COMPARISON OF TWO IMMORTALIZED HUMAN CELL LINES TO
STUDY NUCLEAR RECEPTOR MEDIATED CYP3A4 INDUCTION


Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences,
Division of Biomedical Analysis, Sorbonnelaan 16, 3584 CA Utrecht, The
Netherlands (SH; JHB; JHMS; IM)

Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences,
Division of Pharmacology and Pathophysiology, Sorbonnelaan 16, 3584 CA Utrecht,
The Netherlands (ASK)

Slotervaart Hospital, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066
EC Amsterdam, The Netherlands (JHB)

The Netherlands Cancer Institute, Department of Medical Oncology, Plesmanlaan
121, 1066 CX Amsterdam, The Netherlands (JHMS)
LS180 AS A MODEL TO STUDY CYP3A4 INDUCTION

Corresponding Author:
Drs. S. Harmsen
Division of Biomedical Analysis
Department of Pharmaceutical Sciences
Faculty of Science
Utrecht University
Sorbonnelaan 16
3584 CA Utrecht
The Netherlands
Phone: +31-30-2537377
E-mail: s.harmsen@uu.nl

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CAL, calcitriol
CAR, constitutive androstane receptor
CITCO, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-3,4-dichlorobenzyl) oxime
CYP, cytochrome P450
DMSO, dimethylsulfoxide
hGR, human glucocorticoid receptor
NR, nuclear receptor
PB, phenobarbital
PXR, pregnane X receptor
RIF, rifampicin
RXR, retinoid X receptor
VDR, vitamin D₃ receptor
Abstract

Since cytochrome P450 3A4 (CYP3A4) is responsible for the biotransformation of over 50% of all clinically used drugs, induction results in an increased clearance of many concomitantly administered drugs, thereby decreasing treatment efficacy or, in the case of pro-drugs, lead to severe intoxications. CYP3A4 induction is regulated by the pregnane X receptor, constitutive androstane receptor, and vitamin D receptor. Since these nuclear receptors show large interspecies differences, accurate prediction of nuclear receptor mediated CYP3A4 induction in humans requires the use of human systems. As primary cultures of human hepatocytes or enterocytes have major drawbacks like poor availability and poor reproducibility, human cell lines are a good alternative. In this study, the widely used HepG2 cell line was compared the LS180 cell line to serve as a model to study CYP3A4 induction. There was a clear difference between the cell lines with respect to CYP3A enzyme expression and induction. In LS180 CYP3A4 was expressed and was found to be induced by prototypical nuclear receptor agonists, while in HepG2 CYP3A4 was non-responsive to treatment with rifampicin, CITCO or calcitriol. We subsequently evaluated if these host-cell differences also have an effect on CYP3A4 reporter gene activity. We clearly show that there are differences in CYP3A4 reporter activity between the cell lines, and based on these results and those found on mRNA and protein level, we conclude that LS180 is a more suitable cell line to study CYP3A4 induction than the widely used HepG2.
Introduction

The risk of clinically relevant drug-drug interactions that involve the highly inducible cytochrome P450 (CYP) 3A enzyme CYP3A4 is significant, because CYP3A4 has a wide substrate specificity and is involved in the biotransformation of more than 50% of all clinically used drugs (Anzenbacher and Anzenbacherova, 2001). Therefore, several strategies to study CYP3A4 induction have been developed. Phenotyping studies, such as the erythromycin breath test, can be used directly to measure CYP induction in humans. However, this method has some serious disadvantages like the invasive administration of probe substances to humans, labor-intensive protocols, large interindividual differences and difficulties in processing and interpreting the results. Also the use of animals, for both in vivo and in vitro studies, poses problems, because there are large interspecies differences in the CYP3A4 induction capacity of compounds (Lehmann et al., 1998). Therefore, CYP3A4 induction is mainly studied in vitro in primary cultures of human hepatocytes, which are considered to mostly resemble the in vivo situation. However, the interindividual donor variability, rapid loss of drug metabolizing enzyme expression, poor availability, and the costs are major drawbacks (Brandon et al., 2003).

