., 2007; Rana et al.). For these reasons, we tested whether the stable transfection of HepG2 cells with HNF4α and PGC-1α, separately, restores hepatospecific functions and will produce hepatocyte-like cells.

HepG2 cells stably over-expressing human HNF4α had a strongly increased HNF4α protein level, but the level of HNF4α mRNA was not altered. Since the stable integration of the plasmid in HepG2 cells was proven by *neo*-gene expression analysis, discrepancy between mRNA and protein levels of HNF4α could be due to the multiple mechanisms of feedback control (mRNA stability and functionality etc.). A hallmark of cellular hepatospecificity is the secretion of specific proteins such as albumin or clotting factors. HNF4α regulates the expression of HNF1, which is involved in the expression of albumin and fibrinogen (Maire et al., 1989; Tronche et al., 1997; Yamagata et al., 1996). In addition, a promoter of the α1-antitrypsin gene contains a HNF4α binding site (Monaci et al., 1988). We analysed 9 different clones of HepG2-HNF4α cells for the secretion of albumin, fibrinogen, plasminogen and α1-antitrypsin. We found improved secretion of hepatospecific proteins in the majority of the clones (with the exception of α1-antitrypsin), and each clone displayed an individual expression pattern of secreted proteins.

An important hepatospecific function is the capability of the liver cells to metabolize xenobiotics. Pivotal transcriptional regulators of drug-metabolizing cytochromes P450 are the xenoreceptors PXR and AhR. The role of HNF4α in the regulation of PXR gene during fetal liver development (but not in adult human liver) has been demonstrated (Kamiya et al., 2003). We found that the over-expression of HNF4α positively affects the expression of PXR and AhR proteins, since the levels of PXR and AhR proteins were elevated in HepG2-HNF4α cells. On the other hand, PXR and AhR mRNAs were decreased as compared to parent HepG2 cells. The dioxin-inducible

expression of CYP1A1 was moderately higher than in parent HepG2 cells. The basal expression of CYP3A4 protein was increased, but the rifampicin-inducible expression of CYP3A4 was lost in HepG2-HNF4α cells.

We showed that the stable over-expression of HNF4α in HepG2 cells improved some hepatospecific functions including secretion of plasma proteins or the increased the expression of xenoreceptors PXR and AhR (Novotna et al., 2011). Hence, we tested whether stable over-expression of PGC-1α in HepG2 restores hepatospecific functions. In clones with increased PGC-1α protein levels, we found the HNF4α protein level strongly increased, which is in accordance with findings of other groups (Arpiainen et al., 2008; Li & Chiang, 2006; Martinez-Jimenez et al., 2006a). Hepatospecific features of the cells including the secretion of albumin and clotting factors were analysed. We found the increased secretion of fibrinogen but not albumin and α1-antitrypsin in all tested clones of HepG2-PGC-1α cells. However, the stable over-expression of HNF4α in HepG2 increased the secretion of all these three plasma proteins (Novotna et al., 2011).

The over-expression of PGC-1α, and consequently the increased expression of the HNF4α protein, positively correlated with increased levels of PXR and AhR proteins in HepG2-PGC-1α clones 3, 4, 5 and 6. However, we found the highest expression of PXR protein in clone 1 which expressed lower levels of PGC-1α protein than clones 3, 4, 5 and 6. The dioxin-inducible expression of CYP1A1 mRNA and protein was not significantly altered compared to parent HepG2. The basal expression of CYP3A4 protein was increased in HepG2-PGC-1α cells, but rifampicin-inducible expression of CYP3A4 was consequently lost.

Collectively, we constructed and characterized cell lines derived from human hepatoma HepG2 cells stably transfected with HNF4α or PGC-1α. The resulting cell lines displayed some improved hepatospecific functions, such as secretion of plasma proteins or an increased expression of xenoreceptors PXR and AhR. However, the xenobiotic-inducible expression of CYP1A1 and CYP3A4 was not improved. This implies that the approach of stable over-expression of hepatospecific transcription factors only partly addressed the issue of the generation of hepatocyte-like cell lines for pre-clinical testing and toxicological applications.

6. CONCLUSION

The achievements of my PhD. thesis are four novel transgenic cell lines derived from human cancer cell lines. In particular, I have constructed and functionally characterized:

(i) Stable transfected luciferase reporter gene cell lines AZ-AHR and AZ-GR allowing assessment of transcriptional activity of AhR and GR receptors, respectively. These cell lines have potential use in pre-clinical research, drug discovery, food safety and environmental applications.

(ii) Transgenic human hepatoma cell lines derived from HepG2 cells, over-expressing transcriptional factors HNF4α and/or PGC-1α. These cell lines have potential use in basic research of hepatocyte-specific cell functions.

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8. CURRICULUM VITAE

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Overview of research internship

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June 2010

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Participation in projects (co-investigator)

FRVŠ 10/2012/G3 – Study of protein expression in models of human cancer cell lines in vitro (2012)

Participation in projects (researcher)

1. P303/12/G163 - Centre of Excellence: Centre of Drug-Dietary Supplements Interactions and Nutrigenetics. Czech Scientific Foundation (2012 – 2018)
2. 13-01809S – Enantiospecific interactions between clinically used drugs and regulatory pathways of human cytochromes P450 (2013-2017)
3. GA303/07/0128 - Study on tissue-specific aspects of transcriptional regulation of selected P-450 enzymes and drug transporters (2007 – 2011)
4. GA304/10/0149 - Generation of hepatocyte-derived cellular models for preclinical testing of drugs (2010 – 2012)
5. OPVK CZ.1.07/2.3.00/20.0062 - Antabuse (disulfiram) as a pilot case of nonprofit drug, (2011 – 2014)
6. OPVK CZ 1.07/2.2.00/07.0354 - Innovation studies of molecular and cellular biology (2009 – 2012)

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1. **Novotna A.**, Doricakova A., Vrzal R., Maurel P., Pavek P., Dvorak Z. (2010) Investigation of Orlistat effects on PXR activation and CYP3A4 expression in primary human hepatocytes and human intestinal LS174T cells. Eur J Pharm Sci 41(2):276-280. [IF_{2011}3,291]

2. Vrzal R., Doricakova A., **Novotna A.**, Bachleda P., Bitman M., Pavek P., Dvorak Z. (2010) Valproic acid augments vitamin D receptor-mediated induction of CYP24 by vitamin D3: a possible cause of valproic acid-induced osteomalacia? Toxicol Lett. 2011 Feb 5;200(3):146-53. [IF_{2011} 3,230]

3. **Novotna A.**, Doricakova A., Vrzal R., Pavek P., Dvorak Z. (2011) Construction and characterization of hepatocyte nuclear factor HNF4α1 over-expressing cell line derived from human hepatoma HepG2 cells. Eur J Pharmacol. 669(1-3):45-50. [IF_{2011} 2,516]

4. **Novotna A.**, Pavek P., Dvorak Z. (2011) Novel stably transfected gene reporter human hepatoma cell line for assessment of aryl hydrocarbon receptor transcriptional activity: construction and characterization. Environ Sci Technol. 2011 Dec 1;45(23):10133-9. [IF_{2011} 5,228]

5. **Novotna A.**, Doricakova A., Pavek P., Dvorak Z. (2012) Construction and characterization of peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α) over-expressing cell line derived from human hepatocyte carcinoma HepG2 cells. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012; 156. [IF_{2011} 0,702]

6. **Novotna A.**, Pavek P., Dvorak Z. Construction and characterization of a reporter gene cell line for assessment of human glucocorticoid receptor activation. Eur J Pharm Sci. 2012, 47 :842-7. [IF_{2011} 3,212]

7. Doricakova A., **Novotna A.**, Vrzal R., Pavek P., Dvorak Z. (2012) The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. Arch. Toxicol. 87:291-301 [IF_{2011} 4.674]

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1. Vrzal R., **Novotna A.**, Doricakova A., Bitmann M., Pavek P., Dvorak Z.: Valproic acid potentiates vitamin D receptor-mediated induction of CYP24 gene – A consequence for drug-induced osteomalacia. *FEBS Journal* **277(Suppl 1)**, p153 (2010. 35th FEBS Congress, Molecules of Life, June 26th – July 1st, 2010, Gothenburg, Sweden.

2. Vrzal R., Dořičáková A., **Novotná A.**, Pávek P., Dvořák Z.: Examination of Orlistat effects on PXR-CYP3A4 signaling in hepatic and intestina cells. 15th Interdisciplinary Toxicological Conference – TOXCON2010, Stará Lesná, Slovensko, 8.9.-11.9.2010. *Interdisciplinary Toxicology* **3(3)**:p. A91-A92, 2010.

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8. Doricakova A., **Novotna A.**, Vrzal R., Pavek P., Dvorak Z.: The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. 19th International Symposium on Microsomes and Drug Oxidations; 12th European Regional ISSX Meeting; Noordwijk aan Zee, The Netherlands; 17-21 June 2012; p221-222.

9. **Novotna A.**, Pavek P., Dvorak Z.: Construction and characterization of a novel reporter gene cell line for assessment of human glucocorticoid receptor activation. 17th Interdisciplinary Toxicological Conference – TOXCON 2012, Stará Lesná, Slovensko, 27.8.-31.8.2012. *Interdisciplinary Toxicology* **5 (Suppl.1)**:p. 59, 2012.

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11. **Novotna A.**, Vrzal R., Pavek P., Dvorak Z.: Transgenic reporter cell lines as the tool for identification of candidate drugs: the case of Ahr and GR. Programme and Abstracts from international konference Chemical Biology: Methods and Progress, Vienna, Austria, 11.-12.2.2013; p.15

APPENDIX I.

Novotna A., Pavek P. and Dvorak Z. Novel stably transfected gene reporter human hepatoma cell line for assessment of aryl hydrocarbon receptor transcriptional activity: construction and characterization. *Environ Sci Technol. 2011*, **45**:10133-9.

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Novel Stably Transfected Gene Reporter Human Hepatoma Cell Line for Assessment of Aryl Hydrocarbon Receptor Transcriptional Activity: Construction and Characterization

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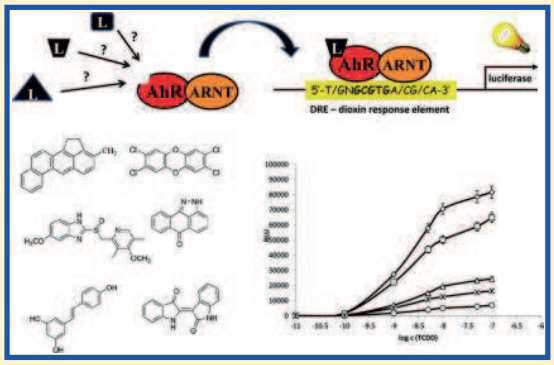
ABSTRACT: We constructed stably transfected gene reporter cell line AZ-AHR, allowing measurement of aryl hydrocarbon receptor (AhR) transcriptional activity. Human hepatoma HepG2 cells were transfected with a construct containing several AhR binding sites upstream of luciferase reporter gene. We prepared 12 clones and we characterized the best five in responsiveness to TCDD. Dose−response analyses were performed for various AhR ligands, including TCDD, 3-methylcholanthrene, indirubin, resveratrol, omeprazole, and SP600125. The EC_{50} values were similar in all tested clones. Induction of luciferase was time-dependent, and treatment for 6 h with 5 nM TCDD was sufficient to evaluate AhR transcriptional activity in 96-well plate format (8−24 fold induction). Response to AhR ligands of cryopreserved cells after thawing was not significantly different from that of fresh cells. Cell line remained fully responsive to AhR ligands over 15 passages and 30 days in culture without significant alterations. Overall, we have developed novel human luciferase reporter cell line AZ-AHR for monitoring AhR transcriptional activity. The sensitivity of the assay allows high throughput format (96-well plate) and evaluation of luciferase activity as soon as after 6 h of incubation, which has potential implication for studies of cytotoxic compounds.



1. INTRODUCTION

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor. It is activated by a variety of exogenous ligands, including synthetic compounds (polyaromatic amines, polyaromatic hydrocarbons, polychlorinated aromatic compounds, dioxins, SP600125 = 1,9-Pyrazoloanthrone) natural compounds (resveratrol, berberine, flavonoids) and drugs (omeprazole, lansoprazole, TSU-16). Endogenous AhR ligands are bilirubin, biliverdin, indirubin, indole, and tryptophan derivatives and arachidonic acid derivatives.[1] AhR regulates genes involved in drug metabolism such as CYP1A1, CYP1A2, CYP1B1, GSTs, UGTs, and NADHP reductase, but also many other genes, for example, AhRR, TGFβ, p27, IL-1β, Jun, and Bax. AhR is pivotal factor in regulation of drug metabolism, but it plays various endogenous functions such as regulating the cell cycle and proliferation, immune response, circadian rhythm and tumor promotion.[2]

In its resting state, AhR is sequestered in the cytosol in multiprotein complex with chaperones. Upon the binding of a lipophilic ligand, AhR translocates to the nucleus, where it forms heterodimer with AhR-nuclear translocator (ARNT). Heterodimer AhR/ARNT binds to promoter sequence called dioxin responsive element (DRE) (consensus sequence 5′-T/GNGCGTGA/CG/CA-3; core sequence 5′-GCGTG-3′) and triggers the expression of target genes.[1]

Given the roles and functions of AhR described above, this is of topical interest to have a potent and reliable tool for identification of AhR ligands and activators, with respect to environmental applications, drug−drug interactions and chemically induced carcinogenesis. Indeed, endogenous tumor-promoting AhR ligand was just identified and published in Nature.[3] Classical approach is the measurement of expression of AhR target genes, for example, CYP1A1 at the level of mRNA, protein and catalytic activity. However, it is rather approach for studying the mechanisms of AhR-CYP1A1 signaling pathway than fast and robust assessment of AhR activation by xenobiotics. A sophisticated technique is gene reporter assay, usually carried out in cancer cell lines transiently transfected with appropriate reporter plasmid harboring AhR response sequences.

Alternatively, cell lines can be stably transfected with reporter plasmid, usually using selection pressure by antibiotics. Construction of stably transfected cell line is time and material consuming process, but experimentation with stably transfected cells has several advantages: (i) There is no need to transfect cells

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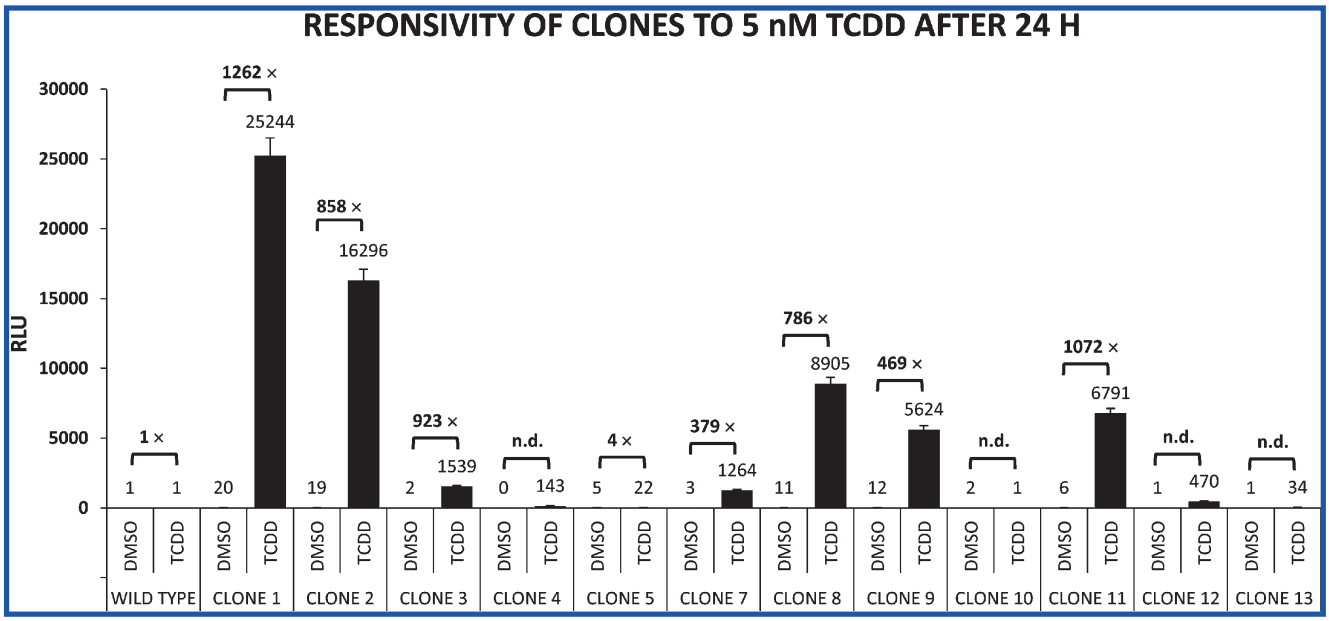

Figure 1. Response of AZ-AHR cells to TCDD. Parent HepG2 cells and clones of AZ-AHR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated with TCDD (5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are mean from triplicate measurements and are expressed as relative luciferase units (RLU). For each clone was calculated fold induction for TCDD over DMSO treated cells, and the value is indicated above the pair DMSO-TCDD in the bar graph. Similar data were obtained from two independent experiments (two passages).

before each experiment, which saves time and materials; (ii) There is no need to normalize data per β-Gal due to the variable transfection efficiency; hence, the data are more homogeneous (iii) There is much higher sensitivity of the assay, because all the cells are transfected; (iv) Taking together points i—iii, stable cell lines allow high through-put measurements in 96-well plate format.

