

Generation of cytochrome P450 3A4-overexpressing HepG2 cell clones for standardization of hepatocellular testosterone 6 β -hydroxylation activity

N. Herzog¹, N. Katzenberger¹, F. Martin, K.-U. Schmidtke and J.-H. Küpper*

Faculty of Science, Brandenburg University of Technology Cottbus-Senftenberg, Germany

Abstract.

Detoxification of xenobiotics including drugs is catalyzed by liver phase I and phase II enzymes. There are three main families of phase I cytochrome P450 (CYP450) monooxygenases that introduce polar groups on drugs. These phase I metabolites can then be further conjugated by transferases during phase II reaction. Liver biotransformation can also lead to toxic drug metabolites, the most common cause of drug failure during clinical investigation. CYP3A4 is considered to be the most important enzyme in drug metabolism. Drug development relies on the use of human liver cells in order to investigate drug metabolism and potential toxicity. With primary human liver cells as the gold standard several problems have to be solved, *i.e.* scarcity of functional human liver tissue, donor variation of CYP activity and rapid dedifferentiation processes during primary cell cultivation. These features make it difficult to use primary human liver cells as standard to measure CYP activity. To avoid problems with primary human liver cells, many attempts have been undertaken to establish liver carcinoma cell lines, non-transformed proliferating human liver cell systems and induced pluripotent stem cell-derived hepatocytes. Due to different problems with these surrogate systems, the one cell line that could be used as convenient standard cell system to benchmark CYP3A4 enzyme activity has not been established yet.

Based on the widely used hepatocellular carcinoma cell line HepG2 and a lentiviral vector system, we generated cell clones for stable CYP3A4 overexpression. Here we present data on a new HepG2 cell clone (clone 9) showing higher than 10,000-fold overexpression of CYP3A4 compared to HepG2 parental cells. As measured by conversion of testosterone into 6 β -hydroxytestosterone, we found an enzyme activity of about 600 pmol per minute per mg total cellular protein, which ranges at the upper end reported for primary human liver cells. This enzyme activity appeared to be kept stable in clone 9 cells, because there was no influence detectable when cells were treated with 5-azacytidine, a drug that interferes with epigenetic silencing processes. Prototypic CYP3A4 inducer rifampicin led to significant increase of CYP3A4 testosterone hydroxylase activity in HepG2 clone 9 cells. Altogether, HepG2 clone 9 strongly and stably overexpressed CYP3A4 leading to a physiological enzyme activity, which apparently was unaffected by epigenetic processes. Thus, HepG2 clone 9 could be a useful reference cell clone for CYP3A4 enzyme activity.

Keywords: 5-azacytidine, biotransformation, CYP3A4, drug development, HepaRG, hepatocytes, HepG2, rifampicin, testosterone, Upcytes

¹Both authors contributed equally to this work.

*Corresponding author: Jan-Heiner Küpper, Faculty of Science, Brandenburg University of Technology Cottbus–Senftenberg, Großenhainer Str. 57, 01968 Senftenberg, Germany. Tel.: +49 3573/ 85 930; Fax: +49 3573/ 85 809; E-mail: jan-heiner.kuepper@b-tu.de.

Abbreviations

Aza	5-azacytidine
CMV	cytomegalovirus
CYP	cytochrome P450
6 β -OH-T	6 β -hydroxytestosterone
KHB	Krebs-Henseleit-Buffer
qRT-PCR	quantitative real-time PCR
Rif	rifampicin

1. Introduction

Hepatotoxicity is the most common cause of drug failure during clinical investigation. Six different cytochrome P450 (CYP) isoenzymes, *i.e.* CYP1A2, -2C9, -2C19, -2D6, -2E1 and -3A4 are responsible for more than 90% of oxidative biotransformation activity on human drugs [2, 26]. Enzymes of the CYP3A family are of particular importance as they alone are involved in the metabolism of more than 50% of human drugs and contribute to 30–60% of the total liver content of CYP enzymes [2, 26, 31]. CYP3A4 is the most important enzyme of the CYP3A family concerning drug metabolism in adult human liver.

Conversion of testosterone to 6 β -hydroxytestosterone is generally accepted to prototypically determine enzyme activity of CYP3A4. In order to predict biotransformation of drugs by CYP enzymes, freshly isolated primary human hepatocytes are considered to be the gold standard. However, overall CYP enzyme activity rapidly drops down by up to 90% during the first two days of cell culture (reviewed in: [5]). In addition, CYP3A protein amount can differ between individual donors by a factor of at least 20-fold [27]. Both features may explain that the reported spectrum of CYP3A4 activities varies between less than 1 pmol (metabolite formation) \times min⁻¹ \times mg⁻¹ (total cellular protein) [30] to higher than 700 pmol \times min⁻¹ \times mg⁻¹ (total cellular protein) [8, 9].