The finding that the highly promiscuous pregnane X receptor (PXR; NR1I2) (Moore et al., 2000) is one of the main regulators of CYP3A4 induction has led to the development of CYP3A4 reporter gene assays. These assays are based on the transient transfection of a cell line with a CYP3A4 reporter construct containing the response elements of PXR located in the proximal (-362/+53) and distal (-7836/-7208) promoter regions of the CYP3A4 gene linked to a firefly-luciferase or other reporter genes such as alkalinephosphatase (Goodwin et al., 1999). In contrast to hepatocytes, many cell lines express lower levels or even no PXR (Thummel et al., 2001; Swales et
al., 2003; Phillips et al., 2005). Therefore cell lines used for the reporter gene assay are often co-transfected with a PXR expression plasmid to increase the levels of this nuclear receptor. Since two other nuclear receptors, the constitutive androstane receptor (CAR; NR1I3) (Kawamoto et al., 1999) and the vitamin D receptor (VDR; NR1I1) (Kolars et al., 1992), are also known to regulate CYP3A4 induction, cells can also be co-transfected with CAR or VDR instead of PXR. At the moment cell-based reporter gene assays have become suitable alternatives to primary cultures of human hepatocytes to study nuclear receptor mediated CYP3A4 induction. Although nuclear receptor expression levels in these reporter gene assays are raised artificially, a good correlation between PXR-mediated CYP3A4 induction measured in a reporter gene assay, and CYP3A4 mRNA (Roymans et al., 2005) and protein (Luo et al., 2004) expression in primary cultures of human hepatocytes was reported. Furthermore, by comparing CYP3A4 reporter gene assay data and known data on CYP3A4 induction in vivo, Persson et al. (2006) showed that a CYP3A4 reporter gene assay is a reliable screening method for the assessment of drug-induced CYP3A4 expression (Persson et al., 2006).

Currently, the cell line most often used in CYP3A4 reporter gene assays is the human hepatocarcinoma-derived HepG2 cell line (Ogg et al., 1997; Ogg et al., 1999; El-Sankary et al., 2001; Luo et al., 2004; Trubetskoy et al., 2005; Noracharttiyapot et al., 2006; Sinz et al., 2006; Huang et al., 2007). However, HepG2 mainly expresses the fetal enzyme CYP3A7 instead of CYP3A4 (Schuetz et al., 1993). In addition, despite the presence of endogenous PXR, CYP3A4 expression is not enhanced by rifampicin treatment in HepG2 cells (Ogino et al., 2002). Since CYP3A4 is also present in the gastrointestinal tract, and has been shown to contribute significantly to the pre-hepatic metabolism of drugs (Kolars et al., 1992), other groups use the human
colon carcinoma-derived LS180 cell line as a host for their CYP3A4 reporter gene assay (Synold et al., 2001; Zhou et al., 2004). In contrast to HepG2, rifampicin was shown to increase CYP3A4 expression in LS180 cells (Schuetz et al., 1996). Moreover, in a comparison between three colon carcinoma cell lines (LS180, CaCo-2 and TC-7) only LS180 showed inducible CYP3A4 expression (Pfrunder et al., 2003).

The aim of this study was to investigate the suitability of LS180 cells as a model to study CYP3A4 induction in comparison with the widely used HepG2 cell line. Therefore, CYP3A mRNA and protein expression levels in both cell lines were determined after treatment with prototypical nuclear receptor agonists (rifampicin (PXR), CITCO (CAR), calcitriol (VDR)) that are known inducers of CYP3A4. Furthermore, the use of these cell lines as hosts for CYP3A4 reporter gene assays was evaluated.
Materials and methods

Materials

All cell culture media and supplements were purchased from Invitrogen (Breda, The Netherlands). 6-(4-chlorophenyl)imidazo [2,1-b] [1,3] thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)-oxime (CITCO; purity >98%) was obtained from BIOMOL International (Plymouth Meeting, PA, USA). All other chemicals were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).

Plasmids

The pGL3-CYP3A4-XREM (proximal (-362/+53) and distal (-7836/-7208)) luciferase reporter construct and the pEF-hCAR expression plasmid were a kind gift from Dr. Richard Kim (Vanderbilt University, Nashville, USA), the pCDG-hPXR expression vector was generously provided by Dr. Ron Evans (The Howard Hughes Medical Institute, La Jolla, USA), the pSG5-hVDR expression plasmid was kindly donated by Dr. Bandana Chatterjee (Department of Molecular Medicine/Institute of Biotechnology, University of Texas Health Science Centre, San Antonio), the pRL-TK control plasmid was obtained from Promega (Madison, WI, USA). Plasmids were checked by enzyme restriction and agarose gel electrophoresis and purified using Promega’s Pureyield Midi-prep (Madison, WI, USA) according to the instructions of the manufacturer.