The first attempt to develop the stable reporter system for assessment of AhR transcriptional activity was performed by Postlind et al who transfected reporter plasmid pLUC1A1 containing CYP1A1 promoter to HepG2 cells. The resulting cell line referred as 101 L, was used to establish dose—response relationships for TCDD and polycyclic aromatic hydrocarbons.[4] Cell line 101 L was also used as high-volume screening system for identifying CYP1A inducers, such as flavonoids, resverarol, apigenin, curcumin, kaempferol, quercetin, etc.[5] Soon after 101 L cells, an in vitro bioassay called CALUX (chemical-activated luciferase expression) was invented. A rat hepatoma (H4IIE) cell line was stably transfected with a construct containing DRE sequence and the luciferase reporter gene.[6] The assay was developed for detection of AhR active compounds in sediments and pore water, but it was modified for many other applications.[7,8] Similar high-throughput reporter-gene system based on H4IIE cells stably transformed with luciferase gene under control of CYP1A1 promoter was later developed by others.[9] Stable cell line called DR-EcoScreen was derived from mouse hepatoma cells Hepa1c1c7 transfected with a reporter plasmid containing seven copies of DRE.[10] This line was used for sensitive, rapid and simple identification of AhR agonists among a large number of environmental chemicals.[10] Sato et al. constructed the stable hepatoma reporter cell line to simultaneous assessment of the transcriptional activation of CYP1A1 and CYP1A2 genes. They incorporated dual reporter plasmid containing the 23-kb region between CYP1A1 and CYP1A2 genes in human hepatoma cells HepG2.[11] Very recently, third generation of CALUX cell line derived from mouse hepatoma cells Hepa1c1c7 stably transfected with reporter plasmid containing multiple DRE was described.[12]

In the current paper, we describe construction and characterization of stably transfected human reporter cell line AZ-AHR, responsive to transcriptionally active AhR. Cells were responsive to various AhR ligands, yielding dose- and time- dependent induction of luciferase activity. The assay was very sensitive allowing high throughput format (96-well plate) and evaluation of luciferase activity as soon as after 6 h. AZ-AHR cells remained fully functional over 15 passages and 30 days in culture, and the response of cryopreserved cells after thawing to AhR ligands was not significantly different from that of fresh cells.

2. MATERIALS AND METHODS

2.1. Materials. Lipofectamine 2000 transfection reagent were from Invitrogen (California). 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (Rhode Island). DMSO, 1,9-pyrazoloanthrone (SP600125), resveratrol (RVT), omeprazole (OME), indirubin (IND), 3-methylcholanthrene (3MC) and hygromycin were from Sigma-Aldrich (Prague, Czech Republic). Luciferase lysis buffer was from Promega (Hercules, CA). All other chemicals were of the highest quality commercially available.

2.2. Plasmid. Reporter plasmid pGL-4.27-DRE was constructed as follows: Two copies of F site sequences and one copy of B site and D site sequences of mice *Cyp1a1*gene[13] were synthesized and inserted using *Kpn*I-XhoI enzymes into the multiple cloning region of pGL4.27 [luc2P/minP/Hygro] vector (Cat. Nuber E8451) from Promega (Hercules, CA), upstream of the minimal promoter and *luc2*P reporter gene sequence.

2.3. Human Hepatoma HepG2 Cells. Human caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430)

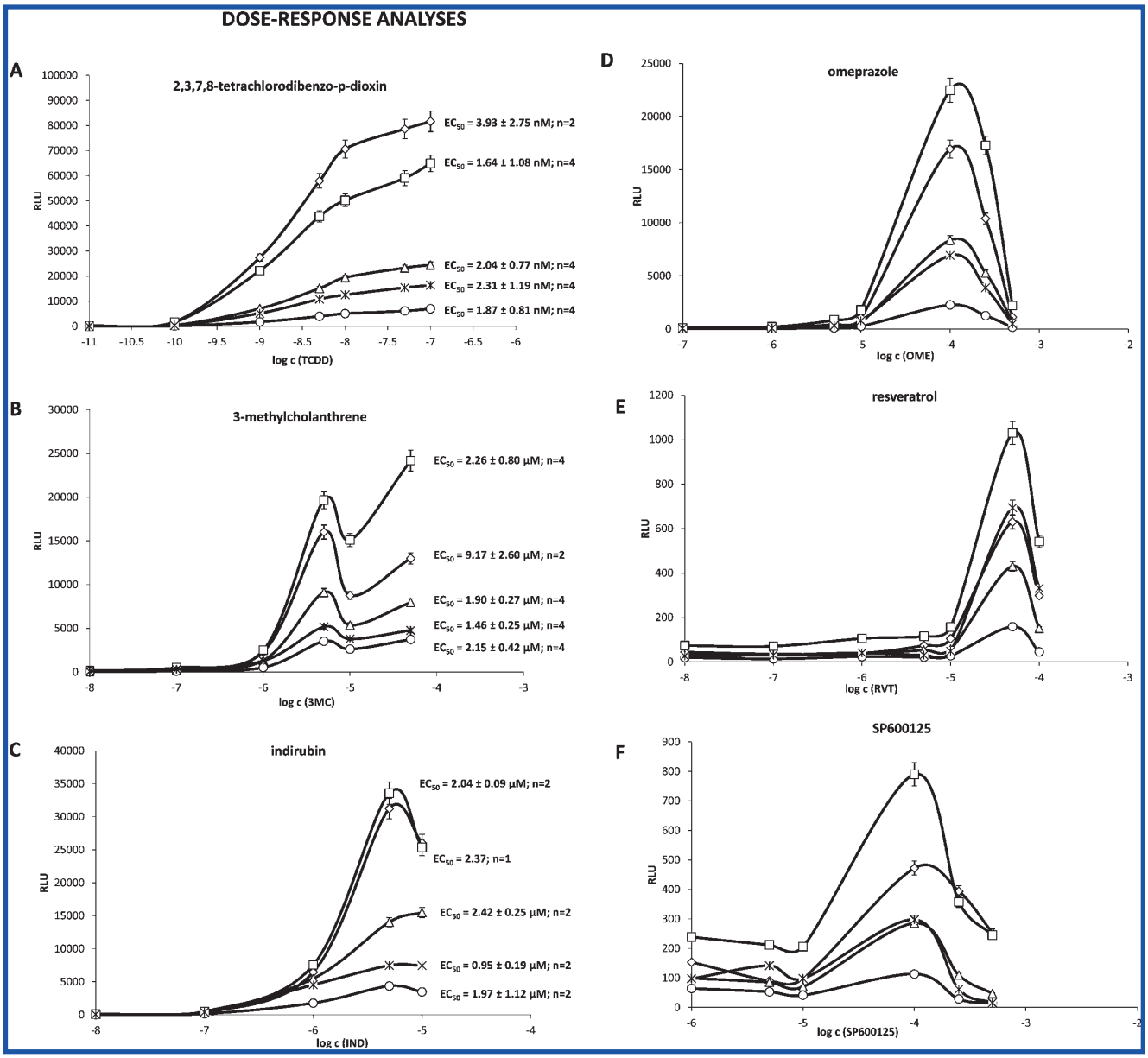

Figure 2. Dose−response analyses of AZ-AHR cells treated with AhR activators. Clones 1, 2, 8, 9, and 11 of AZ-AHR cells were treated for 24 h with: Panel A: TCDD (0.01−100 nM); Panel B: 3MC (0.01−50 μM); Panel C: IND (0.01−10 μM); Panel D: OME (0.1−500 μM); Panel E: RVT (0.01−100 μM); Panel F: SP600125 (1−500 μM). The data are mean from triplicate measurements and are expressed as relative luciferase units (RLU). Number of independent experiments (cell passages) for each clone treated with TCDD, 3MC and IND is indicated in graph legend. EC_{50} values are expressed as mean ± SD. Analyses of SP600125, RVT, and OME were performed in three independent experiments for each clone. Legend to plots: Clone 1 (◊); Clone 2 (□); Clone 3 (Δ); Clone 4 (○); Clone 5 (×).

were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Transfection of HepG2 Cells and Selection Process. HepG2 cells were seeded at the density of 1.3×10^6 cells in 60 mm culture dishes in 5 mL of the DMEM culture medium and transfected with pGL-4.27-DRE (10 μg) reporter plasmid using Lipofectamine 2000 reagent. After 36 h of incubation, the culture medium was replaced by the selection medium supplemented with hygromycin (0.6 mg/mL). The medium was renewed every 3−4 days for the period of 9 weeks, until a polyclonal population was selected. Thereafter, the cells were transferred to 10 cm culture dishes at the density of 500−1000 cells and cultured for additional 4 weeks under hygromycin resistance until small colonies were visible. Twelve colonies were subcloned into a 48-well tissue culture plate to obtain monoclonal populations (termed AZ-AHR cells). Hygromycin resistant clones were analyzed for response to AhR ligands, including TCDD, 3-MC, IND, RVT, OME, and SP600125. The use of GMO at Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (ref 91997/ENV/10).

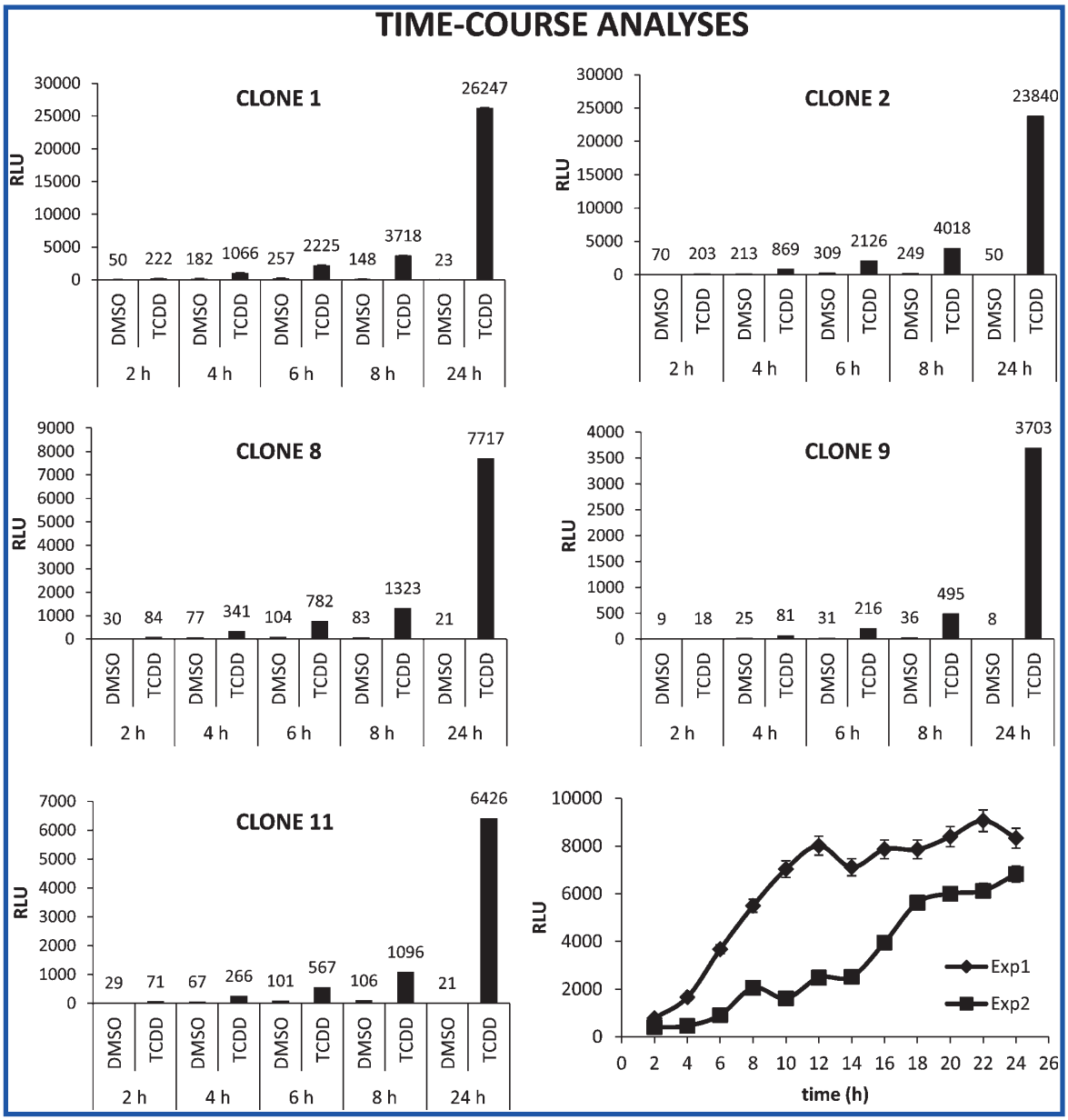

Figure 3. Time-course analyses of AZ-AHR cells treated with TCDD. Bar graphs: Clones 1, 2, 8, 9, and 11 of AZ-AHR cells were treated for 2, 4, 6, 8, and 24 h with 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V). Experiments were performed in three independent passages for each clone. Bottom right plot: Clone 2 was treated for 2 h, 4, 6, 8, 10, 12, 14, 18, 20, 22, and 24 h with TCDD and/or DMSO. Showed are data from two independent experiments. The data are mean from triplicate measurements and are expressed as relative luciferase units.

2.5. Gene Reporter Assay. AZ-AHR cells were seeded on 96-well plates in density 20 000 cells per well. Following 16 h of incubation, cells were treated with tested compounds as described in detail in figure legends. After the treatments, cells were lysed by reporter lysis buffer (Promega) and luciferase activity was measured in 96-well plate format, using Tecan Infinite M2000 and commercial reagents (Promega).

2.6. Cytotoxicity Testing. AZ-AHR cells were treated with the tested compounds for 24 h, using multiwell culture plates of 96 wells. In parallel, the cells were treated with vehicle (DMSO; 0.1%, v/v) and Triton X-100 (1%, v/v) to assess the minimal (i.e., positive control) and maximal (i.e., negative control) cell damage, respectively. The MTT assay was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments LLC). The treatments were performed in quadruplicates and in two independent cell passages.

2.7. Statistical Analyses. Results were expressed as mean ± standard deviation. In all measurements, SD were lower than 5% of the data value, which is indicated in the graphs. Student's pair test was applied. The values of EC_{50} were determined using freeware program BioDataFit 1.02: Data Fit For Biologists (http://www.changbioscience.com/stat/ec50.html)

3. RESULTS

3.1. Response of AZ-AHR Cells to TCDD. Human hepatocellular carcinoma cells HepG2 were transfected with pGL-4.27-DRE luciferase reporter plasmid responsive to transcriptionally

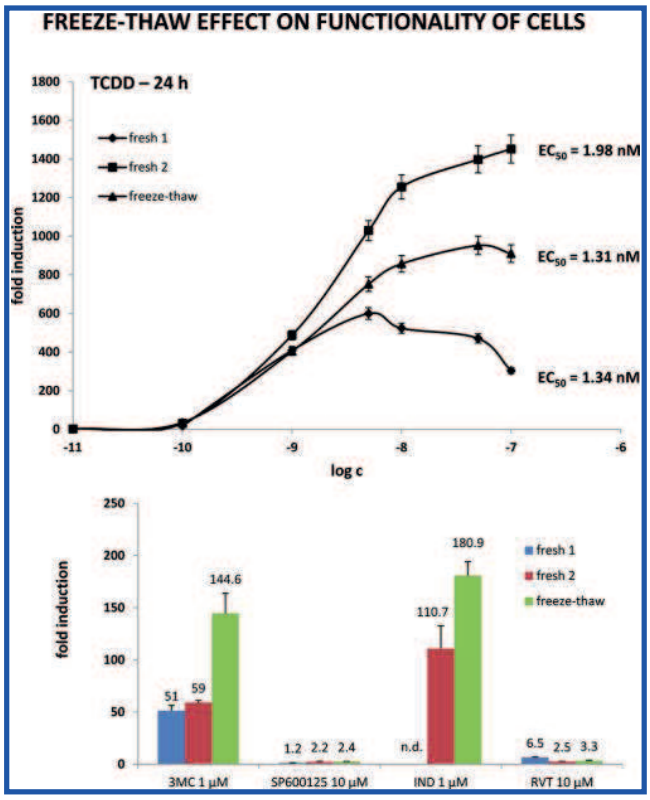

Figure 4. Response of AZ-AHR cells to AhR ligands after freeze−thaw cycle. Experiments were performed in clone 1 of AZ-AHR fresh cells (8th passage = *fresh 1* and 12th passage = *fresh 2*), and in clone 1 of AZ-AHR cells (freeze−thaw) after cryopreservation. Cells were treated with TCDD (0.01−100 nM), 3MC (1 μM), SP600125 (10 μM), RVT (10 μM), IND (1 μM) and DMSO (0.1% V/V) for 24 h. The data are mean from triplicate measurements and are expressed as fold inductions over DMSO-treated cells. Upper panel: dose−response curves for fresh and cryopreserved AZ-AHR cells treated with TCDD; Lower panel: Effects of 3MC, SP600125, RVT, IND on luciferase activity in fresh and cryopreserved AZ-AHR cells.

active aryl hydrocarbon receptor AhR (for details see Sections 2.2. and 2.4.). We isolated 12 clones of AZ-AHR hygromycin-resistant cells. We tested response of AZ-AHR clones to model AhR ligand TCDD. For this purpose, parent HepG2 cells and AZ-AHR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated with 5 nM TCDD and vehicle (DMSO; 0.1% V/V) for 24 h, and luciferase activity was measured in 96-well format. There was no induction of luciferase activity in parent HepG2 cells. Out of 12 clones examined, significant induction of luciferase activity was reached in eight clones (1, 2, 3, 5, 7, 8, 9, 11), with fold induction ranging from 4× to 1262× . For further analyses, the most responsive clones (1, 2, 8, 9, 11), yielding both high relative luciferase units (RLU) and high fold induction over the vehicle-treated cells, were used (Figure 1).