To circumvent the scarcity and donor variation of primary human hepatocytes, hepatocellular carcinoma cell lines such as HepG2 and HepaRG are widely used [8, 12, 14]. Since CYP3A4 expression in HepG2 cells is strongly down-regulated compared to primary hepatocytes, there have been attempts to overexpress human CYP3A4 cDNA in HepG2 cells. This has been accomplished by using stable plasmid transfection [6], or transient CYP3A4 overexpression with adenoviral vectors [13, 29]. In both cases, the transgene was controlled by the strong cytomegalovirus promoter. Surprisingly, also these groups reported varying CYP3A4 activities in those genetically engineered HepG2 cells ranging between 7–600 pmol \times min⁻¹ \times mg⁻¹ (total cellular protein). While recombinant adenovirus infection normally results in transient overexpression of the gene of interest and its gradual loss over several population doublings, plasmid transfection followed by antibiotic selection can lead to stable chromosomal integration of the gene of interest. However, stably integrated plasmid cDNAs controlled by the cytomegalovirus promoter are often epigenetically silenced [11]. Thus, both approaches may lead to unpredictable and varying CYP3A4 expression levels and enzyme activities.

In comparison, differentiated HepaRG cells have been reported to display basal CYP3A4 activity between 15–30 pmol \times min⁻¹ \times mg⁻¹ [1, 8], which is at the lower limit of primary human hepatocytes. In case of treating differentiated HepaRG cells with CYP3A4 inducer rifampicin, CYP3A4-mediated conversion of testosterone can be induced up to values of 800–1200 pmol \times min⁻¹ \times mg⁻¹ [8, 17].

Furthermore, it has been reported by several groups that hepatocyte-like cells can be differentiated from induced pluripotent stem cells. It appears, however, that current technology on iPS-derived hepatocytes leads to embryonic rather than fully differentiated hepatocytes [19]. Along with this, CYP3A4 enzyme activities in the range of $1 \text{ pmol} \times \text{min}^{-1} \times \text{mg}^{-1}$ protein were observed [30], which is at the lowest limit of adult human hepatocytes. Interestingly, it could be shown that iPS-derived human hepatocytes co-cultured *in vitro* with endothelial cells and mesenchymal stem cells to form liver buds, further differentiate into a mature phenotype upon liver bud transplantation into mice [28].

For many of these studies a benchmark cell line could help to classify such new hepatocyte systems with respect to CYP3A4 enzyme activity independent of primary hepatocyte properties like donor variation or cultivation time.

In order to meet the requirement for a convenient and reliable cell culture system to standardize human CYP3A4 enzyme activity by using its testosterone hydroxylase activity, we generated novel HepG2 cell clones with CYP3A4 overexpression leading to stable enzyme activities in the physiological range of human primary hepatocytes.

2. Materials and methods

2.1. Cell culture and generation of CYP3A4-overexpressing HepG2 clones

Human hepatocellular carcinoma (HepG2) cells (ATCC: HB-8065) were routinely cultivated in growth medium, *i.e.* Dulbecco's MEM medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 2 mM L-Alanyl-L-Glutamine (Biochrom AG, Berlin, Germany) at 37°C and 5% CO₂ in a humidified incubator.

To generate CYP3A4-overexpressing HepG2 clones, human CYP3A4 cDNA (NCBI reference sequence: NM.017460) was subcloned into a lentiviral expression vector (Life Technologies GmbH, Darmstadt, Germany). In this vector, cDNA expression is controlled by the human cytomegalovirus promoter. Recombinant lentiviruses were generated according to standard methods and used to infect HepG2 cells. Infected cells were selected with 3 µg/ml blasticidin (PAA, GE Healthcare, Austria,) to allow for the formation of recombinant cell clones. Clones were isolated and individually screened for CYP3A4 overexpression by using qRT-PCR and immunofluorescence as described below. In order to maintain stable CYP3A4 expression, clones were cultivated in growth medium supplemented with blasticidin.

2.2. Spheroid cultures of HepG2-CYP3A4 clone 9

Three-dimensional cultures of HepG2 CYP3A4-overexpressing clone 9 were performed in a cell reactor (cellstar® Greiner, Kremsmünster; Austria). Cells (2×10^6) were seeded into the cell reactor and cultured in growth medium with blasticidin at 37°C and 5% CO₂ with shaking at 50 rpm for 2 weeks. This allows for the organization of multicellular spheroids. Subsequently, spheroids were prepared for cryosection (Microm HM 560, Thermo Fisher Scientific, Dreieich, Germany) as described below.

2.3. Immunocytochemistry

For immunocytochemical analysis, cells were grown in 96-well plates for 24 hours. Cells were fixed with -20°C cold methanol for 2 min followed by washing with phosphate-buffered saline (PBS) and

Table 1
Primer sequences for qRT-PCR

Primer name	Sequence	Amplicon size (bp)
GAPDH Forward	TGCACCACCAACTGCTTAGC	87
GAPDH Reverse	GGCATGGACTGTGGTCATGAG	
CYP3A4 Forward	GTGGGGCTTTTATGATGGTCA	272
CYP3A4 Reverse	GCCTCAGATTTCTACCAACACA	

blocking with 3% bovine serum albumin (BSA, *w/v*) in PBS at room temperature for 20 min. Antibodies were diluted in 0.2% (*w/v*) BSA in PBS. Primary antibodies were incubated at 37°C for 60 min, and secondary antibodies were applied at 37°C for 30 min, respectively. Finally, cells were analyzed by CKX41 fluorescence microscope (Olympus, Tokyo, Japan) equipped with Color View II camera (Olympus, Tokyo, Japan). Primary rabbit anti-human CYP3A4 antibody was obtained from Biomol GmbH (Hamburg, Germany). Secondary goat anti-rabbit antibody (Dianova, Hamburg, Germany) was Cy3-conjugated.