Cell culture

The human colon adenocarcinoma-derived cell line, LS180 (used between passage 12 to 14) and the human hepatoma-derived cell line, HepG2 (used between passage 13 and 15) were purchased from ATCC (Manassas, VA, USA). The cell-
lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 ++ medium (with 25 mM HEPES and L-glutamine, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin), at 37°C under a humidified atmosphere of 5% CO₂.

Treatment

HepG2 and LS180 cells were plated at a density of 1x10⁶ cells/well in 6 well plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) in 2ml RPMI 1640 medium++. After reaching 80-90% confluency, medium was replaced with medium containing different concentrations of rifampicin (1 µM or 10 µM), CITCO (10 nM or 250 nM) or calcitriol (1 nM or 100 nM) and refreshed after 24 hours. The final solvent concentrations did not exceed 0.1%. After 48 hours cells were washed with phosphate buffered saline (PBS). The cells were further used for immunoblot analysis or qPCR as described below.

RNA Extraction and RT-PCR

Total RNA was extracted using the GeneElute Mammalian total RNA miniprep kit (SigmaAldrich, Zwijndrecht, The Netherlands). RNA integrity and quantity were determined using a Nanodrop Diode Array Spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). 1 µg of total RNA was reverse transcribed according to the manufacturer guidelines concerning the random hexamer primer (RevertAid™ First Strand cDNA synthesis kit, Fermentas, St.Leon-Rot, Germany).
**Quantitative RT-PCR**

The CYP3A4, CYP3A5, CYP3A7, and 18S mRNA expression levels were analyzed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). All reactions were singleplexed with 18S. Oligonucleotide primers and a Taqman probe for CYP3A4 were as follows: forward, TCAATAACAGTCTTTCCATTCCTCAT; reverse, CTTCGAGGCGACTTTCTTTCA; and probe, TGTTTCCAAGAGAAGTTACAAA. The primers and probe used for 18S, CYP3A5 (Hs00241417_m1), and CYP3A7 (Hs00426361_m1) real-time PCR were commercially available Assay on Demand kits (Applied Biosystems, Foster City, CA, USA). According to manufacturer guidelines, data were expressed as threshold cycle value (ct) and used to determine dct values. Fold changes in expression were calculated according to the transformation: fold increase = 2-(difference in dct).

**Immunoblot analysis**

After 48 hours, cells were harvested and lysed in 250 µl PBS containing 1% Triton X-100, 0.1% SDS, 1 mM dithiothreitol, and 1% protease inhibitor cocktail tablet (Roche, Basal, Switzerland). Protein concentrations were determined by a Pierce BCA protein assay (Pierce, Rockford, IL, USA) and 10 µg of total protein was separated by SDS-polyacrylamide gel electrophoresis on NuPage Novex Bis-Tris precast gradient gels (4-12%) (Invitrogen, Breda, The Netherlands). Human CYP3A4 protein (Gentest, Becton Dickinson, Woburn, MA, USA) was used as a control. Proteins were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA, USA). After overnight blocking in 3% bovine serum albumin, the membranes were incubated with a murine monoclonal anti-human CYP3A primary antibody (1:500; Gentest, Becton
Dickinson, Woburn, MA, USA). This antibody is known to cross-react with both CYP3A4 and CYP3A7, but not with CYP3A5. Next, the blot is incubated with a bovine anti-mouse IgG coupled to horse radish peroxidase (HRP) secondary antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The proteins were visualized by a chemiluminescence-based detection reagent (Supersignal West Femto, Pierce, Rockford, IL, USA) and the intensities of the CYP3A bands were determined on a Gel Doc Imaging system equipped with a XRS camera with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