3.2. Dose−response Analyses of AZ-AHR Cells Treated with AhR Activators. In the first series of functional tests, we performed dose−response experiments in five clones (1, 2, 8, 9, 11) of AZ-AHR cell line. Cells were treated in 96-well plates with increasing concentrations of TCDD, RVT, 3MC, SP600125, OME, IND, and vehicle (DMSO; 0.1% V/V) for 24 h, and luciferase activity was measured in cell lyzates. In parallel, we have tested cytotoxicity of AhR activators using standard MTT assay after 24 h of treatment. The values of IC_{50} were 10.9 μM and 398 μM for IND and OME, respectively, whereas IC_{50} were not reached within the tested concentration range for TCDD, RVT, 3MC, and SP600125.

Treatment of AZ-AHR cells with TCDD (10 pM to 100 nM), the most potent AhR ligand, yielded typical sigmoid logarithmic dose−response curves for each tested clone. With exception of clone 1 (EC_{50} = 3.93 nM), the values of EC_{50} were homogeneous between the clones, ranging from 1.64 to 2.31 nM (Figure 2A). Sigmoid-shaped curves were also obtained in all clones of AZ-AHR cells treated with 3MC (10 nM to 50 μM), with EC_{50} values ranging from 1.46 to 2.26 μM. Similarly as in case of TCDD, clone 1 yielded higher EC_{50} (9.17 μM) as compared to other clones. Interestingly, we have systematically observed local minimum in 10 μM concentration of 3MC (18 independent dose−response experiments), indicating more than one mechanism of 3MC action involved in signal transduction in high concentrations of 3MC, such as activation of Nrf2-ARE pathway[14] or AhR-independent induction of p21[15] (Figure 2B).

Indirubin, an endogenous ligand of AhR, induced luciferase activity in dose dependent manner in all tested clones with EC_{50} ranging from 0.95 to 2.42 μM. In concentrations of IND 10 μM and higher, the activity of luciferase descended, suggesting inhibition of luciferase catalytic activity, cytotoxicity (IC_{50} 10.9 μM) or feed-back mechanisms in supra-physiological concentrations (Figure 2C). The efficacy of TCDD, 3MC and IND were of the same order of magnitude (Figure 2A−C).

Omeprazole, a drug inhibiting proton pump, is an activator of AhR and inducer of CYP1A genes, but it does not bind to AhR in vitro and the mechanism of AhR activation by OME is unknown.[16] In gene reporter assays with 5 clones of AZ-AHR cells, OME displayed atypical dose−response profile of U-shaped curve, with maximum induction of luciferase activity (efficacy comparable to 3MC) at 100 μM concentration of OME (Figure 2D). Further decrease in luciferase activity could be due to the OME cytotoxicity (IC_{50} = 398 μM). We have also tested the effects of SP600125 (specific inhibitor of JNK) and RVT (natural antioxidant) on luciferase expression in AZ-AHR cells, since both compounds were described as partial agonists AhR.[17] Both RVT (Figure 2E) and SP600125 (Figure 2F) caused dose-dependent induction of luciferase activity in all tested clones, with maximal luciferase activity at 50 μM and 100 μM, respectively. Further increase in SP600125 and RVT concentrations caused decrease of luciferase activity, probably due to the interactions with other cell targets and inhibition of luciferase activity.

Collectively, we observed dose-dependent induction of luciferase activity in five clones of AZ-AHR cells by six structurally different AhR activators.

3.3. Time-Course Analyses of AZ-AHR Cells Treated with TCDD. In the next series of functional tests, we examined time effects of TCDD on luciferase expression in AZ-AHR cells. Clones 1, 2, 8, 9, and 11 were seeded in 96-well plates and following 16 h of stabilization, cells were treated for 2 , 4, 6, 8, and 24 h with TCDD (5 nM) and DMSO (0.1% V/V). In all clones tested, TCDD caused time-dependent increase of luciferase activity. The assay was very sensitive and significant induction of luciferase activity was attained after 6 h of treatment with TCDD, with fold induction ranging from 8 to 24 fold. This implies potential application of AZ-AHR cell line in testing cytotoxic compound which may kill cells in longer time periods. There were not substantial differences between individual clones,

Table 1. Maintenance of Luciferase Inducibility by 5 nM TCDD

passage number	days in culture	clone 1		clone 2		clone 8		clone 9		clone 11	
		RLU	fold	RLU	fold	RLU	fold	RLU	fold	RLU	fold
3	1	16565	1273	16088	567	11570	788	9578	522	6307	787
5	7	25244	1262	16296	858	8905	786	5624	469	6791	1072
7	12	40016	599	5451	149	3608	1503	11750	746	9065	339
8	14	19815	417	21605	483	12709	643	4693	335	13803	746
9	16	nd	nd	36338	689	18931	653	5567	445	7629	254
12	22	57912	1059	43785	431	15131	496	3975	370	10827	274
13	26	27544	599	16428	345	7423	244	2817	352	8359	237
14	28	28698	2870	17583	440	6920	706	2368	592	9084	966
15	30	26247	944	23840	475	7717	371	3703	411	6426	300

with exception of clone 1 that displayed much higher fold induction of luciferase after 24 h as compared to other clones (Figure 3, bar graphs). In clone 2, we performed more detailed analysis, where the induction of luciferase was measured every 2 h in the period of 24 h. The maximal luciferase activity by TCDD was attained between 12 and 18 h of the treatment (Figure 3, bottom right plot).

3.4. Response of AZ-AHR Cells to AhR Ligands after Freeze−thaw Cycle. We tested functionality of AZ-AHR cells (clone 1) after cryopreservation. We treated fresh cells (8th passage = *fresh 1* and 12th passage = *fresh 2*) with TCDD (0.01−100 nM), 3MC (1 μM), SP600125 (10 μM), RVT (10 μM), IND (1 μM), and DMSO for 24 h. Thereafter, cells were frozen according to a general procedure, using fetal calf serum and DMSO as cryoprotectant. Cells were stored in liquid nitrogen for three weeks. After the thawing, the treatments (vide supra) were repeated in thawed cells.

Dose−response curves and EC_{50} values for TCDD were similar for fresh cells and cells after freeze−thaw cycle (Figure 4, top). There were not substantial differences between fold induction of luciferase activity by AhR ligands (3MC, IND, RVT, SP600125) in fresh and cryopreserved cells (Figure 4, bottom). Collectively, novel reporter AZ-AHR cell line is fully functional and responsive to AhR ligands after the cryopreservation process.

3.5. Maintenance of Luciferase Inducibility in AZ-AHR cells by TCDD. Finally, we tested the ability of five clones (1, 2, 8, 9, 11) of AZ-AHR cell line to respond to TCDD in long-term period. We checked response of cells to TCDD (5 nM; 24 h) after each passage of the cells. The induction of luciferase activity by TCDD was stable during 30 days of AZ-AHR cells in culture, which corresponds to 15 passages. Even though there was a variability between passages, there was no systematic decline or decrease in luciferase induction in both absolute RLU values and fold induction magnitude (Table 1).

DISCUSSION

Stably transfected reporter cell lines are mainly used for large scale detection of various activators, based on the plasmid transfected. The construction of stable cell line is time and material consuming process, but in long-term perspective, this experimental approach saves time and expenses, because there is no further need of transfection procedure before experimentation. In the population of cells selected on antibiotic, each cell (100%) is transfected with reporter plasmid. Therefore, this is not necessary to deal with transfection efficiency, and the assays are highly sensitive.

Several stably transfected gene reporter cells lines were developed for an assessment of transcriptional activity of AhR. The majority of these lines were derived from rodent cancer cells. Probably the most known system is an in vitro bioassay CALUX (chemical-activated luciferase expression), which is rat hepatoma H4IIE cell line stably transfected with a construct containing DRE sequence and the luciferase reporter gene.[6] The assay was used for environmental applications, but it was modified for many other purposes.[7,8] Rat cells H4IIE were also used by others.[9] The examples of stable cell lines derived from mouse hepatoma cells Hepa1c1c7 are DR-EcoScreen cells[10] and third generation of CALUX.[12] Since there are substantial differences between AhR signaling in rodent and human cells, for example the different effects of glucocorticoids on AhR signaling,[18] this is of great interest to establish cell line based on human cells, responding to AhR activation. The examples of reporter cell lines derived from human hepatoma HepG2 cells are 101 L cells[4] and cells allowing simultaneous assessment of the transcriptional activation of CYP1A1 and CYP1A2 genes.[11]

In the current paper, we describe construction and characterization of stably transfected human gene reporter cell line allowing assessment of AhR activators. This AZ-AHR cell line was prepared from HepG2 transfected with reporter plasmid pGL-4.27-DRE, containing two copies of F site sequences, one copy of B site and one copy of D site sequences.[13] Resulting cells yielded dose- and time- dependent induction of luciferase activity in response to treatment with various AhR ligands, including TCDD, 3MC, IND, RVT, OME and SP600125. The assay was very sensitive allowing high throughput format (96-well plate) and evaluation of luciferase activity as soon as after 6 h, which allows to test highly toxic compounds. AZ-AHR cells remained fully functional over 15 passages and 30 days in culture, and the response of cryopreserved cells after thawing to AhR ligands did not decline. This line is suitable for various environmental applications and screenings and in drug discovery.

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NOMENCLATURE

AhR	aryl hydrocarbon receptor
AhRR	AhR repressor
ARNT	AhR-nuclear translocator
CYP	Cytochrome P450
DRE	dioxin responsive element
GST	glutathione-S-transferase
HepG2	human hepatocellular carcinoma cells
IL-1β	interleukin 1 beta
IND	indirubin
3MC	3-methylcholanthrene
n	number of repeat experiments
OME	omeprazole
RVT	resveratrol
SP600125	
1,9-pyrazoloanthrone	
TCDD	2,3,7,8- tetrachlorodibenzo-*p*-dioxin
TGFβ	tumor growth factor beta
UGT	UDP-glucuronyl transferase

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APPENDIX II.

Novotna A., Pavek P. and Dvorak Z. Construction and characterization of a reporter gene cell line for assessment of human glucocorticoid receptor activation. *Eur J Pharm Sci. 2012*, **47** :842-7.

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Construction and characterization of a reporter gene cell line for assessment of human glucocorticoid receptor activation

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ABSTRACT

Glucocorticoids are widely used drugs in human pharmacotherapy. There is an increasing demand for tools allowing detection of the ligands for glucocorticoid receptor (GR), with regard to pre-clinical drug testing and environmental applications. We constructed human luciferase reporter gene cell line AZ-GR derived from HeLa human cervix carcinoma cells, which were stably transfected with reporter plasmid containing three copies of glucorticoid response element (GRE) upstream of luciferase reporter gene. We isolated five dexamethasone-responsive clones, and we further characterized two most responsive ones (AZ-GR). Dose–response analyses were performed with 22 different natural and synthetic steroids and the values of EC_{50} were calculated. AZ-GR cells displayed high specificity and sensitivity to glucocorticoids, very low responsiveness to mineralocorticoids, but no responsiveness to estrogens, gestagens or androgens. Time-course analyses revealed that AZ-GR cells allow detection of GR activators soon after 14 h of the treatment (6–10-fold induction by 100 nM dexamethasone). Functionality of AZ-GR cells was not affected with cryopreservation. Generated reporter gene cell lines fully maintained responsiveness to glucocorticoids for 32 days in the culture and over 16 passages without significant alterations. The sensitivity of the assay allows high throughput format using 96-well plates.

Collectively, we present here glucocorticoid-responsive stable reporter gene cell line that allows high throughput, rapid, sensitive and selective detection of GR activators, with possible use in pre-clinical research and environmental applications.

1. Introduction

Glucocorticoid receptor (GR) is a ligand-inducible transcriptional factor that belongs to the subfamily of nuclear hormone receptors. It exists in two isoforms, GRα and GRβ that are generated by different splicing of exon 9 (Hollenberg et al., 1985). Transcriptionally active is isoform GRα that binds glucocorticoids, whereas GRβ does not bind the ligands and inhibits GRα-mediated gene transcription (Kino et al., 2009). In the resting state, GRα is localized in the cytosol in the complex with chaperone proteins. Upon the ligand binding, it translocates to the nucleus where it binds to glucocorticoid response elements in DNA as a homodimer GRα/GRα (Dvorak and Pavek, 2010). Glucocorticoids are massively used drugs for their anti-inflammatory and immunosuppressive effects. Synthetic (exogenous) glucocorticoids include dexamethasone, beclomethasone, betamethasone, triamcinolone etc. The most important physiological (endogenous) ligand of human GR is cortisol (hydrocortisone).

There is a need for the reliable, selective, sensitive and high throughput tools for monitoring glucocorticoid activity, at least for two purposes: (i) Pre-clinical applications, i.e. in search of new glucocorticoids. (ii) Environmental applications, i.e. monitoring of steroid hormones in soils and waters. Gene reporter assays are routinely used tools for assessment of activation of transcription factors or receptors. In the current paper, we used HeLa cells that have fully functional endogenous GR, and we generated AZ-GR cells stably transfected with the plasmid containing multiple GREs fused to luciferase reporter gene.

Several luciferase reporter gene assays for assessment of steroid receptor transcriptional activity have been developed. Israel and Kaufman transfected reporter gene plasmid containing multiple GREs to Chinese hamster ovarian cells. The resulting cell line showed high induction by dexamethasone (Israel and Kaufman 1989). Stably transfected cell line called MDA-kb2 was derived from MDA-MD-453 cells that were transfected with MMTV.luciferase.neo reporter gene construct. The MDA-kb2 cell line was used to screen chemicals for androgen receptor and GR-mediated activity (Wilson et al., 2002). Mori et al. constructed reporter cell line

Abbreviations: DEX, dexamethasone; DMSO, dimethylsulfoxide; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HeLa, human negroid cervix epitheloid carcinoma cells.

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hGR-Luc for the purpose of lipophilic chemical screening. This cell line was constructed by genomic recombination in stable genetic modified HeLa55 cells using a uniquely designed donor vector harboring an exchange cassette comprised of the human GR gene, its response element, and a luciferase reporter gene (Mori et al., 2008).

In the current paper, we have developed stably transfected human luciferase reporter gene cell lines AZ-GR allowing measurement of GR transcriptional activity. The cell lines are selective for glucocorticoids and the luciferase activity is possible to measure after 14 h of incubation. The assay is very sensitive and allows high throughput format (96-well plates). AZ-GR cell line remained fully functional over 16 passages and 32 days in culture. The responsiveness to glucocorticoids was retained in cryopreserved cells after thawing as compared to fresh cells.

2. Materials and methods

2.1. Compounds and reagents

Fugene HD transfection reagent was from Roche (Basel, Switzerland). DMSO, hygromycin B, dexamethasone, beclometasone, betamethasone, cortisol, corticosterone, prednisolone, methylprednisolone, testosterone, estradiol, diethylstilbestrol, 4-hydroxyta- moxifen, spironolactone, aldosterone, 17α-hydroxyprogesterone and progesterone were purchased from Sigma–Aldrich (Prague, Czech Republic). Raloxifene hydrochloride, tamoxifen citrate salt, genistein, cyproterone acetate, danazol, triamcinolone and mifepristone were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Luciferase lysis buffer was from Promega (Hercules, CA). All other chemicals were of the highest quality commercially available.

2.2. Plasmid

Reporter plasmid pGL-4.27-GRE was constructed as follows: Three copies of tandem GREs (**GGTACA**TTT**TGTTCT GGTACA** GTA **CGTCCT** GTTCT **GGTACA**AAC**TGTTCT**) were synthesized and inserted using KpnI-XhoI enzymes into pGL4.27 [luc2P/minP/Hygro] vector (Cat. Nuber E8451) from Promega (Hercules, CA).