2.4. Immunohistochemistry analysis of spheroids

Spheroids generated as described above were embedded in Neg-50TM frozen section medium (Thermo Fisher Scientific). Cryosections (9 μm) were prepared by MICROM HM560 microtome. Slices were fixed with 4% (*v/v*) formalin at 4°C for 10 min, followed by treatment with –20°C methanol/acetone (1:1) for 10 min. Subsequently, antibody incubation and immunofluorescence analysis were performed as described above.

2.5. Quantitative real-time PCR

Total RNA from 1×10^6 cells was extracted with peqGOLD TriFastTM extraction buffer (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. 2 μg of isolated total RNA were used for reverse transcription reaction with RevertAid H minus reverse transcriptase (Thermo Fisher Scientific). cDNA templates were processed for gene expression analysis by using the primers shown in Table 1 and iQTM5 Thermo cycler (Bio-Rad, Hercules, USA). All data were normalized to GAPDH. Values were expressed as fold change of controls.

2.6. Microsomal assay

200 μl reaction mixture containing 0.5 mg/ml human liver microsomal protein (prepared microsomes pooled from 50 different individual donors, Life Technologies), 1 mM NADPH and 250 μM testosterone in 100 mM sodium phosphate buffer were incubated at 37°C for 2 h. Reactions were terminated by addition of 200 μl acetonitrile. The samples were analyzed with HPLC as described below.

2.7. Analysis of drug metabolites

For analysis of substrate conversion capability of HepG2-CYP3A4 clones, 0.5×10^6 cells per well were seeded into 24-well plates. After 24 hours in culture, Krebs Henseleit buffer containing 250 μM

testosterone (Applichem, Darmstadt, Germany) was added. Drug incubation was performed for 2 h at 37°C in the routine cell culture incubator. Supernatants were used for HPLC and LC-MS analysis. HPLC analysis of testosterone and its respective metabolites was accomplished with a VWR-Hitachi Elite LaChrom series HPLC (VWR International GmbH, Darmstadt, Germany) with photodiode array L-2455 (column: Phenomenex Kinetex C18, 4.6 × 150 mm, 5 μm particle size, 100 Å; security guard CK18, 4.6 × 2 mm). Eluted substances were detected in the wavelength range 210–400 nm. Reaction products were identified, relative to authentic standards, based on their retention times, UV absorption spectra, and mass spectra $[M+H]^+$. Quantification of the compounds was carried out with calibration curves at corresponding $\lambda = 245$ nm. HPLC method: injection volume 20 μl; flow rate 1.0 ml/min; column temperature 25°C; mobile phase A 10 mM KH_2PO_4 pH3; mobile phase B acetonitrile; HPLC gradient profile: 0–3 min eluent B 30%, 20.5 min eluent B 55%, 22.5 min eluent B 55%, 23 min eluent B 95%, 26 min eluent B 95%, 26.5 min eluent B 20%, and 29 min eluent B 20%. LC-MS analysis was carried out on a Waters alliance HPLC system with ZMD SingleQuad-MS (Waters GmbH, Eschborn, Germany; ESI+; cone voltage: 30 eV; column: Phenomenex Luna C18 [2] 2 × 150 mm, 5 μm particle size, 100 Å; security guard: C18 [2], 4 × 3 mm). 6β-hydroxytestosterone and androstenedione (both from Sigma-Aldrich, St. Louis, USA) served as standards. For the determination of the enzyme activity related to total cellular protein, cells were lysed using extraction buffer (0.1% SDS (w/v); 1 mM PMSF; 62.5 mM Tris-HCl; pH 6.8). Supernatants were assayed for protein amounts using the Pierce BCA protein assay kit (Thermo Fisher Scientific p/a Perbio Science, Bonn, Germany).

2.8. Rifampicin treatment

Cells (0.5×10^6) were seeded into a 24-well plate and incubated overnight in medium. Thereafter, 50 μM rifampicin (Sigma-Aldrich, St. Louis, USA) in routine medium was added for three days with medium change every day. After rifampicin incubation time was finished, CYP3A4 activity was determined by performing 250 μM testosterone incubation in Krebs Henseleit buffer for 2 hours and HPLC analysis as described above.

2.9. Treatment with 5-azacytidine

To rescue possible epigenetically silencing of the cytomegalovirus promoter, experiments with 5-azacytidine (Sigma-Aldrich, St. Louis, USA) were performed. Cells (0.5×10^6) were seeded into a 24-well plate and incubated in medium for overnight. Thereafter, medium was exchanged by medium containing 0–15 μM 5-azacytidine. 5-azacytidine treatment was performed for three days with daily medium change. After incubation time was finished, CYP3A4 activity was determined by performing 250 μM testosterone incubation in Krebs Henseleit buffer for 2 hours and HPLC analysis as described above.