**CYP3A4-reporter gene assay**

LS180 or HepG2 cells were seeded (2 × 10^5 cells/well and 5 × 10^5 cells/well respectively) in 96 well plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) in 200 µl RPMI 1640 ++ medium and incubated overnight in 5% CO₂-humidified, 37°C atmosphere. Following incubation, the cells were transfected with 75 ng/well of nuclear receptor expression vector (pCDG-hPXR, pEF-hCAR, or pSG5-hVDR), 210 ng/well of the CYP3A4 luciferase reporter construct (pGL3-CYP3A4-XREM), and 15 ng/well of the renilla luciferase expression controle vector (pRL-TK), using 0.99 µl/well Exgen500 in vitro transfection reagent (Fermentas, St.Leon-Rot, Germany) in 150 mM NaCl. In addition, to study the effect of endogenously expressed PXR, CAR or VDR on CYP3A4 reporter activity, transfections were performed in which the nuclear receptor expression plasmids were replaced by an empty plasmid (pSG5; 75 ng/well). After overnight transfection, the medium was removed, cells were washed with phosphate buffered saline (PBS), and fresh medium (200 µl) containing different concentrations of the inducers rifampicin, CITCO or calcitriol, were added to the wells. Rifampicin and CITCO were dissolved in DMSO.
while calcitriol was dissolved in ethanol. The final solvent concentration did not exceed 0.1%. After 48 hours, the medium was removed, cells were washed with PBS, and lysed with 20 µl/well Passive Lysis Buffer (Promega, Medison, WI, USA) for 15 minutes on a shaker. The cell lysates (10 µl) were transferred to a white half area 96-well plate (Corning BV, Schiphol-Rijk, The Netherlands) and the reporter activities of *firefly* luciferase and *renilla* luciferase were determined using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Medison, WI, USA) according to the manufacturer’s manual, with reagent volumes adjusted to the cell-lysate volume (Promega, Madison, WI, USA). Luminescence was recorded on a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). The fold induction was calculated by normalization of the *firefly*-luciferase signal to the *renilla*-luciferase signal.

**Statistical analysis**

Data were analyzed by using one-way ANOVA and the means were compared by applying the Bonferroni post-hoc test and were considered statistically significant when $P < 0.05$. The correlation between the data obtained from the CYP3A4 reporter gene assays and the protein expression levels were determined using the Pearson correlation coefficient. All statistical determinations were performed with SPSS v14.
Results

Inducible expression of CYP3A mRNA in LS180 and HepG2

First we considered which CYP3A enzymes are induced following treatment with rifampicin, CITCO and calcitriol in LS180 and HepG2. As shown in figure 1, in LS180 a significant increase of CYP3A4 mRNA expression was observed after exposure to rifampicin and calcitriol, while a less marked induction of CYP3A5 mRNA expression was observed following treatment with these compounds. CITCO however did not affect CYP3A4, CYP3A5 or CYP3A7 mRNA expression in LS180. In HepG2 no significant increase in CYP3A mRNA expression was detected after exposure to rifampicin, CITCO or calcitriol.

Inducible expression of CYP3A protein in LS180 and HepG2

The CYP3A protein response profile after exposure to rifampicin, CITCO or calcitriol was examined in LS180 and HepG2 using Western immunoblotting. As shown in figure 2, inducible expression of CYP3A protein was detected after exposure to rifampicin and calcitriol in LS180. CITCO did not increase CYP3A protein expression in LS180 cells. In HepG2 the protein expression of CYP3A was very low and was not induced after treatment with the prototypical nuclear receptor agonists rifampicin, CITCO and calcitriol (data not shown).

Effect of endogenously expressed nuclear receptors on CYP3A4 reporter activity

To evaluate the effect of endogenously expressed nuclear receptors on the CYP3A4 reporter gene activity, LS180 and HepG2 cells were transiently transfected with the CYP3A4 reporter construct and an “empty” nuclear receptor expression plasmid. The transfected cells were treated with the corresponding nuclear receptor
agonists. Treatment of LS180 with 100 nM calcitriol for 48 hours resulted in a major increase in CYP3A4 reporter activity (figure 3). In HepG2 also an increase in CYP3A4 reporter gene activity was observed after treatment with calcitriol, but it was much lower compared to the CYP3A4 reporter activity in LS180. This indicates that both cell lines express functional endogenous VDR. Exposure to rifampicin led to a small, but significant ($P<0.05$) increase in CYP3A4 reporter gene activity in LS180, but not in HepG2, indicating the presence of functional endogenous PXR in LS180. CITCO did not significantly increase CYP3A4 reporter activity in both LS180 and HepG2 compared to the vehicle 0.1% DMSO, which indicates that both cell lines do not express high levels of functional CAR.

