

Blood monocyte derived Neo-Hepatocytes as *in vitro* test system for drug-metabolism

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Neo-Hepatocytes as alternative in vitro drug testing system

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Abbreviations:

AHMC, 3-(2-(N,N-diethylamino)ethyl)-7-hydroxy-4-methylcoumarin

AMMC, 3-(2-N, N-diethyl-N-methylaminoethyl)-7-methoxy-4-methylcoumarin

BFC, 7-Benzoyloxy-4-(trifluoromethyl)coumarin

CEC, 3-Cyano-7-ethoxycoumarin

CHC, 3-Cyano-7-hydroxycoumarin

CYP, Cytochrome P450

DBF, Dibenzylfluorescein

EFC, 7-Ethoxy-4-(trifluoromethyl)coumarin

EPHX1, microsomal epoxide hydrolase 1

GST, glutathione-S-transferase

HC, 7-Hydroxycoumarin

HFC, 7-Hydroxy-4-(trifluoromethyl)coumarin

hrFGF4, human recombinant fibroblast growth factor 4

hrIL-3, human recombinant interleukin 3 (hrIL-3)

hrM-CSF, human recombinant macrophage colony-stimulating factor

KET, Ketoconazole

3-MC, 3-Methylcholanthrene

MCB, Monochlorobimane

MFC, 7-Methoxy-4-(trifluoromethyl)coumarin

n, number of replicates

N, number of individual experiments/donors

NAT1, N-acetyltransferase 1

NIF, Nifedipine

NMO, NAD(P)H menadione oxidoreductase 1

PBMC, peripheral blood monocytes

PCMO, programmable cell of monocytic origin

QUE, Quercetin dehydrate

RES, Resorufin

RIF, Rifampicin

SRB, Sulforhodamine B

SULT1, Sulfotransferase 1A1

UGT1A6, UDP-glucuronosyltransferase 1A6

VER, Verapamil

Abstract

The gold standard for human drug metabolism studies is primary hepatocytes. However, availability is limited by donor organ scarcity. Therefore, efforts have been made to provide alternatives, e.g. the hepatocyte-like (NeoHep) cell type, which was generated from peripheral blood monocytes (PBMCs). In this study, expression and activity of phase I and phase II drug-metabolizing enzymes were investigated during trans-differentiation of NeoHep cells and compared to primary human hepatocytes. Important drug metabolizing enzymes are cytochrome P450 (CYP) iso-forms (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 3A4), microsomal epoxide hydrolase 1 (EPHX1), glutathione-S-transferase (GST) A1 and M1, N-acetyltransferase 1 (NAT1), NAD(P)H menadione oxidoreductase 1 (NMO1), Sulfotransferase 1A1 (SULT1A1) and UDP-glucuronosyltransferase 1A6 (UGT1A6). Monocytes and programmable cells of monocytic origin (PCMOs) expressed only a few of the investigated enzymes. Throughout differentiation, NeoHep cells showed a continuously increasing expression of all drug-metabolizing enzymes investigated, resulting in a stable basal activity after approximately 15 days. Fluorescence based activity assays indicated that NeoHep cells and primary hepatocytes have similar enzyme kinetics, although the basal activities were significantly lower in NeoHep cells. Stimulation with 3-Methylcholanthrene (3-MC) and Rifampicin (RIF) markedly increased CYP1A1/2 or CYP3A4 activities, which could be selectively inhibited by Nifedipine (NIF), Verapamil (VER), Ketoconazole (KET) and Quercetin (QUE). Our data reveal similarities in expression, activity, induction and inhibition of drug-metabolizing enzymes between NeoHep cells and primary human hepatocytes and hence suggest that NeoHep cells are useful as an alternative to human hepatocytes for measuring bio-activation of substances.