3. Results

By using a lentiviral vector system for CYP3A4 overexpression and human hepatoma cell line HepG2, we intended to generate a reliable and convenient cell system to benchmark this enzyme activity.

In comparison to the parental cell line, we focused on cell clones showing (I) clear-cut increase of CYP3A4 expression, (II) an unchanged phenotype with respect to morphology and proliferation

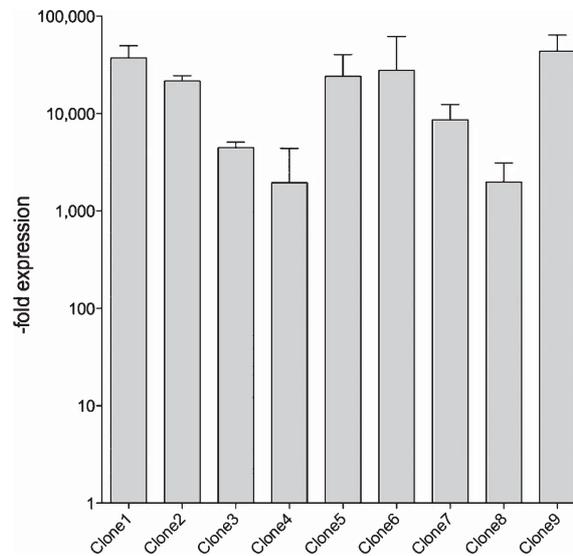


Fig. 1. Gene expression analysis of CYP3A4 in recombinant HepG2 clones. Subconfluent HepG2 cells and clones derived thereof were lysed and processed for qRT-PCR as described in Materials and Methods. CYP3A4 cDNA-specific primers were used, data were normalized to GAPDH housekeeping gene expression. Mean values and standard deviations from three experiments were calculated.

characteristics, (III) testosterone hydroxylase activities in the physiological range of primary human hepatocytes, and (IV) reproducible enzyme activities.

Following recombinant lentivirus infection of HepG2, we isolated blasticidin-resistant clones and analyzed them for CYP3A4 overexpression using quantitative real-time PCR. Figure 1 shows that clones 1–9 displayed levels of CYP3A4 expression, which were several orders of magnitude higher than those of parental HepG2 cells. Since clone 9 displayed the highest CYP3A4 gene expression, it was utilized

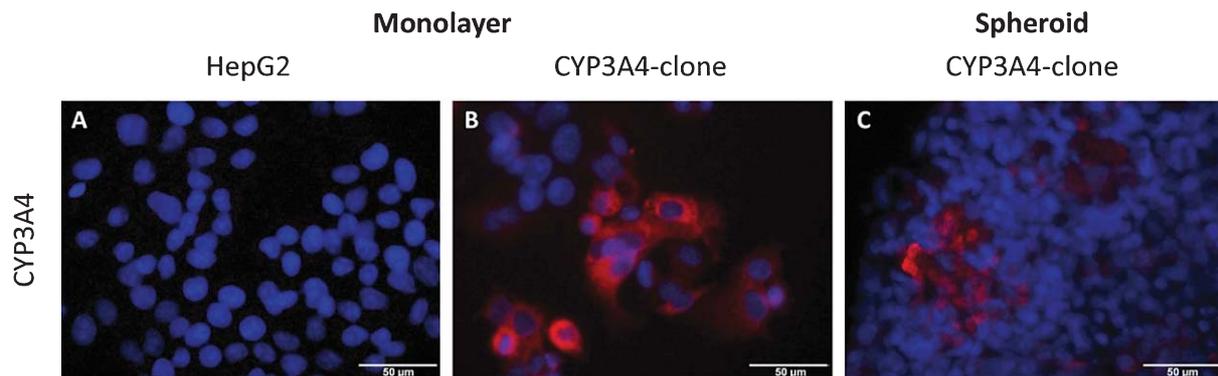


Fig. 2. Immunofluorescence analysis of CYP3A4 overexpression in HepG2 clone 9. Cells were grown as monolayers or spheroids as described in the Materials and Methods section. Upon fixation for monolayer cells, or cryostat sectioning and fixation for spheroids, samples were stained with anti-CYP3A4 antibody and Cy3-conjugated secondary antibodies (red). Nuclei were stained with DAPI (blue). (A) HepG2 parental cells; (B) HepG2 CYP3A4-expressing clone 9 as monolayer; (C) clone 9 spheroid. Scale bar 50 μ m.