CYP3A4 reporter gene activity in LS180 and HepG2

To examine the influence of the host cell line on the activity of the CYP3A4 reporter gene assay in the presence of exogenous PXR, CAR or VDR, LS180 and HepG2 were transiently transfected with the CYP3A4 reporter construct combined with PXR-, CAR- or VDR-expression plasmids, followed by exposure to different concentrations of the corresponding agonists. The CYP3A4 reporter activities in both LS180 and HepG2 as a result of nuclear receptor activation are shown in figure 4.

In LS180 rifampicin, CITCO and calcitriol were able to significantly increase CYP3A4 reporter gene activity compared to the control, while in HepG2 only rifampicin and calcitriol gave a significant increase in CYP3A4 reporter gene activity compared to the control. Moreover, the increase of PXR- and VDR-mediated CYP3A4 reporter activity following treatment with respectively rifampicin and calcitriol in HepG2 was significantly lower (~2 fold) compared to the increase of CYP3A4 reporter gene activity in LS180 after treatment with the same nuclear
receptor agonists. In contrast to rifampicin and calcitriol, CITCO only increased CAR-mediated CYP3A4 reporter gene activity in LS180 and not in HepG2.

Relationship between CYP3A4 protein expression and reporter gene activity

Statistical correlation analysis revealed a significant correlation between the protein expression and CYP3A4 reporter gene data in LS180. For PXR ($r^2=0.87$, $n=18$, $p<0.001$) and VDR ($r^2=0.86$, $n=18$, $p<0.001$) a good correlation was found, while for CAR ($r^2=0.04$, $n=27$, $p<0.31$) no correlation was found.
**Discussion**

In the present study, we explored the suitability of the colon carcinoma-derived LS180 cell line for its use in CYP3A4 induction studies compared to the hepatoma-derived HepG2 cell line. HepG2 is still the most widely used cell line in CYP3A regulation and induction studies. Although several groups have already shown that the HepG2 cell line expresses high levels of the fetal CYP3A enzyme CYP3A7 (Schuetz et al., 1993; Wilkening et al., 2003). In contrast, LS180 has been shown to express CYP3A4 (Kolars et al., 1992; Schuetz et al., 1996).

To evaluate the difference in CYP3A enzyme inducibility between HepG2 and LS180, both cells were challenged with the prototypical nuclear receptor agonists rifampicin, CITCO or calcitriol, and mRNA and protein expression were determined. mRNA analysis of the different CYP3A enzyme expression levels in both cell lines revealed that CYP3A4 and CYP3A5 were induced after treatment with rifampicin or calcitriol in LS180, while none of the CYP3A mRNA enzyme levels in HepG2 were induced after treatment with these agents. Protein expression analysis showed that the expression of CYP3A protein was inducible in LS180 only. The protein levels increased after treatment with the prototypical CYP3A4 inducers rifampicin and calcitriol. Although the antibody used could not discriminate between the CYP3A4 and CYP3A7 enzymes, it can be assumed that CYP3A4 protein expression levels in this cell line were induced based on the results of the mRNA analysis, which clearly show that only CYP3A4 mRNA expression levels are inducible following treatment with these compounds.

In HepG2, CYP3A protein was hardly detectable even after treatment with rifampicin, CITCO or calcitriol. This is consistent with the mRNA analysis data, which also show no CYP3A induction after treatment with the same compounds.
Furthermore, as mentioned before HepG2 expresses CYP3A7, which is not induced by rifampicin (Krusekopf et al., 2003; Usui et al., 2003). Krusekopf only observed pronounced CYP3A7 induction in HepG2 after treatment with typical human glucocorticoid receptor (hGR) agonists indicating an important role for the hGR in CYP3A7 regulation. Furthermore, the promoter sequence of the CYP3A7 gene has two mutations in the proximal ER6 repeat, which has implications for binding of nuclear receptors of the NR1I family (e.g. PXR, CAR, VDR). These mutations lead to less pronounced binding of liganded VDR to the CYP3A7 promoter resulting in a loss of gene activation after calcitriol treatment (Hara et al., 2004). Since PXR also recognizes and binds to ER6 repeats, the mutations may affect the binding of PXR to the CYP3A7 promoter as well, which could explain the lack of response to rifampicin treatment of HepG2 cells with respect to CYP3A7 induction.