Introduction

Drug metabolism is a major determinant of drug clearance and most frequently responsible for inter-individual differences in drug pharmacokinetics (Donato et al., 2004). Adverse pharmacokinetics can result in altered or even inadequate responses to the drug, affecting its use as therapeutic (Lin and Lu, 1997). *In vitro* screening became an invaluable tool to identify the metabolic profile of drug candidates, potential drug interactions or the role of polymorphic enzymes before starting clinical trials.

Drug metabolism in the liver can be divided into two phases (Williams, 1959). Phase I metabolism adds a functional group (e.g. OH, SH or NH₂) to the substrate by oxidative, reductive and hydrolytic pathways. Phase II metabolic enzymes modify the newly introduced functional group to O- and N-glucuronides, sulfate esters, various α -carboxy-amides, and S-glutathionyl adducts in order to increase their polarity (Parkinson, 1996), making elimination from the cells more rapid. Thus, hepatocytes mediate detoxification by activation of phase I and II enzymatic pathways.

All members of the cytochrome P450 super-family, belonging to phase I drug-metabolizing enzymes, can be identified by a highly conserved haem-thiolate functionality, responsible for their catalytic mechanism (Nelson et al., 2004). Amino acid variations in their substrate binding sites confer compound-, region- and stereo-selectivity of the enzymes (Guengerich and MacDonald, 1990) representing the rate limiting step of drug biotransformation processes. Experimental data suggest that most biotransformation of xenobiotics is done by enzymes of the first three families (CYP1, 2 and 3) while other CYPs are involved in “house-keeping” metabolism of endogenous molecules (Parkinson, 1996; Pelkonen et al., 1998).

To date, primary human hepatocyte cultures are the most powerful tool for *in vitro* studies (Hewitt et al., 2007), although they have major limitations due to donor organ scarcity and rapid cellular changes during culture (Guillouzo et al., 1993), resulting in a strong demand for

alternative *in vitro* systems. The human hepatoma cell line HepG2, secreting low levels of many plasma proteins characteristic for normal human liver cells (Knowles et al., 1980), is best used to study induction of drug-metabolizing enzymes, as their basal expression is significantly lower than in primary human hepatocytes (Rodriguez-Antona et al., 2002; Wilkening et al., 2003) and strongly varies during culture time (Wilkening and Bader, 2003). In recent years, numerous reports described the generation of hepatocytes or “hepatocyte-like” cells from various types of extra-hepatic cells (Hengstler et al., 2005; Ruhnke et al., 2005a; Ruhnke et al., 2005b; Nussler et al., 2006). In this study, we wanted to investigate the usefulness of “hepatocyte-like” (NeoHep) cells, derived by trans-differentiation of peripheral blood monocytes (PBMCs), as *in vitro* test system for drug screening purposes. Therefore, we analyzed expression and activity of CYPs involved in xenobiotic metabolism with high abundance in the liver, namely 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1 and 3A4 (Shimada et al., 1994; Rendic and Di Carlo, 1997; Hewitt et al., 2007). There exist various modes of regulation, defining the relative abundance and activity of each CYP iso-form, that include induction (CYP1A1, 1A2, 2A6, 2E1, 2C and 3A4), inhibition (all CYPs) and genetic polymorphism (CYP2A6, 2C9, 2C19 and 2D6) (Rendic and Di Carlo, 1997), which are important to ascertain to minimize potential drug-drug interactions when developing drug candidates (Lin and Lu, 1998; Hewitt et al., 2007). Therefore, we investigated the effect of model inducers (3-MC and RIF) and inhibitors (NIF, VER, KET and QUE) on CYP expression and activity.

Most reports only describe the expression of CYPs and neglect the expression of phase II enzymes, which are also important for activation and detoxification of many xenobiotics (Cantelli-Forti et al., 1998). To obtain a more complete view about the drug-metabolizing potential of NeoHep cells, expression and activity of phase II enzymes, EPHX1, GST A1 and M1, NAT1, NMO1, SULT1A1 and UGT1A6 were determined in the present work.