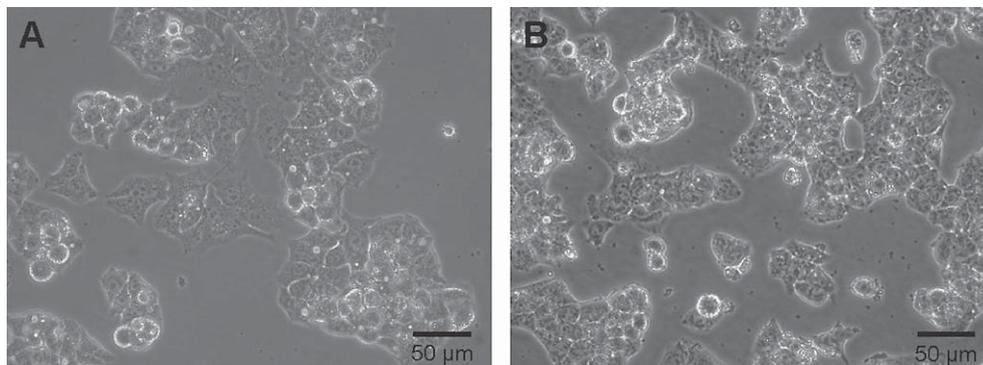


Fig. 3. Cell morphology of HepG2 clone 9 is not different from parental HepG2. Phase contrast microscopy of HepG2 cells and clone 9 cultivated as monolayers to semi-confluence. (A) HepG2 cells (B) HepG2-3A4 clone 9 cultivated in the presence of blasticidin. Scale bar 50 μ m.

for following experiments. By using indirect immunofluorescence, we found CYP3A4 overexpression, which is even more pronounced in monolayer cell cultures of clone 9 than in cells that were cultivated to form spheroids (3D cell cultures). By contrast, there was no endogenous CYP3A4 expression detectable in parental HepG2 cells (Fig. 2).

Apparently, stable and constitutive overexpression of CYP3A4 in clone 9 did not affect cell morphology. HepG2 clone 9 showed the same cobblestone-like morphology and formation of cell agglomerates than parental cell line HepG2 (Fig. 3). Cultivation of clone 9 in medium with blasticidin increased the population doubling time to 35 hours compared to 27 hours for HepG2 parental cells, which were cultivated in the absence of any antibiotics. Both, HepG2 and HepG2 clone contain about 5% of cells in S-phase (data not shown). Thus, the cell cycle was slightly prolonged in clone 9, most likely due to the blasticidin effect, but the ratio of proliferating cells remained unaffected.

Furthermore, we tested whether this strong overexpression of CYP3A4 results in measurable enzyme activity in these cells. To measure CYP3A4 activity, conversion of testosterone into 6 β -hydroxytestosterone is widely used as standard reaction. In addition to this testosterone hydroxylase activity of CYP3A4, testosterone is also metabolized into androstenedione by other CYP enzymes (Fig. 4). As demonstrated in Fig. 5A, we found that CYP3A4 overexpression indeed led to a stable and reproducible enzyme activity of about 600 pmol \times min⁻¹ \times mg⁻¹ (total cellular protein), which is at the upper range known for human primary hepatocytes. In case of parental HepG2 cells, endogenous CYP3A4 activity was below our detection limit. Since recombinant CYP3A4 cDNA is under transcriptional control of the human cytomegalovirus promoter / enhancer (CMV promoter), which is prone to epigenetic down-regulation, we were interested to see whether enzyme activity can be further increased by using the substance 5-azacytidine. As shown in Fig. 5, treatment with 5-azacytidine for 72 h did not lead to a significant increase of CYP3A4 enzyme activity. Apparently, there was no epigenetic influence on recombinant CYP3A4 expression in HepG2 clone 9.

We further compared total cellular CYP3A4 activity with that of microsomes, subcellular membrane preparations enriched for CYP enzymes. Microsomes are often employed to reference CYP3A4 activity. Due to a relative increase of CYP protein versus cellular background protein, commercially available microsomes showed higher CYP3A4 enzyme activities than CYP3A4-overexpressing HepG2 clone 9. However, as is evident from Fig. 5A, microsomal CYP3A4 activity was reduced by about 50% with only one additional cycle of freeze-thawing.

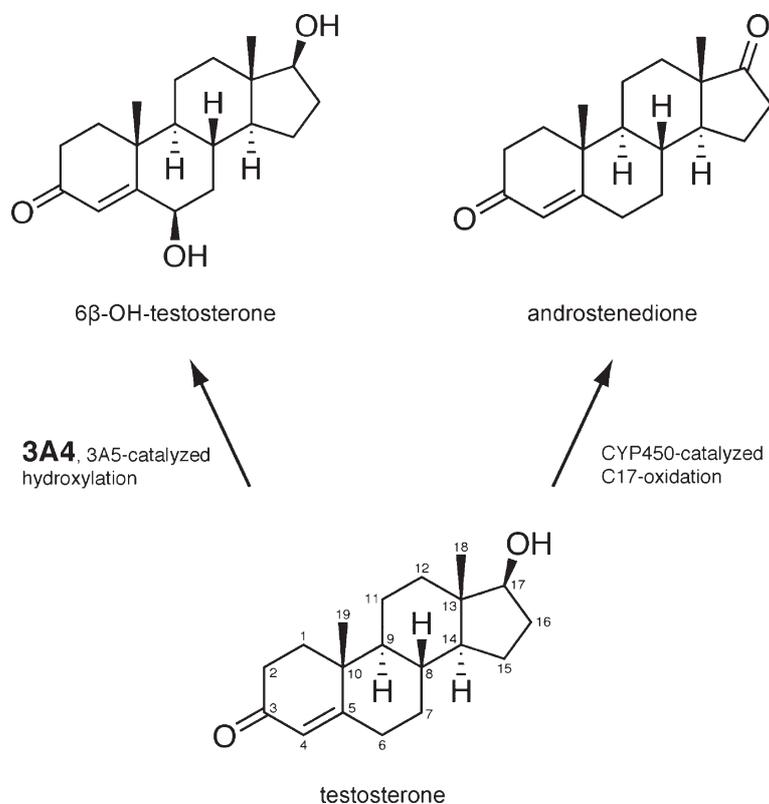


Fig. 4. 6β-hydroxytestosterone is the main biotransformation product of testosterone. Steroid hormone testosterone is involved in carbohydrate, fat and protein metabolism. In human liver, the predominant metabolite of testosterone is 6β-hydroxytestosterone, mainly produced by CYP3A4. Androstenedione formation and other testosterone hydroxylations are catalyzed by various CYP enzymes, but normally occur at much lower ratio than the main product.