The results discussed above clearly show that HepG2 and LS180 have distinct CYP3A induction profiles. We therefore evaluated if this alternate expression pattern also affects CYP3A4 reporter activity. Indeed, in the CYP3A4 reporter gene assay, both cell lines clearly showed distinct responses after treatment with the prototypical nuclear receptor agonists. Treatment of LS180 and HepG2 that were transfected with CYP3A4 reporter constructs without co-transfection of nuclear receptor expression plasmids, resulted in a significant ($p<0.05$) increase of reporter activity after treatment with rifampicin and calcitriol in the LS180 cell line. These results indicate that functional PXR and VDR are endogenously expressed in this cell line. The most pronounced effect on CYP3A4 reporter activity was found when both cell lines were co-transfected with the nuclear receptor expression plasmids of PXR or VDR and subsequently treated with the corresponding agonists. Comparison of the CYP3A4 reporter activities in LS180 and HepG2 cells after PXR or VDR activation
revealed that there was a significant difference in the increase of CYP3A4 reporter activity between the cell lines. The fold induction of CYP3A4 reporter activity in LS180 cells was about 2 fold higher for both nuclear receptors than the fold induction in HepG2.

The CYP3A4 reporter activities in the presence of PXR expression plasmid in LS180 showed a good correlation with CYP3A4 protein expression levels, which in turn were in concordance with CYP3A4 protein expression levels found in primary cultures of human hepatocytes that were treated with rifampicin (Hariparsad et al., 2004). In the case of calcitriol, however, the CYP3A4 protein levels were twice as high in LS180 as in human hepatocytes (Kolars et al., 1992). This difference is probably a result of the high endogenous expression of VDR in LS180. The high endogenous VDR expression may cause problems with respect to CYP3A4 reporter gene assays that are co-transfected with other nuclear receptors such as PXR, because PXR and VDR bind to the same response elements within the promoter region of the CYP3A4-luciferase construct. However, in contrast to the highly promiscuous PXR, VDR has a very narrow ligand specificity and only bile acids and vitamin D derivatives are known to activate this receptor (Makishima et al., 2002; Hara et al., 2004). Therefore, although endogenous VDR expression is high in LS180 cells, it still is a suitable cell line to study the effect of xenobiotics on CYP3A4 induction, as most compounds exert their effect on CYP3A4 expression through the more promiscuous PXR (Lehmann et al., 1998; Goodwin et al., 1999; El-Sankary et al., 2001).

In addition to PXR and VDR, CAR is also able to cross regulate CYP3A4 (Qatanani and Moore, 2005). Therefore the role of CAR activation on the induction of CYP3A4 was investigated. CAR is, just like PXR (Squires et al., 2004) and VDR (Racz and Barsony, 1999), located in the cytoplasm in vivo. In contrast to PXR and
VDR, CAR translocation is stimulated in a ligand-independent manner. Phenobarbital, phenytoin and bilirubin have been shown to trigger CAR nuclear translocation without binding to the LBD of CAR. Due to the constitutive activity of CAR, translocation results in transcriptionally activation of its target genes. In HepG2 cells it has been reported that CAR spontaneously translocates to the nucleus due to a lack of cytoplasmic CAR retention protein (CCRP) (Kobayashi et al., 2003). As a consequence, CAR transfection may automatically result in increased nuclear accumulation and enhanced transcription of its target genes (e.g. CYP3A4) in a ligand-independent manner due to its constitutive activity. This might explain the high ligand-independent CYP3A4 reporter activation in the HepG2 cell line after co-transfection with CAR. Co-transfection of CAR in the LS180 cell line also resulted in increased CYP3A4 reporter gene activity, but additional treatment with CITCO resulted in a dose-dependent increase of the CYP3A4 reporter gene activity. CITCO is the only known agonist of CAR, and causes transcriptional activation as a result of direct binding to the ligand binding domain of CAR. This triggers ligand-dependent nuclear accumulation and results in increased CYP3A4 reporter gene activity. As a consequence, LS180 cells could be used to evaluate the potential of compounds to bind directly to the LBD of CAR and subsequently activate transcription in a ligand-dependent manner. Currently, however, no validated systems are available to screen compounds for their capacity to activate CAR either ligand-dependently or independently.