Methods

Chemicals

The following chemicals were used: hrFGF-4, hrIL-3 and hrM-CSF (R&D Systems, Minneapolis, USA); AHMC, AMMC, anti- β -actin antibody, BFC, CEC, CHC, Coumarin, DBF, EFC, HC, HFC, Histopaque-1077, KET, MCB, 2-Mercaptoethanol, 3-MC, MFC, NIF, QUE, RES, RIF, SRB, Trizol, VER and William's Medium E (Sigma, München, Germany); FBS (EC approved – S. America), L-Glutamine, human serum type AB, Penicillin/Streptomycin and RPMI 1640 Medium (Cambrex, Taufkirchen, Germany). β -Glucuronidase/Arylsulfatase mix (Roche, Mannheim, Germany); anti-CYP2C8/9/19, -CYP2E1, -CYP3A4 antibodies (CHEMICON, Chancellors Ford, UK); anti-CYP1A1/2, -CYP2A/B6 and -CYP2D6 antibodies (Santa Cruz Biotechnology, Santa Cruz, USA); horseradish peroxidase-conjugated secondary antibodies (Cell Signaling, Danvers, USA); NuPage Bis-Tris gels (Invitrogen; Karlsruhe, Germany).

Generation of programmable cells of monocytic origin (PCMOs)

PCMOs were generated from peripheral blood of healthy volunteers as described (Ruhnke et al., 2005a; Ruhnke et al., 2005b). Withdrawal of blood was approved by the local ethics committee of the Medical Faculty at Mannheim, University of Heidelberg (Proposal No 164/05). Briefly, peripheral blood monocytes (PBMCs) were isolated by density gradient centrifugation (Histopaque-1077). The resulting mononuclear cell fraction was allowed to adhere to tissue culture plastic (1.0×10^6 cells/cm²) for 2 h in RPMI 1640 medium (10 % human AB serum, 2 mM L-Glutamine, 100 U/ml Penicillin and 100 μ g/ml Streptomycin). Non-adherent cells were removed by aspiration and remaining cells were cultured for 6 days in RPMI 1640 based medium (see above) supplemented with 5 ng/ml hrM-CSF, 0.4 ng/ml hrIL-3 and 0.1 mM 2-mercaptoethanol.

Generation and culture of NeoHep cells

For differentiation into NeoHep cells, PCMOs (day 6) were cultured in hepatocyte conditioning medium (RPMI 1640, 10 % FBS, 2 mM L-Glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 3 ng/ml hrFGF-4) as reported (Ruhnke et al., 2005a; Ruhnke et al., 2005b). Culture medium was replaced every third day.

Isolation of primary human hepatocytes

Human liver tissue was obtained according to the institutional guidelines from liver resections of tumor patients with primary or secondary liver tumors. Hepatocytes were isolated by a two-step collagenase perfusion technique followed by a Percoll gradient centrifuge for purification, as described (Dorko et al., 1994). Hepatocyte viability was consistently above 90 %, as assessed by the trypan blue exclusion test. Freshly harvested hepatocytes were cultured on rat-tail collagen-coated culture plates in Williams' E medium (10 % FCS, 1 M Insulin, 15 mM HEPES, 1.4 M Hydrocortisone, 100 U/ml Penicillin and 100 mg/ml Streptomycin).

Treatment of cells

Prior to all experiments, the cells were serum starved overnight in William's Medium E (2 mM L-Glutamine). Treatment with the different substances was performed in serum free medium for indicated times and concentrations. Control conditions included cells maintained for the same period in serum-free medium supplemented with the solvent chemical.

Conventional RT-PCR

Total cellular RNA was isolated using Trizol reagent according to the manufacturers' protocol. First-strand cDNA was synthesized from 1 µg total RNA using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Primer sequences and the corresponding annealing temperatures are summarized in Table 1. Appropriate cDNA dilutions and PCR

cycling numbers were determined for each gene to ensure that the PCR did not reach saturation. PCR products resolved by gel electrophoresis in a 1.5 % (w/v) agarose gel (in TBE) were visualized by ethidiumbromide.