Since CYP3A4 overexpression in HepG2 clone 9 is controlled by a heterologous promoter (*i.e.* CMV promoter), it would have been expected that prototypic CYP3A4 inducer rifampicin would have no effect on recombinant CYP3A4 enzyme activity. Interestingly, we found significant increase of this enzyme activity if HepG2 clone 9 cells were exposed to rifampicin (Fig. 5B). By contrast, we were not able to measure any CYP3A4 activity in parental HepG2 cells treated with rifampicin or with 5-azacytidine, as mentioned above. Thus, observed increase of CYP3A4 activity in rifampicin-treated clone 9 very likely was caused by exogenous rather than endogenous CYP3A4.

4. Discussion

During drug development, primary human hepatocytes are widely used to identify drug metabolites and to investigate possible liver toxicity. Because of the primary human hepatocyte scarcity, substantial donor variation and rapid dedifferentiation processes of primary hepatocytes during their cultivation, many attempts have been undertaken to establish substitutes for those cells. These are new hepatoma cell lines with improved features such as HepaRG cells [1, 12, 21], the Hepatocyte upcytes[®] of Medicyte, *i.e.* non-transformed proliferating human liver cells [3, 24], or new approaches to improve the

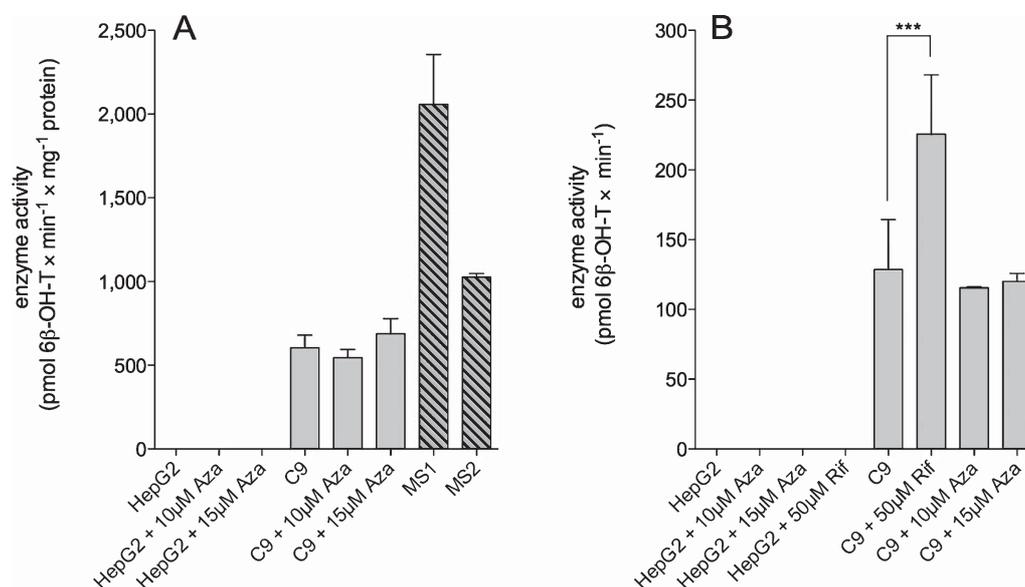


Fig. 5. CYP3A4 testosterone hydroxylation activity in HepG2 clone 9 is unaffected by 5-azacytidine, but can be raised by rifampicin. 0.5×10^6 HepG2 or clone 9 (C9) cells were seeded into 24-well plates and cultivated for three days in the presence or absence of 5-azacytidine (Aza) or rifampicin (Rif) as indicated. Medium was changed by Krebs Henseleit buffer containing 250 μ M testosterone and incubated for two hours to produce the main metabolite 6 β -hydroxytestosterone (6 β -OH-T). In parallel, CYP3A4 testosterone hydroxylase activity was assayed with microsomal preparations that were used directly (MS1) or subjected to one additional cycle of freeze-thawing (MS2). Substrate and metabolite analyses were performed as described in Materials and Methods. (A) CYP3A4 enzyme activity normalized to total cellular or microsomal protein content by BCA assay; (B) CYP3A4 enzyme activity per well. Mean values and standard deviations from independent experiments, each with triplicates, were calculated. Student's *t*-test compares C9 cells with and without 50 μ M rifampicin treatment as indicated; ***, $p < 0.001$.

differentiation characteristics of iPS-derived hepatocytes by the formation of liver buds [28]. One of the basic features these primary hepatocyte substitutes should meet, is their biotransformation activity, *i.e.* a physiological spectrum of phase I and phase II reactions, including CYP3A4 enzyme activity. In order to compare CYP3A4 enzyme activities obtained with these cell systems, we suggest using the new HepG2-3A4 clone 9 described here as reference cell line. As we show here, HepG2 clone 9 stably and reproducibly overexpress CYP3A4 leading to enzyme activities at the upper range reported for primary human hepatocytes [9].