In conclusion, there is a clear difference in the inducible protein expression of CYP3A between both cell lines. We clearly show that HepG2 is inferior to LS180 with respect to CYP3A4 induction. The alternate CYP3A enzyme expression and gene regulation in HepG2 compromises the use of this cell line for CYP3A4 induction.
studies. Based on our results we therefore recommend the use of the LS180 cell line to study CYP3A4 induction instead of the widely used HepG2.
References


Footnotes

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LEGENDS TO THE FIGURES

Figure 1 Inducible expression of CYP3A enzymes in HepG2 and LS180

The inducibility of the different CYP3A enzyme mRNA levels were determined after 2 × 24 hours treatment of the cells with 0.1% DMSO, rifampicin (1 µM and 10 µM), CITCO (10 nM and 250 nM), and calcitriol (1 nM and 100 nM). Total RNA was isolated and converted to cDNA, which was assessed at least in triplicate using singleplexed quantitative Taqman real-time PCR (CYP3A4, CYP3A5, CYP3A7) and normalized to 18S. The results are expressed as fold induction over the control (0.1% DMSO/EtOH). Statistical analysis on real-time PCR data were performed on mean dct values (and not fold changes) to exclude potential bias attributable to averaging data that had been transformed through the equation 2^(−ΔΔCt) (Quinkler et al., 2005) (significance * p<0.05, ** p<0.01, *** p<0.001).

Figure 2 CYP3A induction in LS180 after treatment with prototypical nuclear receptor agonists

Proteins (10µg) were separated using SDS-PAGE, transferred to Immobilon-P membrane and probed with mouse-antihuman CYP3A4/7 antibody. Blots were subjected to densitometric analysis. The level of induction is expressed as an increase in CYP3A protein levels. The results are derived from a representative experiment and data are the means ± SD from three separate determinations and are expressed as the relative induction compared to the vehicle (significance * p<0.05).

Figure 3 Effect of endogenous nuclear receptor expression on CYP3A4 reporter activity
LS180 and HepG2 cells were transfected with the CYP3A4-reporter construct and an empty nuclear receptor expression plasmid. Cells were treated for 48 hours with the nuclear receptor agonists rifampicin (RIF; 10 µM), CITCO (250 nM) or calcitriol (CAL; 100 nM). Only in LS180 calcitriol induced CYP3A4 reporter activity, which indicates that LS180 expresses high endogenous levels of VDR (significance * \( p<0.05 \)).

**Figure 4 Differential response in CYP3A4 reporter activity upon activation of three distinct nuclear receptors in LS180 and HepG2**

LS180 and HepG2 cells (2x10^5 cells/ml and 5x10^5 cells/well, respectively) were transfected with the pGL3-CYP3A4-XREM reporter construct, and the nuclear receptor expression vectors pCDG-hPXR, pEF-hCAR or pSG5-hVDR and the pRL-TK control vector. After 24 hours of transfection, cells were exposed to the corresponding nuclear receptor agonists serially diluted in DMSO (rifampicin: 1, 5, 10, 25 µM; CITCO: 10, 50, 100, 250 nM; calcitriol: 1, 10, 50, 100 nM) with a final solvent concentration of 0.1%. After 48 hours, luciferase activity was measured. These results are derived from a representative experiment and data are the means ± SD from three separate determinations and is expressed as absolute reporter activity (significance (* \( p<0.05 \))).
Figure 3

The figure shows a bar graph comparing the CYP3A4 reporter activity (in relative light units) across different treatments. The x-axis represents various treatments: CYP3A4 + 0.1% DMSO, CYP3A4 + 10 uM RIF, CYP3A4 + 250 nM CITCO, and CYP3A4 + 100 nM CAL. The y-axis represents the CYP3A4 reporter activity. The graph includes data points for LS180 and HepG2 cell lines, indicated by different symbols. Significant differences are indicated by asterisks.