2.3. HeLa Cells

Human Negroid cervix epitheloid carcinoma cells HeLa (ECACC No. 93021013) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 μg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Transfection of HeLa cells and selection process

HeLa cells were transfected with pGL-4.27-GRE (2 μg) reporter plasmid using Fugene HD reagent and seeded at the density of 8×10^5 cells in 60 mm culture dishes in 5 ml of the DMEM culture medium. Following 36 h of incubation, the culture medium was replaced by the selection medium supplemented with hygromycin B (0.2 mg/mL). The medium was changed every 3–4 days for the period of 3 weeks, until a polyclonal population was selected. Thereafter, the cells were transferred to 10 cm culture dishes at the density of 500–1000 cells per dish and cultured for additional 2 weeks in the presence of hygromycin B until small colonies were visible. 17 colonies were subcloned into a 48-well tissue culture plate to obtain monoclonal populations (two best clones 2 and 15 are termed AZ-GR cells). Hygromycin B resistant clones were treated with glucocorticoids, mineralocorticoids, estrogenes, androgens and gestagens. The use of GMO at Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (Ref. 91997/ENV/10).

2.5. Gene reporter assay

AZ-GR cells were seeded on 96-well plates in density 20.000 cells per well. Following 16 h of incubation, cells were treated with tested compounds as described in detail in figure legends. After the treatments, cells were lysed and luciferase activity was measured in 96-well plate format, using Tecan Infinite M2000 plate luminometer.

2.6. Statistical Analyses

Student's pair *t*-test was applied. The values of EC_{50} were determined using free software ED50plus v1.0 (http://www.free-downloads-center.com/download/ed50plus-v1-0-2434.html). Fitting of dose–response curves was performed by GraphPad software.

3. Results

3.1. Selection process and generation of AZ-GR clones

HeLa cells were transfected with pGL-4.27-GRE reporter plasmid and stably transfected clones were selected under the pressure of hygromycin B, as described in Methods section. We found slight morphological differences between parent HeLa cells and stably transfected cells. HeLa cells have an epithelial like morphology and elongated shape, while transfected cells were rather oval (Fig. 1).

We isolated seventeen hygromycin B - resistant clones and we tested the responsiveness of the clones to synthetic glucocorticoid dexamethasone. For this purpose, stably transfected clones cells

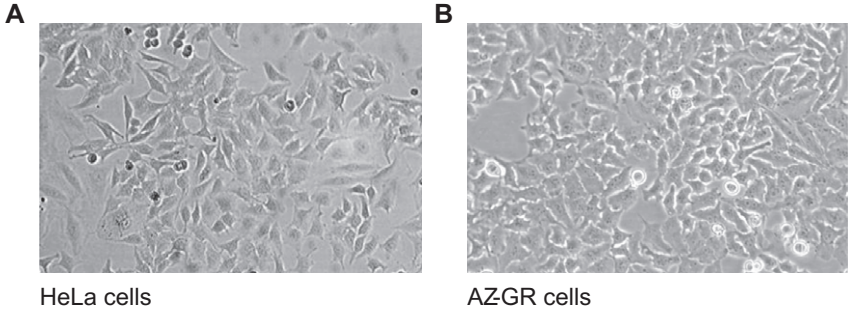

Fig. 1. Morphology of AZ-GR cells and HeLa cells Phase contrast micrographs of parent HeLa cells (at 7th passage) and AZ-GR cells (5th passage, clone 2).

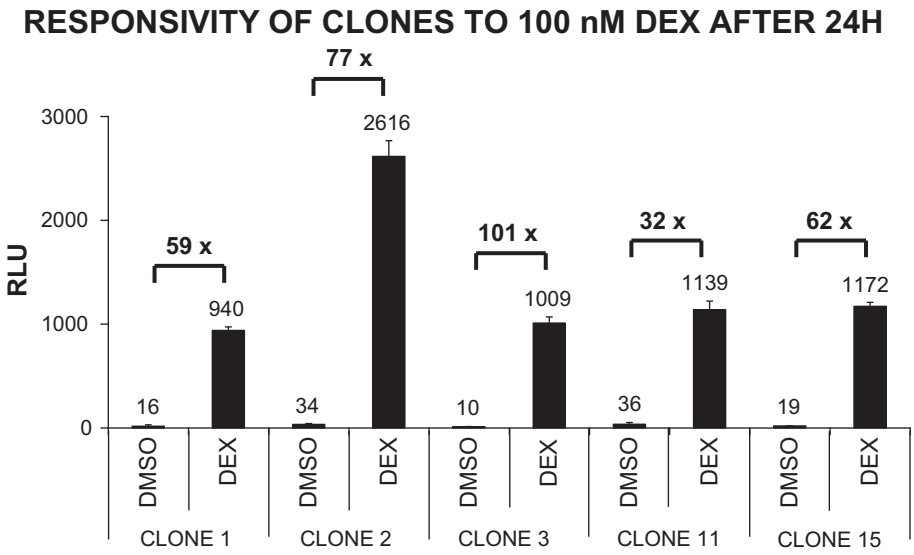

Fig. 2. Response of stably transfected GR-responsive clones to dexamethasone. Clones were seeded in 96-well plates and following 16 h of stabilization, cells were treated with dexamethasone (DEX; 100 nM) and vehicle (DMSO; 0.1% V/V) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are mean from triplicate measurements and are expressed as relative luciferase units (RLU). Differences in RLU units within the triplicates were lower than 5%. Fold induction by dexamethasone over DMSO-treated cells was calculated and the value is placed above the pair DMSO-DEX in the bar graph. Similar data were obtained from three independent experiments (three passages).

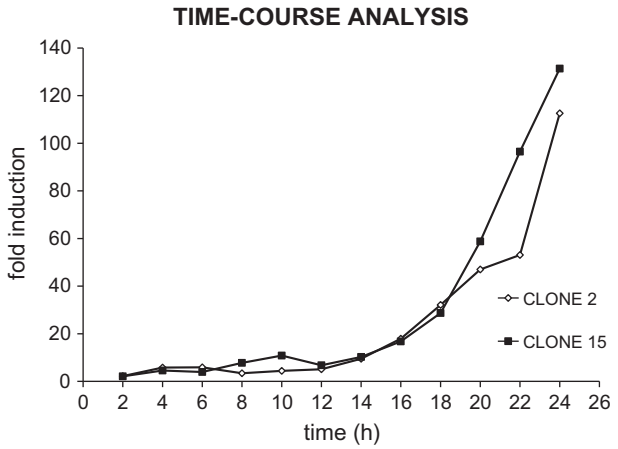

Fig. 3. Time-course analyses of AZ-GR cells treated with dexamethasone. Clones #2 and #15 of AZ-GR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h with dexamethasone (DEX; 100 nM) and vehicle (DMSO; 0.1% V/V). After the treatments, cells were lysed and luciferase activity was measured. The data are mean from triplicate measurements and time-course curves are expressed as fold induction over DMSO-treated cells. Differences in RLU units within the triplicates were lower than 5%. Experiments were performed in three independent passages for each clone.

were seeded in 96-well plates and following 16 h of stabilization, cells were treated with 100 nM DEX and vehicle (DMSO; 0.1% V/V) for 24 h. The fold induction of luciferase activity by dexamethasone in five strongly responsive clones (clones #1, #2, #3, #11, #15) ranged from 32 × to 101 × (Fig. 2). The most responsive clones #2 and #15 (AZ-GR cells), yielding both high relative luciferase units (RLU) and high fold induction over the vehicle-treated cells, were used for further detailed analyses.

3.2. Time-course analyses of AZ-GR cells treated with dexamethasone

In the next series of experiments, we examined the time course of luciferase induction by dexamethasone in AZ-GR cells. The cells were treated with 100 nM dexamethasone, and luciferase activity was measured after 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h of incubation. In both clones, dexamethasone caused time-dependent induction of luciferase activity. The significant induction was attained after 14 h of the treatment with dexamethasone, with fold induction ranging from 7-fold to 10-fold (Fig. 3).

3.3. Dose–response analyses of AZ-GR cells treated with steroids

We performed dose–response analyses with 22 well-established natural and synthetic ligands for steroid receptors, including glucocorticoids, mineralocorticoids, estrogens, androgens and gestagens. AZ-GR cells were treated in 96-well plates with increasing concentrations of tested compounds and vehicle (DMSO; 0.1% V/V) for 24 h, and luciferase activity was measured in cell lyzates. The values of E_{MAX}, $LogEC_{50}$ and efficacies were calculated from dose–response curves (Fig. 4; Table 1).

Treatment of AZ-GR cells with glucocorticoids, including dexamethasone, beclometasone, betamethasone, cortisol, prednisolone, methylprednisolone and triamcinolone (0.01 nM–10 μM) yielded typical sigmoid logarithmic dose response curves for both clones (Fig. 4). The sigmoid-shaped curves were also obtained for mineralocorticoids corticosterone (0.01 nM–10 μM) and aldosterone (1 nM–100 μM). However, the potency (higher EC_{50} values) and efficacy (lower RLU and fold induction) of mineralocorticoids were substantially lower than those for glucocorticoids (Fig. 4; Table 1). Aldosterone binds to GR (Fuller et al., 2000), which is probably reason for slightly increased luciferase activity in AZ-GR cells after treatment with mineralocorticoids.

Mifepristone, the synthetic antagonist (partial agonist) of glucocorticoid receptor and progesterone receptor, induced luciferase activity in dose dependent manner. The potency of mifepristone was much higher ($logEC_{50}$-10.65) as compared to potent glucocorticoid agonist dexamethasone ($logEC_{50}$-8.02). On the other hand, the efficacy of mifepristone was negligible (Fig. 4; Table 1).

Estrogens, androgens and gestagens did not induce luciferase activity in AZ-GR cells. Collectively, these results reveal the selectivity of novel cell line AZ-GR for glucocorticoids.

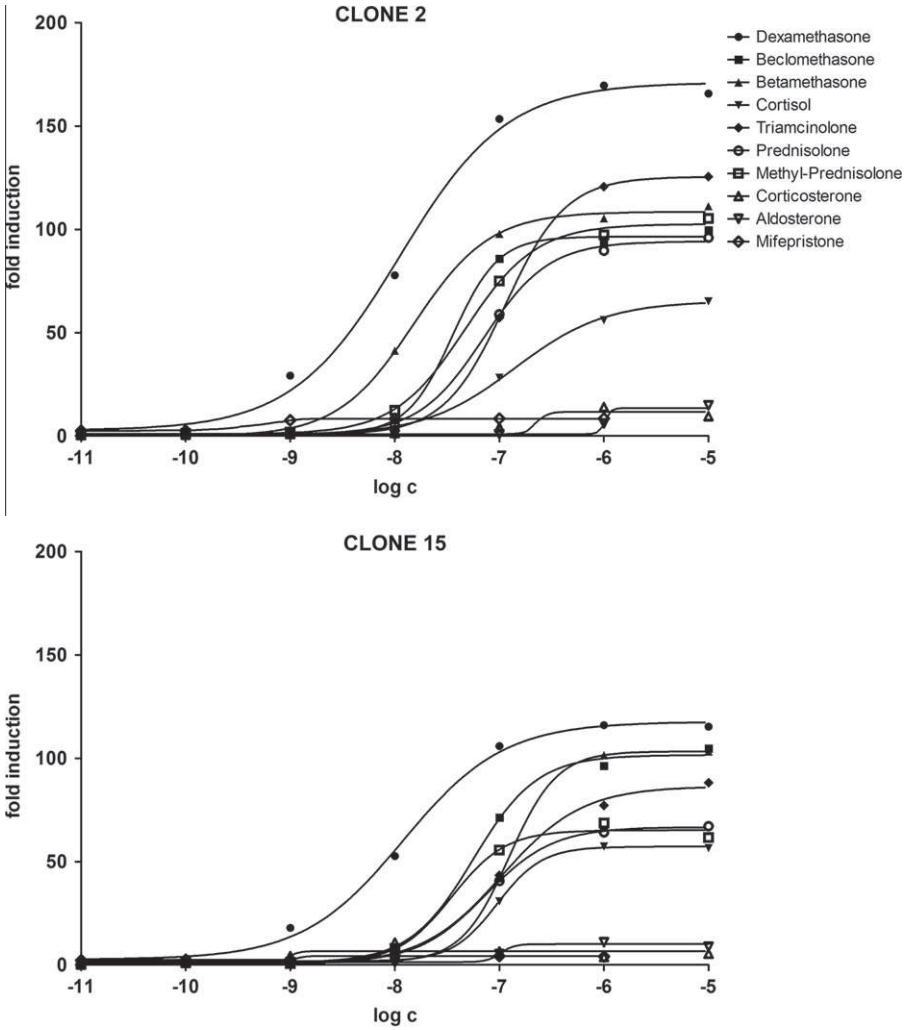

Fig. 4. Dose–response analyses of AZ-GR cells treated with ligands for steroid receptors. AZ-GR cells were seeded in 96-well plates and following 16 h of stabilization, cells were treated for 24 h with: dexamethasone, beclometasone, betamethasone, cortisol, prednisolone, methylprednisolone, triamcinolone, mifepristone, corticosterone (0,01 nM–10 μM) and aldosterone (1 nM–100 μM). **Panel A:** Clone #2; **Panel B:** Clone #15. After the treatments, cells were lysed and luciferase activity was measured. The data are mean from triplicate measurements from three independent experiments (cell passages) for each clone and are expressed as fold induction over DMSO-treated cells. Differences in RLU units within the triplicates were lower than 5%.

3.4. Response of AZ-GR cells to steroid compounds after cryopreservation

AZ-GR cells were frozen according to a general procedure, using fetal calf serum and DMSO as cryoprotectant. Cells were stored in liquid nitrogen for two weeks. After the thawing, we tested functionality of AZ-GR cell lines. The cells were seeded in 96-well plates and following 16 h of stabilization, treated with increasing concentrations of steroids and vehicle (DMSO; 0.1% V/V) for 24 h, and luciferase activity was measured in cell lyzates. From dose response curves, we calculated values of log EC_{50} for individual compounds. The fold inductions and the values of log EC_{50} obtained in cryopreserved cells were not significantly different from those obtained in fresh cells (Fig. 5; Table 1). Taking together, novel reporter AZ-GR cell lines are fully functional and responsive to glucocorticoids after the cryopreservation process.

3.5. Maintenance of luciferase inducibility in AZ-GR cells by dexamethasone

Finally, we tested the ability of AZ-GR cell lines to respond to dexamethasone in long term period. We checked response of cells to dexamethasone (100 nM; 24 h) after each passage of the cells. The induction of luciferase activity by dexamethasone was stable over 32 days of AZ-GR cells in culture, which corresponds to 16 passages (Table 2).

4. Discussion

Stably transfected gene reporter cell lines are used for profiling of a wide range of compounds affecting transcriptional factors and nuclear receptors, including steroid hormone receptors. While generation of a stable cell line is a time consuming process (weeks to months), they have several advantages, such as good reproducibility of results due to the homogenous transfection, high sensitivity because 100% of cells are transfected etc. Basically, two approaches are used for generation of stable reporter gene cell lines:

(i) Cell line is derived from cells that do not contain the receptor of interest (glucocorticoid receptor) and they are usually rapidly growing, low-differentiated cell lines such as CHO and CV-1. The cell lines are then constructed in two steps; the first step is transfection with reporter vector (GRE-luc) and in the second step the transfection with expression plas-

Table 1
Characteristics of AZ-GR cells in comparison with published data. Cryo = cells after freeze–thaw cycle; EC50 – half maximal effective concentration; EMAX – maximal effective concentration – expressed as maximal fold activation; Efficacy – percentage of E_{MAX} attained by cortisol.