It is well known that methylation processes at CpG islands within transcriptionally active regions of genomic DNA can lead to epigenetic silencing of gene expression [4, 16]. The human cytomegalovirus promoter / enhancer is widely used in gene expression studies and can be gradually down-regulated by such processes [18, 20, 23]. We were thus interested to see whether recombinant CYP3A4 enzyme activity can be increased by interfering with possible epigenetic silencing of the heterologous CMV promoter. We employed the epigenetic modifier substance 5-azacytidine, a cytosine analogue that is incorporated into the DNA of proliferating cells and by this acts as an inhibitor of DNA methyltransferases. This drug is already used in cancer therapy to rescue the expression of silenced tumor suppressor genes [15, 22, 25]. Also cultivated cells that express a gene of interest leading to some cytotoxicity can down-regulate heterologous promoter activity by methylation at CpG islands and subsequent conversion of euchromatin into heterochromatin. Ectopic gene expression can thus be rescued by 5-azacytidine, which interferes

with this methylation process [11]. From our experiments we conclude that there is no such silencing process in recombinant CYP3A4-overexpressing HepG2 clone 9, because we did not find an increase of enzyme activity following 5-azacytidine treatment. By contrast, we recently could find in a stable cell clone of larynx carcinoma cell line HEp-2 that recombinant CMV promoter-driven expression of muscle specific kinase, a protein targeted by autoantibodies in Myasthenia gravis, is strongly down-regulated over several passages. This protein expression can be rescued by using an epigenetic substance mixture [7]. Apparently, CYP3A4 overexpression did not exhibit such a cytotoxic effect and thus enzyme activity kept stable in HepG2 clone 9.

In contrast to 5-azacytidine, rifampicin treatment did increase recombinant CYP3A4 enzyme activity in clone 9 significantly (Fig. 5B). Rifampicin is widely used as the prototypical inducer of CYP3A4 transcription and subsequent increase of enzyme activity. It was found that this inducing effect of rifampicin is mediated by pregnane X receptor acting on the endogenous CYP3A4 promoter [10]. We don't know the mechanism by which rifampicin could lead to induction of enzyme activity in a heterologous expression system based on the cytomegalovirus promoter. To our knowledge, such an effect has not been described yet.

Altogether, HepG2 clone 9 strongly and stably expressed CYP3A4 leading to a physiological enzyme activity, which apparently was unaffected by epigenetic processes. Thus, HepG2 clone 9 meets all criteria defined above to be a reference cell clone for CYP3A4 enzyme activity.

Acknowledgments

This work was funded by Ministerium für Arbeit, Soziales, Frauen und Familie, Land Brandenburg, Germany, and of Europäischer Sozialfonds (ESF 1398125).

References

- [1] Antherieu S, Chesne C, Li R, Camus S, Lahoz A, Picazo L, Turpeinen M, Tolonen A, Uusitalo J, Guguen-Guillouzo C, Guillouzo A. Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. *Drug Metab Dispos* 2010;38:516-25.
- [2] Arimoto R. Computational models for predicting interactions with cytochrome p450 enzyme. *Curr Top Med Chem* 2006;6:1609-18.
- [3] Burkard A, Dahn C, Heinz S, Zutavern A, Sonntag-Buck V, Maltman D, Przyborski S, Hewitt NJ, Braspenning J. Generation of proliferating human hepatocytes using upcyte® technology: Characterisation and applications in induction and cytotoxicity assays. *Xenobiotica* 2012;42(10):939-56.
- [4] Deaton AM, Bird A. CpG islands and the regulation of transcription. *Genes Dev* 2011;25:1010-22.
- [5] Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, Rogiers V. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Curr Drug Metab* 2006;7:629-60.
- [6] Frederick DM, Jacinto EY, Patel NN, Rushmore TH, Tchao R, Harvison PJ. Cytotoxicity of 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT) and analogues in wild type and CYP3A4 stably transfected HepG2 cells. *Toxicol In Vitro* 2011;25:2113-9.
- [7] George S, Paulick S, Knutter I, Rober N, Hiemann R, Roggenbuck D, Conrad K, Kupper JH. Stable expression of human muscle-specific kinase in HEp-2 M4 cells for automatic immunofluorescence diagnostics of myasthenia gravis. *PLoS One* 2014;9:e83924.
- [8] Gerets HH, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, Atienzar FA. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biol Toxicol* 2012;28:69-87.

- [9] Gomez-Lechon MJ, Donato MT, Castell JV, Jover R. Human hepatocytes in primary culture: The choice to investigate drug metabolism in man. *Curr Drug Metab* 2004;5:443-62.
- [10] Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 1999;56:1329-39.
- [11] Grassi G, Maccaroni P, Meyer R, Kaiser H, D'Ambrosio E, Pascale E, Grassi M, Kuhn A, Di NP, Kandolf R, Kupper JH. Inhibitors of DNA methylation and histone deacetylation activate cytomegalovirus promoter-controlled reporter gene expression in human glioblastoma cell line U87. *Carcinogenesis* 2003;24:1625-35.
- [12] Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F, Guguen-Guillouzo C. The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem Biol Interact.* 2007;168:66-73.
- [13] Hosomi H, Fukami T, Iwamura A, Nakajima M, Yokoi T. Development of a highly sensitive cytotoxicity assay system for CYP3A4-mediated metabolic activation. *Drug Metab Dispos* 2011;39:1388-95.
- [14] Jennen DG, Magkoufopoulou C, Ketelslegers HB, van Herwijnen MH, Kleinjans JC, van Delft JH. Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. *Toxicol Sci* 2010;115:66-79.
- [15] Johnson AA, Akman K, Calimport SR, Wuttke D, Stolzing A, de Magalhaes JP. The role of DNA methylation in aging, rejuvenation, and age-related disease. *Rejuvenation Res* 2012;15:483-94.
- [16] Jones PA. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012;13:484-492.
- [17] Josse R, Aninat C, Glaise D, Dumont J, Fessard V, Morel F, Poul JM, Guguen-Guillouzo C, Guillouzo A. Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. *Drug Metab Dispos* 2008;36:1111-8.
- [18] Kamensek U, Sersa G, Vidic S, Tevz G, Kranjc S, Cemazar M. Irradiation, cisplatin, and 5-azacytidine upregulate cytomegalovirus promoter in tumors and muscles: Implementation of non-invasive fluorescence imaging. *Mol Imaging Biol* 2011;13:43-52.
- [19] Kondo Y, Iwao T, Nakamura K, Sasaki T, Takahashi S, Kamada N, Matsubara T, Gonzalez FJ, Akutsu H, Miyagawa Y, Okita H, Kiyokawa N, Toyoda M, Umezawa A, Nagata K, Matsunaga T, Ohmori S. An efficient method for differentiation of human induced pluripotent stem cells into hepatocyte-like cells retaining drug metabolizing activity. *Drug Metab Pharmacokinet* 2014;29:237-43.
- [20] Kong Q, Wu M, Huan Y, Zhang L, Liu H, Bou G, Luo Y, Mu Y, Liu Z. Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs. *PLoS One* 2009;4:e6679.
- [21] Lubberstedt M, Muller-Vieira U, Mayer M, Biemel KM, Knospel F, Knobloch D, Nussler AK, Gerlach JC, Zeilinger K. HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment *in vitro*. *J Pharmacol Toxicol Methods* 2011;63:59-68.
- [22] Lund K, Cole JJ, VanderKraats ND, McBryan T, Pchelintsev NA, Clark W, Copland M, Edwards JR, Adams PD. DNMT inhibitors reverse a specific signature of aberrant promoter DNA methylation and associated gene silencing in AML. *Genome Biol* 2014;15:406.
- [23] Mehta AK, Majumdar SS, Alam P, Gulati N, Brahmachari V. Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. *Gene* 2009;428:20-24.
- [24] Norenberg A, Heinz S, Scheller K, Hewitt NJ, Braspenning J, Ott M. Optimization of upcyte(R) human hepatocytes for the *in vitro* micronucleus assay. *Mutat Res* 2013;758:69-79.
- [25] Rajendran G, Shanmuganandam K, Bendre A, Muzumdar D, Goel A, Shiras A. Epigenetic regulation of DNA methyltransferases: DNMT1 and DNMT3B in gliomas. *J Neurooncol* 2011;104:483-94.
- [26] Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: Studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* 1994;270:414-23.
- [27] Snawder JE, Lipscomb JC. Interindividual variance of cytochrome P450 forms in human hepatic microsomes: Correlation of individual forms with xenobiotic metabolism and implications in risk assessment. *Regul Toxicol Pharmacol* 200;32:200-9.
- [28] Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang RR, Ueno Y, Zheng YW, Koike N, Aoyama S, Adachi Y, Taniguchi H. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* 2013;499:481-4.
- [29] Tolosa L, Gomez-Lechon MJ, Perez-Cataldo G, Castell JV, Donato MT. HepG2 cells simultaneously expressing five P450 enzymes for the screening of hepatotoxicity: Identification of bioactivable drugs and the potential mechanism of toxicity involved. *Arch Toxicol* 2013;87:1115-27.

- [30] Ulvestad M, Nordell P, Asplund A, Rehnstrom M, Jacobsson S, Holmgren G, Davidson L, Brolen G, Edsbagge J, BJORQUIST P, KUPPERS-MUNTER B, ANDERSSON TB. Drug metabolizing enzyme and transporter protein profiles of hepatocytes derived from human embryonic and induced pluripotent stem cells. *Biochem Pharmacol* 2013;86:691-702.
- [31] WRIGHTON SA, SCHUETZ EG, THUMMEL KE, SHEN DD, KORZEKWA KR, WATKINS PB. The human CYP3A subfamily: Practical considerations. *Drug Metab Rev* 2000;32:339-61.