Clone 2					Literature Log EC50	
Compound	Log EC50	E_{MAX} -fold	Efficacy -%	Log EC50-cryo	Sedlak et al.	Wilkinson et al.
Dexamethasone	−8.02 ± 0.01	170	262	−8.16	−8.78 ± 0.28	−9.81
Beclomethasone	−7.52 ± 0.06	99	152	−7.42	−8.98 ± 0.14	nd
Betamethasone	−7.81 ± 0.00	111	171	−7.85	−8.95 ± 0.15	nd
Cortisol	−7.18 ± 0.07	65	100	−7.17	−7.34 ± 0.35	−8.01
Triamcinolone	−7.27 ± 0.04	125	192	−7.25	−8.33 ± 0.12	nd
Prednisolon	−7.35 ± 0.01	96	148	−7.39	nd	nd
Methyl-prednisolon	−7.52 ± 0.04	105	162	−7.59	nd	−9.37
Mifepristone	−10.65 ± 0.16	8	12	−9.85	−8.23 ± 0.34	−8.16
Corticosterone	−6.62 ± 0.13	14	21	−7.21	−7.52 ± 0.15	−6.36
Aldosterone	−5.95 ± 0.11	13	20	−6.42	−8.61 ± 0.72	>−2.00
17-α-OH-progesterone	>−5.00	1.0	1.5	>−5.00	>−5.00	nd
Progesterone	>−5.00	1.0	1.5	>−5.00	−6.87 ± 0.18	>−2.00
Testosterone	>−5.00	1.3	2.0	>−5.00	−6.89 ± 0.10	>−2.00
Danazol	>−5.00	1.1	1.7	>−5.00	>−5.00	>−2.00
Cyproterone acetáte	>−5.00	1.6	2.4	>−5.00	>−5.00	>−2.00
Spironolactone	>−5.00	0.7	1.1	>−5.00	>−5.30	>−2.00
17-β-estradiol	>−5.00	0.2	0.3	>−5.00	>−5.00	>−2.00
Genistein	>−5.00	1.8	2.8	>−5.00	>−5.00	nd
Diethylstilbetrol	>−5.00	1.3	2.0	>−5.00	>−5.00	nd
4-OH-tamoxifen	>−5.00	0.9	1.4	>−5.00	>−5.00	>−2.00
Raloxifene hydrochloride	>−5.00	1.1	1.7	>−5.00	nd	>−2.00
Tamoxifen citrate	>−5.00	2.2	3.4	>−5.00	>−5.00	>−2.00

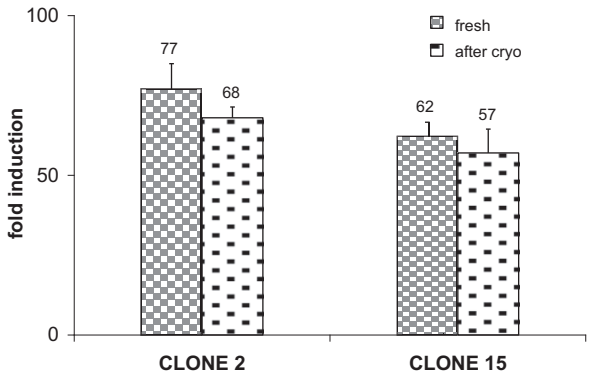

Fig. 5. Response of AZ-GR cells to dexamethasone after cryopreservation. Experiments were performed in clones #2 and #15 of AZ-GR fresh cells (9th passage) and in clones #2 and #15 of AZ-GR cells after cryopreservation (*freeze–thaw cycle*). Cells were treated with DEX (100 nM) and DMSO (0.1% V/V) for 24 h. After the treatments, cells were lysed and luciferase activity was measured. The data are mean from triplicate measurements and are expressed as fold inductions over DMSO-treated cells. Analyses were performed in three independent experiments for each clone.

Table 2
Maintenance of responsiveness of AZ-GR cells to dexamethasone.

Passage number	Days in culture	Clone 2		Clone 15	
		RLU	Fold	RLU	Fold
3	6	2612	72	2896	80
5	10	1371	67	1399	78
6	12	2202	110	2576	161
10	19	1139	68	961	57
11	21	637	31	685	55
13	26	736	35	647	51
14	28	1247	68	1291	88
16	32	971	47	890	68

mid encoding corresponding steroid receptor (GR) follows. Disadvantage of this approach is the length of this process, which is twice longer than for single transfection process and requires selection with two different antibiotics (Sedlak et al., 2011). In addition, resulting cell line may be far away from physiological situation, in term of expression of transcriptional co-activators etc. Advantage of this approach is usually very high selectivity of the cell line for desired group of compounds (glucocorticoids).

(ii) Starting parent cell line expresses fully functional endogenous receptor, which is the case of HeLa cells expressing functional GR. The cells are transfected only with reporter plasmid and only one selection agent is necessary (Mori et al., 2008).

In the current paper, we describe stably transfected luciferase reporter gene cell line AZ-GR derived from HeLa cells transfected with reporter plasmid containing three copies of glucocorticoid response elements. A panel of 22 natural and synthetic steroids was tested in cell line AZ-GR and the values of E_{MAX}, EC_{50} and efficacies were calculated, and they were comparable with the data from literature (Table 1) (Wilkinson et al., 2008). AZ-GR cells were highly specific and sensitive to glucocorticoids, but no responsiveness to estrogens, gestagens or androgens was observed. Cells displayed very low responsiveness to mineralocorticoids, but the potency and efficacy of mineralocorticoids was much lower than those for glucocorticoids. The sensitivity of the assay allowed high throughput format of the analyses, using 96-well plates. Time-course analyses revealed that GR activators may be detected using AZ-GR cells soon after 14 h of the treatment. A functionality of AZ-GR cells was not affected by cryopreservation process and the cells fully maintained responsiveness to glucocorticoids for 32 days in the culture and over 16 passages without significant alterations.

Collectively, we present here glucocorticoid-responsive stable cell line that allows high throughput, rapid, sensitive and selective detection of GR activators. Cell line AZ-GR has possible use in preclinical research for detection of glucocorticoid-active substances and detection of P450 inducers. Environmental applications, such as monitoring of endocrine disruptors and steroid hormones in soils and waters (rivers, sees, lakes) are expected as well.

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Czech Scientific Agency GACR 303/12/G163, GACR 503/10/0579 and GACR 304/10/0149. The authors declare that they have no conflict of interest. The generated cell line is openly available for a non-commercial use within co-operation.

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APPENDIX III.

Novotna A., Doricakova A., Vrzal R., Pavek P. and Dvorak Z. Construction and characterization of hepatocyte nuclear factor HNF4α1 over-expressing cell line derived from human hepatoma HepG2 cells. *Eur J Pharmacol. 2011*, **669**: 45-50.

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Construction and characterization of hepatocyte nuclear factor HNF4alpha1 over-expressing cell line derived from human hepatoma HepG2 cells

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ABSTRACT

Cancer cell lines derived from hepatocytes have an altered phenotype and they lack hepatocyte-specific functions. It is at least partly due to the under-expression of transcription factors such as hepatocyte nuclear factor 4α (HNF4α), steroid receptor co-activator 1 (SRC1) etc. Recently, a strategy of transient transfection of human hepatic cells with HNF4α revealed improved hepatospecific functions, including the expression of drug-metabolizing enzymes. In the current study we established a human cell line derived from HepG2 cells stably transfected with human HNF4α, and we examined this line for hepatospecific markers. Of the 9 clones analyzed, we found an increased secretion of fibrinogen (9 clones), albumin (5 clones) and plasminogen (3 clones), while secretion of alpha1-antitrypsin was not changed. The expression of pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) proteins but not mRNAs was slightly increased. TCDD-dependent induction of CYP1A1 mRNA and protein was augmented in 50% of clones, but there was no correlation between the CYP1A1 inducibility and expression levels of AhR and HNF4α. Induction of CYP3A4 mRNA by rifampicin was about 1.5–2.5 fold (clones 2, 4, 6, 7) and it was not significantly different from CYP3A4 mRNA induction in parent HepG2. The basal expression of CYP3A4 protein was increased in all clones, but rifampicin-induced expression of CYP3A4 protein was in all clones lower than in parent HepG2. Overall, the stable over-expression of HNF4α in HepG2 cells restores some of the hepatospecific functions, but it has a minor effect on the expression of xenobiotic-metabolizing enzymes and their regulators.

1. Introduction

Differentiation of human hepatocytes and functions of adult human liver require transcriptional factors that belong to five families, including the homeodomain homolog HNF1, forkhead homolog HNF3, homeodomain homolog HNF6, basic leucin zipper protein C/EBP and zinc finger protein HNF4 (hepatocyte nuclear factor 4). The subfamily HNF4 comprises more than 150 highly conserved proteins that include receptors for steroid hormones, nuclear receptors (retinoic X receptor, thyroid hormone receptor, vitamin D receptor) and factors HNF4α, HNF4β, HNF4γ and their splice variants (Mangelsdorf et al., 1995). HNF4α is crucial for development of the fetal liver (Chen et al., 1994) and for many physiological functions (Gupta et al., 2005). It is highly expressed in the liver, kidney, small intestine and pancreatic β-cells (Sladek et al., 1990; Yamagata et al., 1996). Initially, HNF4α was considered as an orphan receptor, but it was "deorphanized" with the identification of fatty acyl-CoA thioesters as endogenous ligands of HNF4α (Hertz et al., 1998). Transcriptionally active HNF4α forms a homodimer that binds to direct repeats (AGGTAC) in DNA promoter. SHP (short heterodimer partner) is a negative regulator of HNF4α. It inhibits the activity of HNF4α by competition with co-activators and by direct transcriptional repression (Lee et al., 2000). Target genes for HNF4α comprise blood coagulation factors (Inoue et al., 2006), HNF1α and genes involved in metabolism of lipids, glucose, bile acids and xenobiotics (Chiang, 2009; Guan et al., 2011; Hirota et al., 2008). The importance of HNF4α for the expression of drug-metabolizing hepatic P450s has been demonstrated in mice (Wiwi et al., 2004) and in human hepatocytes (Jover et al., 2001).

Human hepatoma cell line HepG2 lacks functional expression of almost all drug-metabolizing P450s, probably due to malfunction of HNF4α. Several hypotheses to explain low transcriptional activity of HNF4α in HepG2 cells have been proposed, including the imbalanced expression of HNF4α splicing variants (α1, α2, α3, α7), increased the expression of SHP, nuclear receptor co-repressor (NCoR) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). However, the most important reason for malfunction of HNF4α in HepG2 cells is the low expression of transcriptional co-activators PGC1α and SRC-1. Indeed, transient transfection of HepG2 cells with PGC1α (peroxisome proliferator-activated receptor gamma co-

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activator 1-alpha) and SRC-1 (steroid receptor co-activator 1) increased the expression of CYP2C9, CYP1A1 and CYP1A2 genes (Castell et al., 2006; Martinez-Jimenez et al., 2006a,b).

The studies performed to date have employed transient transfection of transcription factors such as HNF4α to cancer cell lines. In the current study we established a human cell line derived from HepG2 cells stably transfected with human HNF4α. Of the 9 clones analyzed, we found an increased secretion of fibrinogen (9 clones), albumin (5 clones) and plasminogen (3 clones), while secretion of alpha1-antitrypsin was not changed. The expression of pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) proteins but not mRNAs was slightly increased. TCDD-dependent induction of CYP1A1 mRNA and protein was augmented in 50% of clones, but there was no correlation between the CYP1A1 inducibility and expression levels of AhR and HNF4α. We concluded that the over-expression of HNF4α in HepG2 restores some of the hepatospecific functions in the resulting cell line, but it has only a minor effect on the expression of xenobiotic-metabolizing enzymes and their regulators.

2. Materials and methods

2.1. Materials

Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCycler FastStart DNA Master^{PLUS} SYBR Green I and FuGENE HD transfection reagent were from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). Rifampicin and DMSO were from Sigma-Aldrich (Prague, Czech Republic). Geneticin (G418) was purchased from Invitrogen (California, USA). All other chemicals were of the highest quality commercially available.

2.2. Human hepatoma HepG2 cells

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

2.3. Cytotoxicity assay

HepG2 cells were treated with G418 (400–1400 μg/ml) for 1, 2, 3, 6 and 7 days, using multi-well culture plates of 96 wells. In parallel, the cells were treated with vehicle (DMSO; 0.1%, v/v) and Triton X-100 (1%, v/v) to assess the minimal and maximal cell damage, respectively. The MTT test was performed using the common protocol. Absorbance was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments LLC). The values of IC_{50} were calculated using data acquired from three independent cell passages.

2.4. Transfection of HepG2 cells and selection process

HepG2 cells were seeded at the density of 2×10^6 cells in 60 mm culture dishes in 5 ml of the DMEM culture medium and transfected with pcDNA3-neo-HNF4α expression vector (2 μg) (Eeckhoute et al., 2004) or empty expressing vector pcDNA3-neo (2 μg) using Fugene HD reagent. After 36 h of incubation, the culture medium was replaced by the selection medium supplemented with G418 (1.1 mg/ml). The medium was renewed every 3–4 days for the period of 8 weeks, until a polyclonal population was selected. Thereafter, the cells were transferred to 10 cm culture dishes at the density of 500–1000 cells and cultured for additional 3 weeks under neomycin resistance until small colonies were visible. Nine colonies were subcloned into a 48-well tissue culture plate to obtain monoclonal populations. G418 resistant clones were analyzed for the secretion of hepatospecific markers and expression of HNF4α, PXR, AhR, CYP1A1 and CYP3A4. The use of GMO at Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (ref. 91997/ENV/10).

2.5. Protein detection and Western blotting

Total protein extracts: 3×10^6 cells were washed twice with ice-cold PBS and scraped into 1 ml of PBS. The suspension was centrifuged (1500×*g*/5 min/4 °C) and the pellet was re-suspended in 150 μl of ice-cold lysis buffer (10 mM Hepes pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; anti-protease cocktail, 0.2% (w/v) sodium dodecyl sulfate). The mixture was incubated for 20 min on ice and then centrifuged (12,000×*g*/10 min/4 °C). The supernatant was collected and the protein content was determined by the bicinchoninic acid method (Pierce, Rockford, IL.). SDS–PAGE gels (8%) were run on a Hoefer apparatus according to the general procedure. Protein transfer onto PVDF membrane was carried out. The membrane was saturated with 5% non-fat dried milk for 2 h at room temperature. Blots were probed with primary antibodies against albumin (mouse monoclonal; sc-70340, 1.B.730), fibrinogen (mouse monoclonal; sc-65966, GMA-035), plasminogen (mouse monoclonal; sc-65967, GMA-039), α1-antitrypsin (mouse monoclonal; sc-73431, 13702), HNF4α (rabbit polyclonal; sc-8987, H-171), PXR (mouse monoclonal; sc-48340, H-11), AhR (rabbit polyclonal; sc-5579, H-211), CYP1A1 (goat polyclonal; sc-9828, G-18), CYP3A4 (mouse monoclonal; sc-53850, HL3) and actin (goat polyclonal; sc-1616, I-19), all purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit.

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

Total RNA was isolated using TRI Reagent®. cDNA was synthesized according to the common protocol, using M-MLV Reverse Transcriptase (Invitrogen) and random hexamers (Amersham Biosciences). qRT-PCR was carried out on a Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The levels of CYP1A1, CYP3A4, AhR, PXR, HNF4α1 and GAPDH mRNAs were determined using primers and conditions as described in Table 1. The measurements were performed in duplicates. Gene expression was normalized per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by the delta–delta method.

Table 1
Oligonucleotides sequences used for RT-PCR analyses.

GAPDH	Forward	5′-TTCAGCAAGAAGAACAAGGACAA-3′
	Reverse	5′-GGTTGAAGAAGTCCTCCTAAGC-3′
CYP3A4	Forward	5′-TCCGGGACATCACAGACAGC-3′
	Reverse	5′-ACCCTGGGGTTCATCACCAA-3′
CYP1A1	Forward	5′-CAAAGTTGTCATGGATGACC-3′
	Reverse	5′-GGTCGGAGTCAACGGATTTGGTCG-3′
PXR	Forward	5′-TCCGGAAAGATCTGTGCTCT-3′
	Reverse	5′-AGGGAGATCTGGTCCTCGAT-3′
AhR	Forward	5′-CATCCCCCACAGCACAACAA-3′
	Reverse	5′-TCCCACTTGGCCAGGACTTC-3′
HNF4α	Forward	5′-GCCTACCTCAAAGCCATCAT-3′
	Reverse	5′-GACCCTCCCAGCAGCATCTC-3′
Neo gene	Forward	5′-GGTCTTGTCGATCAGGATG-3′
	Reverse	5′-ACGAGGAAGCGGTCAG-3′

2.7. Statistical analyses

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

3. Results

3.1. Cytotoxicity of geneticin

First, we determined cytotoxicity of G418 antibiotic in HepG2 cells for the periods of 1, 2, 3, 6 and 7 days. Employing the standard MTT assay, the values of IC_{50} for each time period were determined (data not shown). Based on these results, we chose G418 at concentration of 1.1 mg/ml for a selection process.

3.2. HNF4α expression in the stable HepG2 transfectants

HepG2 cells were transfected with pcDNA3-HNF4α (Suaud et al., 1999) and control empty plasmid pcDNA3 carrying a *neo* selection marker conferring resistance to the antibiotic G418 (*neo* gene). We selected nine G418-resistant HepG2–HNF4α clones and one HepG2–pcDNA3 control clone. We measured the expression of HNF4α mRNA and protein in HepG2–HNF4α, HepG2–pcDNA3 and in parent HepG2 cells. Surprisingly, there was no significant difference in the expression levels of HNF4α mRNA in HepG2–HNF4α cells as compared to HepG2–pcDNA3 and parent HepG2 cells. On the other hand, strongly increased protein levels of HNF4α were observed in all analyzed HepG2–HNF4α clones (except for clone 4) as compared to HepG2–pcDNA3 and parent HepG2 cells (Fig. 1A). To verify the stable integration of expressing vector, we determined the mRNA expression level of *neo* gene in HepG2–HNF4α, HepG2–pcDNA3 and parent HepG2 cells. We found high levels of *neo* gene mRNA in HepG2–HNF4α cells and HepG2–pcDNA3 cells as compared to non-transfected HepG2 cells (Fig. 1B).

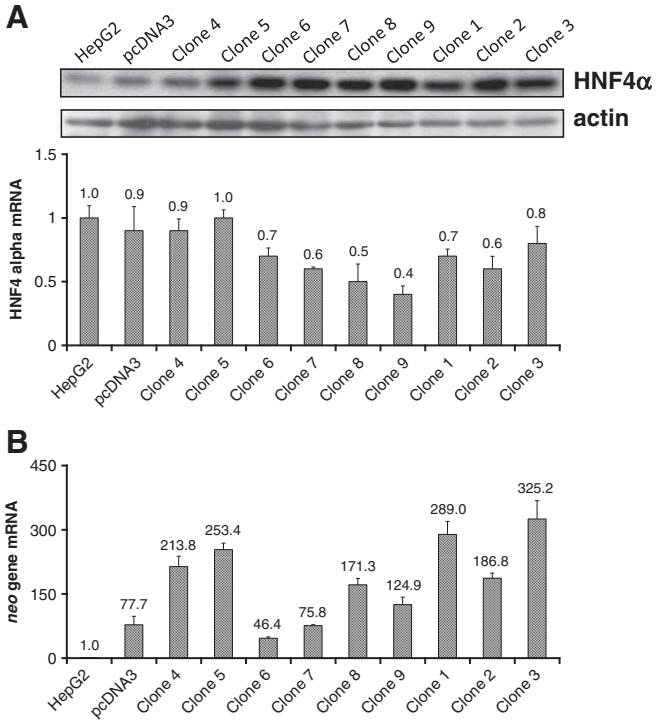

Fig. 1. HNF4α expression in the stable HepG2 transfectants. G418-resistant HepG2–HNF4α cells were obtained as described in the Materials and methods section. The expression of HNF4α and *neo* gene mRNAs and HNF4α protein was measured in 9 clones of HepG2–HNF4α cells, in HepG2–pcDNA3 cells and in parent HepG2 cells. Panel A: representative RT-PCR and western blot analyses of HNF4α mRNA and protein, respectively, are showed. RT-PCR data are shown as mean ± S.D. from duplicate measurements and are expressed as fold induction over parent HepG2 cells. The data were normalized per GAPDH mRNA levels. Similar profiles, both for protein and mRNA, were observed in three independent experiments. Panel B: representative RT-PCR analysis of *neo* resistance gene mRNA in samples corresponding to Panel A is shown.

3.3. Secretion of hepatospecific markers

We analyzed HepG2–HNF4α clones for the secretion of hepatospecific markers including α1-antitrypsin, albumin, fibrinogen and plasminogen. The cells were maintained in a serum-free medium for 48 h. Secretion proteins in the extracellular medium were analyzed by Western immunoblotting. Parent HepG2 and HepG2–pcDNA3 secreted α1-antitrypsin, albumin and fibrinogen, but the secretion of plasminogen was not detectable (Fig. 2). The secretion of fibrinogen was clearly increased in all clones (except of clone 4), which corresponds with the protein level of HNF4α (Fig. 1A). We found no change (clones 4, 5), slight increase (clones 2, 3, 6, 7, 8) or drastic decrease (clones 1, 9) of albumin secretion in HepG2–HNF4α cells. Plasminogen was detected in clones 6, 7 and massively in clone 9. The protein level of α1-antitrypsin was not significantly altered in any clone as compared to HepG2–pcDNA3 and parent HepG2 cells.

3.4. Expression of xenoreceptors PXR and AhR

In next series of experiments, we analyzed the expression of xenoreceptors AhR and PXR in HepG2–HNF4α clones. The level of AhR mRNAs in HepG2–pcDNA3 control cells was decreased by approx. 50%; the levels in clones 1–9 varied between 30% and 90% of the AhR level in parent HepG2 cells (Fig. 3). In contrast, the level of AhR protein in HepG2–pcDNA3 control cells was increased as compared to parent HepG2 cells. The levels of AhR protein in clones 1–9 varied, and they were indirectly proportional to the AhR mRNA levels; the highest expression of AhR protein was in clones 2, 3, 6, 7, 8 and 9 (Fig. 3). The level of PXR mRNAs in HepG2–pcDNA3 control cells was slightly decreased (by approx. 10%); the PXR mRNA levels in clones 1–9 varied between 40% and 100% of the PXR level in parent HepG2 cells (Fig. 3). The levels of PXR protein were elevated in all clones with the exception of clone 4; the strongest expression of PXR protein was observed in clones 6, 7, and 8 (Fig. 3).

3.5. Induction of drug-metabolizing enzymes CYP1A1 and CYP3A4

As a measure of the hepatocyte-like functionality of HepG2–HNF4α cell line, we investigated the ligand-inducible expression (induction) of CYP1A1 and CYP3A4 proteins and mRNAs. The cells were treated with rifampicin (RIF; 10 μM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h (mRNA analyses) and 48 h (protein analyses).

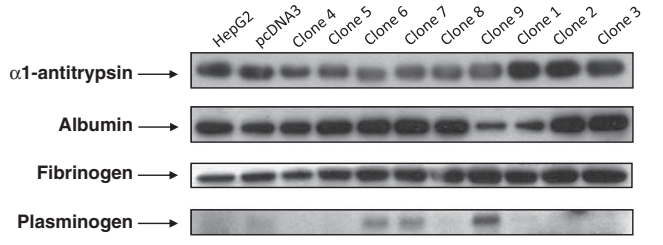

Fig. 2. Secretion of hepatospecific markers in G418-resistant HepG2–HNF4α cells. The secretion of hepatospecific markers was monitored in culture media from G418-resistant HepG2–HNF4α cells, HepG2–pcDNA3 cells and parent HepG2 cells cultured in the absence of serum for 48 h. Representative western blot analyses of α1-antitrypsin, albumin, fibrinogen and plasminogen proteins are showed. Similar profiles were observed in three independent experiments.

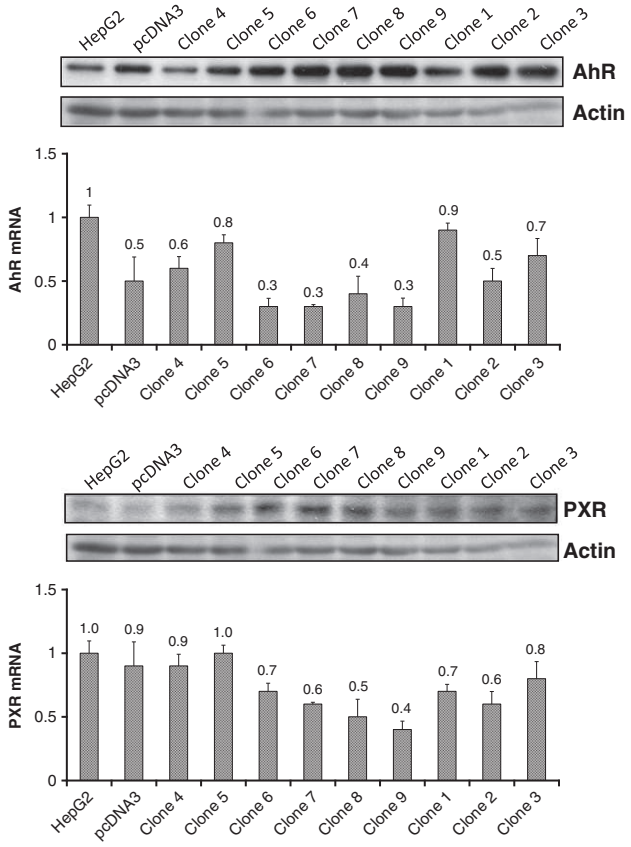

Fig. 3. Expression of xenoreceptors AhR and PXR in G418-resistant HepG2–HNF4α cells. Representative RT-PCR analyses of AhR and PXR mRNAs (bar graphs) and western blots of AhR and PXR proteins (insets in graphs) in G418-resistant HepG2–HNF4α cells, HepG2–pcDNA3 cells and parent HepG2 cells are showed. Similar profiles were obtained from three independent experiments. RT-PCR data are shown as mean ± S.D. from duplicate measurements and are expressed as fold induction over the value of HepG2 parent cells. The data were normalized per GAPDH mRNA levels.

Treatment with TCDD resulted in strong induction of CYP1A1 mRNA (110 fold) and protein in parent HepG2 cells. Induction of CYP1A1 was much weaker in HepG2–pcDNA3 cells, at both mRNA (22 fold) and protein levels. The magnitude of CYP1A1 mRNA induction in HepG2–HNF4α cells varied between 35 fold and 463 fold in individual clones. The expression at the mRNA level was well correlated with CYP1A1 protein levels (Fig. 4A). It is worth noting that CYP1A1 induction was significantly stronger in all tested clones of HepG2–HNF4α cells as compared to HepG2–pcDNA3 cells stably transfected with empty resistance vector. When compared to parent HepG2 cells, the induction of CYP1A1 mRNA and protein was the highest in clone 1 (Fig. 4A).

Induction of CYP3A4 mRNA by RIF in parent HepG2 cells was negligible (1.8 fold). The level of CYP3A4 mRNA in HepG2–pcDNA3 cells treated by RIF was even slightly decreased as compared to vehicle-treated cells; i.e. no induction. Similarly, induction of CYP3A4 mRNA by RIF in HepG2–HNF4α cells varied in individual clones between 0.8 fold and 2.4 fold, and these values were not significantly different from CYP3A4 mRNA induction in parent HepG2 cells (Fig. 4B). The basal level of CYP3A4 protein in parent HepG2 was not nearly detectable, but treatment with RIF resulted in clear induction of CYP3A4 protein. The levels of both basal and RIF-induced CYP3A4 protein in HepG2–pcDNA3 cells were higher as compared with HepG2 cells, which were inconsistent with the expression of CYP3A4 mRNA. While the basal expression of CYP3A4 protein was detected in all clones of HepG2–HNF4α cells, the RIF-induced levels of CYP3A4 protein were in all clones lower than in parent HepG2 and HepG2–pcDNA3 cells (Fig. 4B).

4. Discussion

Primary human hepatocytes are considered as a gold in vitro model for drug metabolism and interactions studies. However, their use is limited due to low availability of liver tissue, in vitro phenotypic instability, limited life-span, insufficient quality of cryopreserved cells etc. Human liver-derived cancer cell lines have many advantages such as availability, unlimited life-span, proliferation in culture, easy cryopreservation; however, their phenotype is altered and they do not exert major hepatospecific functions, including drug-metabolizing capacity. It has been demonstrated that deficient expression of hepatic genes is at least partly caused by a lower level of transcriptional factors and co-activators (PGC1α, SRC-1, SRC-2, c/EBP, HNF4α) in human hepatoma cells. The over-expression of SRC1 and PGC1α led to up-regulation of HNF4α-dependent genes (ApoCIII, ApoAV, PEPCK, AldoB, OTC, CYP7A1) and forced HepG2 cells toward a more differentiated phenotype (Martinez-Jimenez et al., 2006b). The same authors transiently transfected PGC1α to HepG2 cells. They observed increased expression of CYP2C9, CYP1A1, CYP1A2 and CYP7A1 genes, and a moderate activating effect on CYP3A4, CYP3A5, and CYP2D6 (Martinez-Jimenez et al., 2006a). The effects of PGC1α were dependent on HNF4α and a significant increase in the expression of cytochromes P450 was observed only with transient co-transfection of PGC1α together with HNF4α (Martinez-Jimenez et al., 2006a,b). Consistently, silencing of HNF4α by si-RNA in human hepatocytes resulted in down-regulation of CYP3A4, CYP3A5, CYP2A6, CYP2B6, CYP2C9 and CYP2D6 (Jover et al., 2001). It has been reported elsewhere, that the interactions of PXR with HNF4α, SRC-1 and PGC-1α are important for induction of CYP3A4 (Li and Chiang, 2006; Matsumura et al., 2004). The importance of HNF4α in the expression of other drug metabolizing enzymes has also been demonstrated, e.g. for the CYP2A6 (Onica et al., 2008), CYP2B6 (Jover et al., 2001) and CYP2C subfamily (Chen et al., 2005; Kawashima et al., 2006; Pavek et al., 2007; Rana et al., 2010). For these reasons, we tested whether stable transfection of HepG2 cells with HNF4α restores hepatospecific functions. This is the first report on the establishment of HepG2 cells stably over-expressing human HNF4α–HepG2–HNF4α cells.

The clones of HepG2–HNF4α strongly increased HNF4α protein level, but the level of HNF4α mRNA was not altered. Since the stable integration of the plasmid in HepG2 cells was proven by *neo*-gene expression analyses, discrepancy between the mRNA and protein levels of HNF4α could be due to the multiple mechanisms of feedback control (mRNA stability and functionality etc.). The functional expression of HNF4α in this particular case means the over-expression of the protein; hence we did not investigate the mechanism of this discrepancy. A hallmark of hepatospecificity of the cells is the secretion of specific proteins such as albumin or proteins involved in blood coagulation. HNF4α regulates the expression of HNF1 (Yamagata et al., 1996), which is involved in the expression of albumin, fibrinogen and plasminogen (Maire et al., 1989; Tronche et al., 1997). In addition, a promoter of α1-antitrypsin gene contains the HNF4α binding site (Monaci et al., 1988). We analyzed 9 different clones of HepG2–HNF4α cells for the secretion of albumin, fibrinogen, plasminogen and α1-antitrypsin. We found improved secretion of hepatospecific proteins in a majority of the clones (with the exception of α1-antitrypsin), and each clone displayed an individual expression pattern of secreted proteins.

The capability of the liver cells to metabolize xenobiotics is an important hepatospecific function. Xenoreceptors PXR and AhR are pivotal transcriptional regulators of drug-metabolizing cytochromes P450. The role of HNF4α in the regulation of PXR gene during the fetal liver development (but not in adult human liver) has been

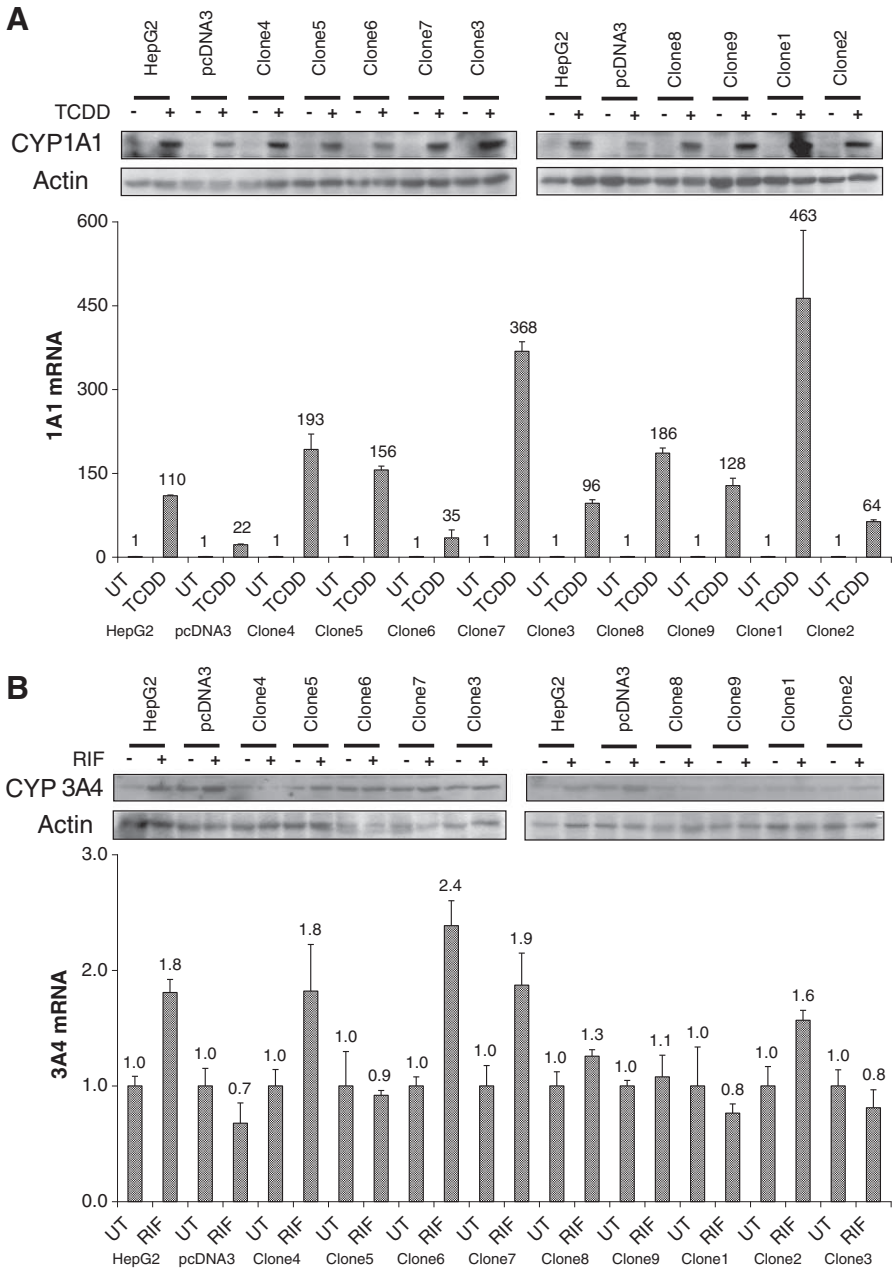

Fig. 4. Induction of CYP1A1 and CYP3A4 in G418-resistant HepG2–HNF4α cells. G418-resistant HepG2–HNF4α cells, HepG2–pcDNA3 cells and parent HepG2 cells were treated with rifampicin (RIF; 10 μM), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h (mRNA analyses) and 48 h (protein analyses). Representative RT-PCR analyses and western blots of AhR (Panel A) and PXR (Panel B) are showed. Similar profiles were obtained from three independent experiments. RT-PCR data are shown as mean ± S.D. from duplicate measurements and are expressed as fold induction over vehicle-treated cells in the each cell type or clone. The data were normalized per GAPDH mRNA levels.

demonstrated (Kamiya et al., 2003). We found that the over-expression HNF4α positively affects the expression of PXR and AhR proteins, since the levels of PXR and AhR proteins were elevated in HepG2–HNF4α cells. On the other hand, the levels of PXR and AhR mRNAs were decreased as compared to parent HepG2 cells. The dioxin-inducible expression of CYP1A1 was moderately stronger than in parent HepG2 cells. The basal expression of CYP3A4 protein was increased, but rifampicin-inducible expression of CYP3A4 was lost in HepG2–HNF4α cells.

In conclusion, we established a cell line derived from human hepatoma HepG2 cells stably transfected with HNF4α. The resulting cell line showed improvement in some hepatospecific functions such as secretion of plasma proteins or increased the expression of xenoreceptors PXR and AhR. However, the xenobiotic-inducible expression of CYP1A1 and CYP3A4 was not improved to an extent to justify the construction of a stably transfected cell line for this purpose.

Acknowledgments

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APPENDIX IV.

Novotna A., Doricakova A., Pavek P. and Dvorak Z. Construction and characterization of peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α over-expressing cell line derived from human hepatocyte carcinoma HepG2 cells). *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012*, [Epub ahead of print].

Construction and characterization of peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α over-expressing cell line derived from human hepatocyte carcinoma HepG2 cells)

Aneta Novotna[a], Aneta Doricakova[a], Petr Pavek[b], Zdenek Dvorak[a]

Aims. The aim was develop stable human cell line stable over-expressing transcription co-activator peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) with restored hepatospecific functions and increased expression of major xenobiotic metabolizing enzymes.
Methods. Six clones of HepG2-PGC-1α and one control clone HepG2-pcDNA3 were isolated and analyzed for secretion of hepatospecific markers, fibrinogen, albumin and alpha1-antitrypsin. Expression levels of protein and mRNA of hepatocyte nuclear factor (HNF4α), pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) were determined. We measured basal and ligand inducible expression of CYP1A1 and CYP3A4.
Results. Stably transfected cell line HepG2-PGC-1α derived from HepG2 cells over-expressing PGC-1α displayed increased secretion of fibrinogen, but not albumin or alpha1-antitrypsin compared to parent HepG2 cells. We found increased levels of HNF4α, PXR and AhR proteins but not their mRNAs in HepG2-PGC1 cells. Basal expression of CYP3A4 protein in HepG2-PGC-1α cells was increased but rifampicin-inducible expression of CYP3A4 protein was lowered in comparison with parent HepG2 cells. Induction of CYP3A4 mRNA varied between 1.3 – 1.9 fold in individual clones. Expression of TCDD-inducible CYP1A1 protein was lower in HepG2-PGC-1α cells than in parent HepG2 cells. Induction of CYP1A1 mRNA by TCDD in HepG2-PGC-1α cells was comparable with that in parent HepG2 cells and ranged between 103 – 198 fold.
Conclusion. Stable expression of PGC-1α in HepG2 cells restores several hepatospecific functions, such as secretion of fibrinogen, expression of HNF4α1 and xenoreceptors PXR and AhR. However, the expression and induction of key drug-metabolizing enzymes (CYP1A1 and CYP3A4) were not improved.

Key words: drug metabolism, cell lines, hepatocytes, cytochrome P450

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INTRODUCTION

Altered phenotype and loss of hepatocyte-specific function of human cancer cell lines derived from liver cells is probably the consequence of diminished expression of key transcriptional factors and their co-activators, such as peroxisome proliferator-activated receptor gamma co-activator (PGC-1α). PGC-1α was identified as the first member of the PGC-1 family co-activators[1]. Its homologs PGC-1β and PGC-1-related co-activator (PRC) display high similarity in the C-terminus, RNA recognition motif, conserved L1 motif and nuclear receptor interaction domain (L2) in N-terminus[2,3]. PGC-1 co-activators form a complex with several HAT-containing proteins (CBP, p300, SRC-1) at their N-terminal regions or dock the protein mediator complex called TRAP/DRIP on the C-terminal region of PGC-1α (ref.[4,5]). PGC-1α transcriptional activator displaces the repressor proteins and consequently increases the gene transcription[6]. PGC-1α plays an important role in the regulation of a wide range of the nuclear receptors such as HNF4α, glucocorticoid receptor, PPARα, and PPARβ and others[7-9]. Depending on the cell type, PGC-1α is regulated in different ways. The major mechanism of PGC-1α induction is activation of the cAMP signaling pathways through cAMP binding to CREB binding site in proximal promoter of PGC-1α (ref.[10]). Another mechanism of PGC-1α induction involves stimulation of activating transcription factor 2 (ATF2) in brown adipose tissue by cold, or calcium signaling in exercised skeletal muscles and heart[11,12]. The expression of PGC-1α is controlled by nutritional and hormonal signals as well as by circadian pacemakers[13]. The highest expression was found in tissues with a high density of mitochondria, i.e. heart, kidney, skeletal muscles, brown adipose tissue, liver and brain[1,14,15]. PGC-1α also plays an important role in adaptive thermogenesis. It is involved in glucose metabolism and maintenance of lipid and energy homeostasis. Fasting induces PGC-1α expression in adult liver, which leads to activation of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase through interactions with the transcription factor HNF4α and forkhead box O1 (FOXO-1)

(ref.[9,16,17]). Moreover, HNF4α is a key transcriptional factor for maintenance of expression of genes encoding the hepatic xenobiotic-metabolizing enzymes P450s (ref.[18,19]). The transactivation effect of PGC-1α on P450 genes mediated by HNF4α was corroborated by transfection experiments using MZ-Hep-1 cells that have low levels of HNF4α. Co-transfection of PGC-1α and HNF4α increased the levels of CYP2C9, CYP1A1 and CYP1A2, whereas transfection of PGC-1α or HNF4α alone had no significant effect on P450s expression[20]. Compared to primary human hepatocytes, the expression of PGC-1α in HepG2 is greatly decreased, whereas the expression of HNF4α is maintained[21]. Transient transfection of HepG2 cells with PGC-1α significantly increased the expression of CYP2C9, CYP1A1 and CYP1A2 genes, and to a lesser extent the expression of CYP3A4, CYP3A5 and CYP2D6 (ref.[20,21]).

In the present study, we describe construction and characterization of a stable cell line derived from HepG2 cells over-expressing transcription co-activator PGC-1α. We observed increased expression of fibrinogen but not albumin or alpha1-antitrypsin in clones over-expressing PGC-1α. The expression of HNF4α, pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) proteins but not their mRNAs was increased. Basal and inducible expression of xenobiotic-metabolizing enzymes CYP1A1 and CYP3A4 was not increased compared to parent HepG2 cells. In conclusion, while stable over-expression of PGC-1α moderately improved hepatospecific functions of HepG2 cells, the resulting cell line did not display higher basal and inducible expression of CYP1A1 and CYP3A4 compared to parent HepG2 cells.

MATERIALS AND METHODS

Materials

Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCycler FastStart DNA Master^{PLUS} SYBR Green I and FuGENE HD transfection reagent were from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). Rifampicin and DMSO were from Sigma-Aldrich (Prague, Czech Republic). Geneticin (G418) was purchased from Invitrogen (California, USA). All other chemicals were of the highest quality commercially available.

Human hepatoma HepG2 cells

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

Cytotoxicity assay

HepG2 cells were treated with G418 (400-1400 μg/mL) for 1, 2, 3, 6 and 7 days, using 96-well plates. In parallel, the cells were treated with vehicle (DMSO; 0.1%, v/v) and Triton X-100 (1%, v/v) to assess the minimal and maximal cell damage, respectively. MTT test was performed using common protocol. Absorbance was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments LLC). The values of IC_{50} were calculated using data acquired from three independent cell passages.

Transfection of HepG2 cells and the selection protocol

HepG2 cells were seeded at a density of 2×10^6 cells in 60 mm culture dishes in 5 mL of DMEM culture medium and transfected with pcDNA3-neo- PGC-1α expressing vector (2 μg) (Dr. Ramiro Jover, Unidad de Hepatología Experimental, Centro de Investigación, Hospital La Fe, Valencia, Spain) or empty expressing vector pcDNA3-neo (2 μg) using Fugene HD reagent. After 36 h of incubation, culture medium was replaced by selection medium supplemented with G418 (1.1 mg/mL). The medium was renewed every 3-4 days for a period of 12 weeks, until polyclonal population was selected. Cells were then transferred in 10 cm culture dishes at density of 500 – 1000 cells and cultured for an additional 5 weeks under neomycin resistance until small colonies were visible. Six colonies were subcloned into a 48-well tissue culture plate to obtain monoclonal populations. G418 resistant clones were analyzed for secretion of hepatospecific markers and expression of PGC-1α, HNF4α, PXR, AhR, CYP1A1 and CYP3A4. The use of GMO at the Faculty of Science, Palacky University Olomouc was approved by the Czech Ministry of Environment (ref. 91997/ENV/10).

Western blotting and protein detection

Total protein extract preparation: 3×10^6 cells were washed twice with ice-cold PBS and scraped into 1mL of PBS. The suspension was centrifuged (4 500 RPM/5 min/4 °C) and the pellet was re-suspended in 170 μL of ice-cold lysis buffer (10 mM Hepes pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; anti-protease cocktail, 0.2% (w/v) sodium dodecylsulfate). The mixture was incubated for 20 min on ice and then centrifuged (13 000 RPM/13 min/4 °C. Supernatant was collected and the protein concentration was determined by the Bradford method (Pierce, Rockford, IL.). SDS–PAGE gels (8%) were run on a Hoeffer apparatus according to the general procedure. Protein transfer onto PVDF membrane was carried out. The membrane was saturated with 5% non-fat dried milk for 2 h at room temperature. Blots were probed with primary antibodies against albumin (mouse monoclonal; sc-70340, 1.B.730), fibrinogen (mouse monoclonal; sc-65966, GMA-035), α1-antitrypsin (mouse monoclonal; sc-73431, 13702), PGC-1α (goat polyclonal; sc-8987, H-171), HNF4α (rabbit polyclonal; sc-8987, H-171), PXR (mouse monoclonal; sc-48340, H-11), AhR (rabbit polyclonal; sc-5579, H-211), CYP1A1 (goat polyclonal; sc-9828, G-18), CYP3A4 (mouse monoclonal; sc-53850,

HL3) and actin (goat polyclonal; sc-1616, I-19), all purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit. Films were scanned and the intensity of the bands was evaluated by densitometry.

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

Total RNA was isolated using TRI Reagent®. cDNA was synthesized according to common protocol, using M-MLV Reverse Transcriptase (Invitrogen) and random hexamers (Amersham Biosciences). qRT-PCR was carried out on Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The primers and conditions for determination of CYP1A1, CYP3A4, AhR, PXR, HNF4α1 and GAPDH mRNAs were described [22]. Primers for PGC-1α were described [20]. The measurements were performed in duplicate. Gene expression was normalized per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by the delta-delta method.

Statistical Analyses

Results were expressed as means ± standard deviation.

RESULTS

Cytotoxicity of geneticin

The dose of geneticin was set at 1.1 mg/mL of culture medium, based on preliminary cytotoxicity experiments (data not shown).

PGC-1α expression in the stable HepG2 transfectants

HepG2 cells were transfected with pcDNA3-PGC-1α and control empty plasmid pcDNA3 carrying a *neo* selection marker conferring resistance to the antibiotic G418 (*neo* gene). We selected six G418-resistant HepG2-PGC-1α clones and one HepG2-pcDNA3 control clone. We measured the expression of PGC-1α mRNA and protein in HepG2-PGC-1α, HepG2-pcDNA3 and in parent HepG2 cells. We found no significant difference in the expression levels of PGC-1α mRNA in HepG2-PGC-1α cells compared to HepG2-pcDNA3 and parent HepG2 cells. On the other hand, we observed greatly increased

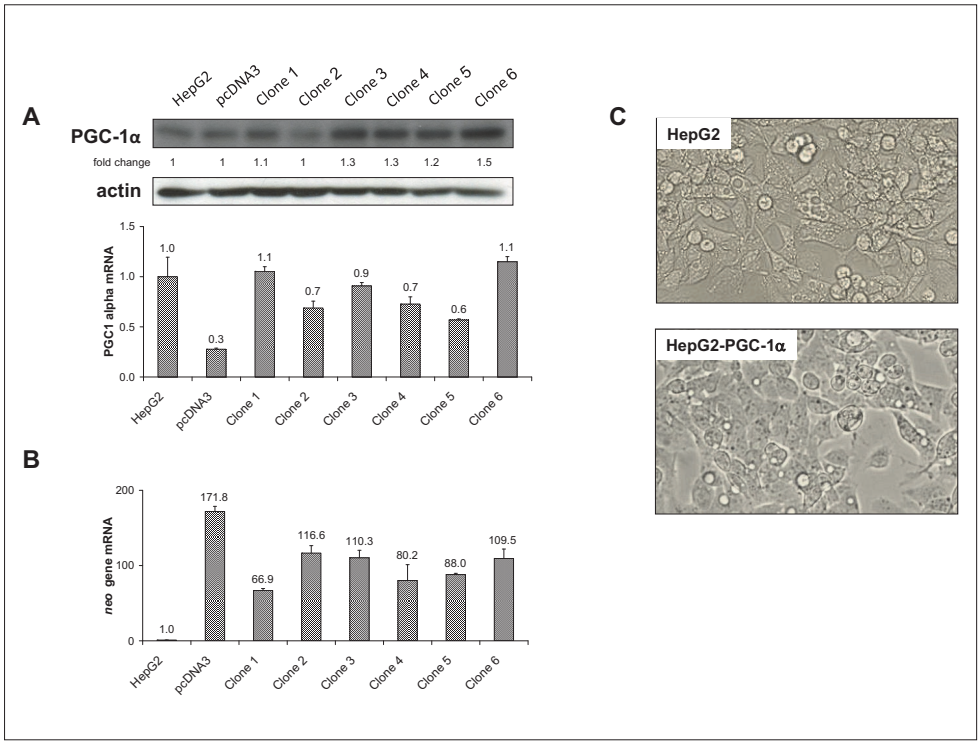

Fig. 1. PGC-1α expression in the stable G-418 resistant HepG2-PGC-1α cells. Cells were obtained as described in Materials and Methods section. **Panel A**: Representative RT-PCR of PGC-1α mRNA is shown in bar graph; western blot analyses of PGC-1α protein is above the graph. RT-PCR data are mean ± SD from duplicate measurements and are expressed as fold induction over the value from parent HepG2 cells. The data were normalized per GAPDH mRNA levels. Similar profiles, both for protein and mRNA, were observed in three independent experiments. **Panel B**: RT-PCR analysis of *Neo* resistance gene mRNA in clones of HepG2-PGC-1α cells. **Panel C:** Morphology of parent HepG2 and HepG2-PGC-1α stable cell line. The picture shows HepG2 at passage 8 and HepG2-PGC-1α stable cell line at passage 21. Numbers indicate fold change in protein expression related to the control cells HepG2 determined by densitometry.

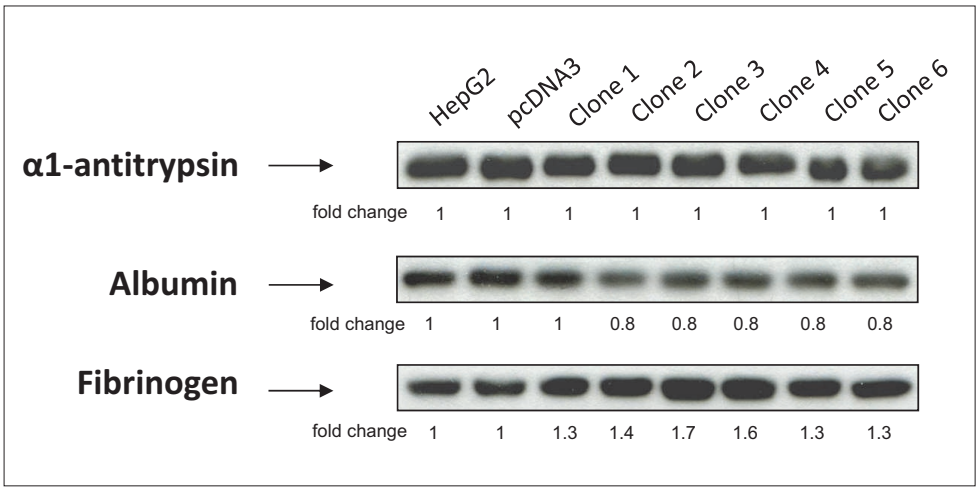

Fig. 2. Secretion of hepatospecific markers in culture of the stable G-418 resistant HepG2-PGC1α cells. HepG2-PGC-1α cells, HepG2-pcDNA3 cells and parent HepG2 cells were maintained in serum free medium. After 48 h, medium was removed and analyzed for secretion of hepatospecific markers. Representative western blot analyzes α1-antitrypsin, albumin and fibrinogen proteins are showed. Similar profiles were observed in three independent experiments. Numbers indicate fold change in protein expression related to the control cells HepG2 determined by densitometry.

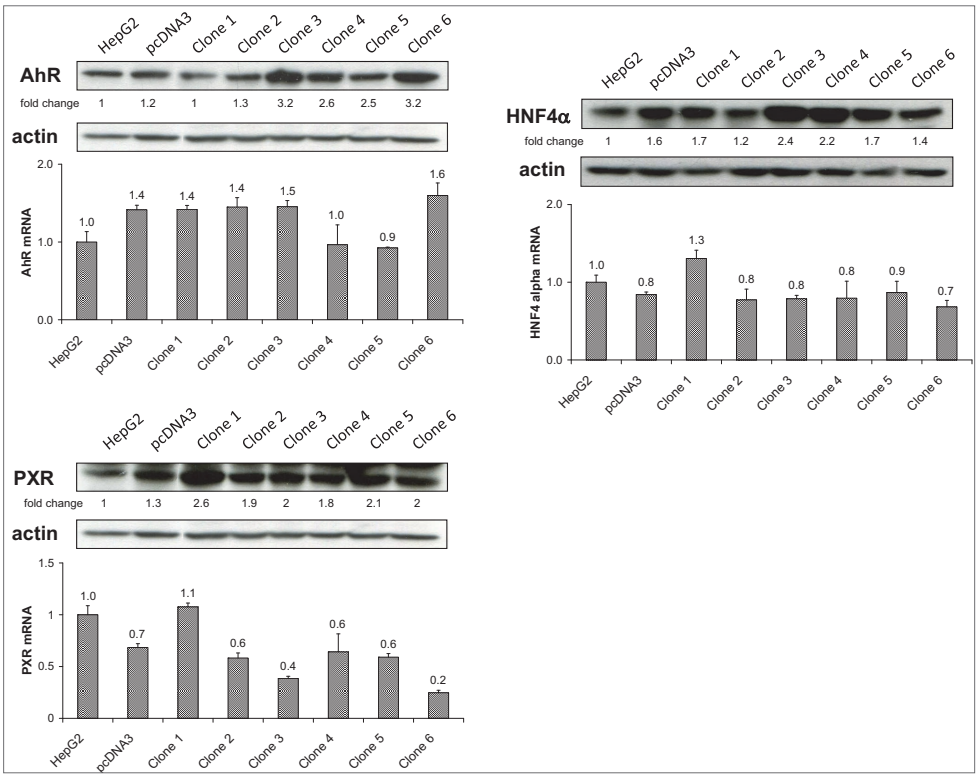

Fig. 3. Expression of AhR, PXR and HNF4α in the stable G-418 resistant HepG2-PGC-1α cells. Total mRNA and total protein extracts were isolated from HepG2-PGC-1α cells, HepG2-pcDNA3 cells and parent HepG2 cells. Representative RT-PCR analyses of AhR, PXR and HNF4α mRNAs are shown in bar graphs; western blots of AhR, PXR and HNF4α proteins are above individual graphs. Similar profiles were obtained from three independent experiments. RT-PCR data are mean ± SD from duplicate measurements and are expressed as fold induction over the value of HepG2 parent cells. The data were normalized per GAPDH mRNA levels. Numbers indicate fold change in protein expression related to the control cells HepG2 determined by densitometry.

PGC-1α protein level in clones 3, 4, 5 and 6 compared to control HepG2-PGC-1α and parent HepG2 cells (Fig. 1A). We determined the mRNA expression level of *neo* gene in HepG2- PGC-1α, HepG2-pcDNA3 and parent HepG2 cells to verify the stable integration of expressing vector (Fig. 1B). The morphology of HepG2-PGC-1α cells was slightly different from parent HepG2 cells (Fig. 1C).

Secretion of hepatospecific markers

We analyzed HepG2-PGC-1α clones for secretion of hepatospecific markers including α1-antitrypsin, albumin and fibrinogen. The cells were maintained in serum-free medium for 48 h. Secretion proteins in extracellular medium were analyzed by Western immunoblotting. Secretion of fibrinogen was increased in all clones (Fig. 2). The greatest increase was observed in clones 3, 4 and 6, which correlates with the protein level of PGC-1α and HNF4α (Fig. 1A and 3). We found no increase in secretion of albumin and α1-antitrypsin in any clone, as compared to HepG2-pcDNA3 and parent HepG2.

Expression of xenoreceptors and transcription factors

Next, we analyzed the expression of xenoreceptors AhR and PXR, and transcription factor HNF4α, the most important regulators of hepatic drug-metabolizing P450s. We found increased expression of HNF4α protein in all clones (except for clone 2) and in control HepG2-pcDNA3 cells as compared to parent HepG2 cells (Fig. 3). The greatest increase was detected in clones 3, 4, 5 and 6 and this correlates with PGC-1α protein level. The expression level of HNF4α mRNA was moderately decreased in HepG2-pcDNA3 control cells in comparison with parent HepG2 cells. HNF4α mRNAs level in tested HepG2-PGC-1α cells ranged between 0.7 fold to 1.3 fold compared to parent HepG2. The level of PXR mRNA in HepG2-pcDNA3 control cells was decreased by approx. 30% and the levels in clones 1-6 varied between 20% to 110% of the PXR level in parent HepG2 cells. On the other hand, the protein level of PXR was increased in all clones as well in control HepG2-pcDNA3 cells compared to parent HepG2 cells (Fig. 3). Expression of AhR mRNAs in the control HepG2-pcDNA3 cells was slightly increased (by approx. 40%) and in clones 1-6 varied between 90% - 160% of the AhR level in parent HepG2 cells. The AhR protein level in HepG2-pcDNA3 was not altered in comparison with parent HepG2 cells. In contrast, we found major increase in AhR protein levels in clones 3, 4 and 6 and moderate increase in clone 5 (Fig. 3), which correlates with PGC-1α protein level (Fig. 1A).

Expression of drug-metabolizing enzymes CYP1A1 and CYP3A4

In the last series of experiments, we measured basal and ligand inducible expression of CYP1A1 and CYP3A4, the most prominent drug-metabolizing cytochromes P450. HepG2-PGC-1α cells were treated with rifampicin (RIF; 10 μM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h (mRNA analyzes) and 48 h (protein analyzes).

Basal expression of CYP3A4 protein in HepG2-PGC-1α cells was markedly increased in all clones, compared to parent HepG2 and control HepG2-pcDNA3 cells. Rifampicin strongly induced CYP3A4 protein in HepG2 and HepG2-pcDNA3 cells. However, found no induction of CYP3A4 protein by rifampicin in HepG2-PGC-1α cells, and the levels of CYP3A4 protein were nearly identical in DMSO- and RIF-treated cells. An apparent loss of CYP3A4 protein induction by RIF was due to the elevated basal levels of CYP3A4 protein in HepG2-PGC-1α cells (Fig. 4A). Induction of CYP3A4 mRNA by RIF ranged from 1.5 to 1.7 fold in parent HepG2 and HepG2-pcDNA3 cells. Basal and RIF-inducible expression of CYP3A4 mRNAs in HepG2-PGC-1α did not significantly differ from CYP3A4 mRNA expression in parent HepG2 cells (Fig. 4A).

The typical AhR agonist TCDD, induced CYP1A1 mRNA with similar potency in all examined cell lines, i.e., in HepG2 cells (approx. 163 fold), in HepG2-pcDNA3 cells (approx. 150 fold) cells, and in HepG2-PGC-1α cell clones (from 65 fold to 198 fold in individual clones) (Fig. 4B). No basal level of CYP1A1 protein was found in either cell line under examination. TCDD-inducible expression of CYP1A1 protein was diminished in control cells HepG2-pcDNA3 compared to parent HepG2 cells. The level of CYP1A1 inducible protein was in all clones of HepG2-PGC-1α cells higher than in control cells HepG2-pcDNA3, but similar to the levels in parent HepG2 cells. In clone 1 we found stronger induction of CYP1A1 protein than in parent HepG2 but this did not correlate with CYP1A1 mRNA level (Fig. 4B).

DISCUSSION

Human hepatocyte-derived cancer cell lines have many advantages such as availability, the unlimited lifespan, proliferation in culture, easy cryopreservation in comparison with primary human hepatocytes. For these properties, hepatoma cell lines would be a suitable model for drug-metabolism studies. However, lower biotransformation activity and lack of hepatospecific functions hinders wide use of cancer cell lines for metabolic studies. The loss of hepatic phenotype of cancer cells is at least partly caused by the under-expression of transcription factors or co-activators such as (PGC-1α, SRC-1, SRC-2, c/EBP, HNF4α) and others. PGC-1α plays a role in adaptive thermogenesis, it is involved in activation of gluconegenesis genes in the liver, and it stimulates genes important in mitochondrial function and oxidative metabolism. PGC-1α activates P450 genes through co-activation of transcription factor HNF4α which is a key factor for expression of hepatic drug-metabolizing P450s in mice, in human hepatocytes and for maintaining of hepatospecific functions[19]. Transient co-transfection of MZ-Hep-1 hepatocyte carcinoma cells with PGC-1α and HNF4α increased the expression of CYP2C9, CYP1A1 and CYP1A2 (ref.[20]). Similarly, transient transfection of HepG2 human hepatoma cells with PGC-1α increased

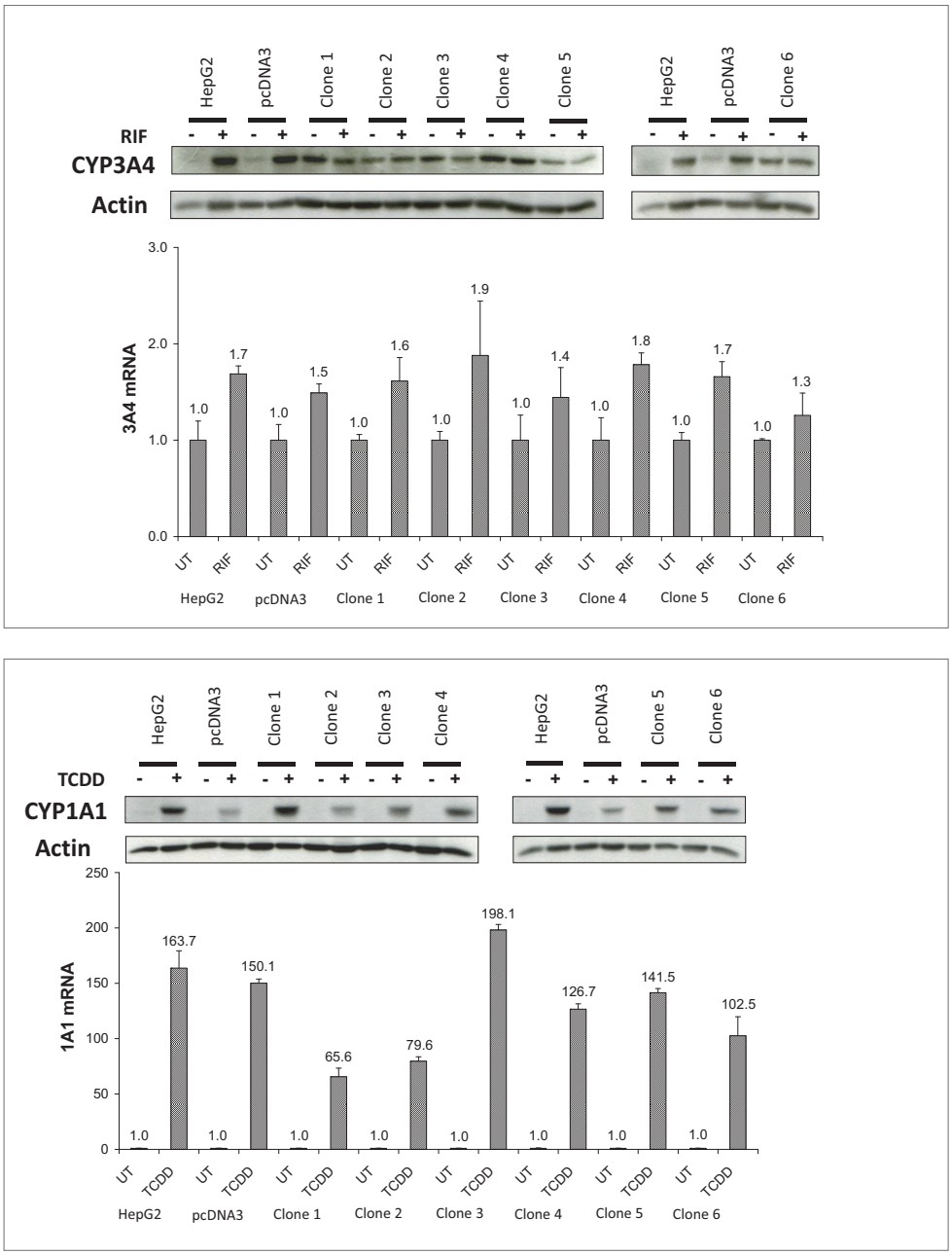

Fig. 4. Expression of CYP1A1 and CYP3A4 in the stable G-418 resistant HepG2-PGC-1α cells. G418-resistant HepG2-PGC-1α cells, HepG2-pcDNA3 cells and parent HepG2 cells were treated with rifampicin (RIF; 10 μM), 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h (mRNA analyzes) and 48 h (protein analyzes). Representative RT-PCR analyzes and western blots of CYP3A4 (**Panel A**) and CYP1A1 (**Panel B**) are showed. Similar profiles were obtained from three independent experiments. RT-PCR data are mean ± SD from duplicate measurements and are expressed as fold induction over vehicle-treated cells in the each cell type or clone. The data were normalized per GAPDH mRNA levels.

the expression of CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP3A4 and CYP3A5 genes[20]. Interaction of PGC-1α with PXR, SRC-1 and HNF4α is essential for CYP3A4 (ref.[23,24]). Another study reported that PGC-1α activates transcription of CYP2A5 gene through the co-activation of HNF4α (ref.[25]).

In a previous study, we showed that stable over-expression of HNF4α in HepG2 cells improved some hepatospecific functions including secretion of plasma proteins or increased expression of xenoreceptors PXR and AhR[22]. Hence, we tested whether stable over-expression of PGC-1α in HepG2 restores hepatospecific functions. In clones with increased PGC-1α protein levels, we found strongly increased HNF4α protein level, which is in accordance with findings of other groups[20,23,25]. Secretion of albumin and proteins involved in the blood coagulation cascade

are hepatospecific features of the cells. HNF4α, regulates the expression of HNF1 (ref.[26]), which is involved in the expression of albumin and fibrinogen[27,28]. In addition, a promoter of the α1-antitrypsin gene contains a HNF4α binding site[29]. We found increased secretion of fibrinogen but not albumin and α1-antitrypsin in all tested clones of HepG2-PGC-1α cells. However, stable over-expression of HNF4α in HepG2 increased the secretion of all these three plasma proteins[22]. Xenoreceptors AhR and PXR are the main transcriptional regulators of drug-metabolizing enzymes CYP1A1 and CYP3A4, respectively. We found that over-expression of PGC-1α, and consequently increased expression of HNF4α protein, positively correlated with increased levels of PXR and AhR proteins in HepG2-PGC1α clones 3, 4, 5 and 6. However, we found the highest expression of PXR protein in clone 1 which expressed lower levels of PGC-1α protein than clones 3, 4, 5 and 6. Dioxin-inducible expression of CYP1A1 mRNA and protein was not significantly altered compared to parent HepG2. Basal expression of CYP3A4 protein was increased in HepG2-PGC1α cells, but rifampicin-inducible expression of CYP3A4 was consequently lost.

In conclusion, this is the first report on the construction of HepG2 cell line stably over-expressing human PGC-1α. The new cell line displayed increased secretion of fibrinogen and higher expression of AhR, PXR and HNF4α as compared to parent cell line. However, the expression of drug-metabolizing enzymes CYP1A1 and CYP3A4 was not restored, implying that the approach of stable over-expression of hepatospecific transcription factors only partly addressed the problem of the generation of hepatocyte-like cell lines.

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CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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