Western blot analysis

Cells were lysed in ice cold RIPA buffer (50 mM TRIS; 250 mM NaCl; 2 % Nonidet P40; 2.5 mM EDTA; 0.1 % SDS; 0.5 % DOC; protease inhibitor and 1.0 % phosphatase inhibitor, pH 7.2) as described (Wiercinska et al., 2006). Protein concentration was measured with the DC Protein Assay Kit (BioRad, Munich, Germany). Total protein lysates were separated by SDS PAGE using NuPAGE Bis-Tris gels and transferred to nitrocellulose membranes (VWR, Darmstadt, Germany). Immunoblotting proceeded as described (Weng et al., 2007).

Cytochrome P450 activity assays

Fluorescence-based P450 assays, were performed by incubation of intact cells (in 96-well-plates) with selected substrates as reported (Donato et al., 2004). Briefly, 100 µl reaction buffer (1 mM Na₂HPO₄; 137 mM NaCl; 5 mM KCl; 0.5 mM MgCl₂; 2 mM CaCl₂; 10 mM O-(+)-Glucose; 10 mM HEPES; pH 7.4) containing the appropriate amount of fluorogenic substrate were added to each well. After incubation at 37 °C, supernatants were transferred to white/black 96-well-plates and cells were fixed for protein quantification by SRB staining. Potential metabolite conjugates formed were hydrolyzed by incubation of supernatants with β-Glucuronidase/Arylsulfatase (150 Fishman units/ml; 1200 Roy units/ml) for 2 h at 37.0 °C. Samples were diluted (1:4) with the appropriate quenching solution. Formation of fluorescent metabolite was quantified by means of a Fluoroskan Ascent fluorescence microplate reader (ThermoLabsystems, Egelsbach, Germany). Results are given as picomoles of metabolite formed per minute normalized to total protein content. Experimental conditions are summarized in Table 2. Methanol fixed cells were used for background subtraction.

Phase II enzyme activity assays

Cells, cultured in 96-well.plates, were incubated at 37 °C with 100 µl reaction buffer (1 mM Na₂HPO₄; 137 mM NaCl; 5 mM KCl; 0.5 mM MgCl₂; 2 mM CaCl₂; 10 mM O-(+)-Glucose; 10 mM HEPES; pH 7.4), containing the appropriate amount of fluorescent substrate (products from CYP assays). After 15, 30, 45, 60, 90 and 120 min the remaining fluorescent signal in the supernatant (Fluoroskan Ascent fluorescence microplate reader) was determined and cells were fixed for protein quantification by SRB staining. Results are given as nanomoles of fluorescent substrate reduced per minute normalized to total protein content. Methanol fixed cells were used as negative control (background subtraction).

SRB staining

SRB staining was performed as reported (Skehan et al., 1990). Briefly, cells were covered with ice cold fixation buffer (95 % Ethanol, 5 % acetic acid) and kept at -20 °C for 1 h. Fixed cells were stained with 0.4 % SRB (w/v) in 1 % acetic acid for 30 min. Unbound dye was removed by washing with 1 % acetic acid. Bound SRB was resolved in 10 mM un-buffered TRIS solution and ODs at 565 nm (SRB) and 690 nm (back-ground) were determined. From the ODs, we calculated the total protein content with the standard curve ($y = 1.0305x - 1.6519$; $R^2 = 0.9868$), obtained by plotting the OD from SRB staining vs. total protein contents measured with the DC Protein Assay Kit.

Statistics

Results are expressed as mean \pm standard error of the mean (s.e.m.). Curve fitting was performed using the GraphPad Prism software (El Camino Real, USA), which allowed determination of R^2 , kinetics parameters and EC₅₀ values. Results were analyzed by analysis of variance (ANOVA) followed by paired comparison (Bonferroni), as appropriate. $P < 0.05$ was taken as the minimum level of significance.

Results

Expression of phase I and II drug metabolizing enzymes during trans-differentiation of NeoHep cells

Conventional RT-PCR was performed in order to assess mRNA levels of human phase I drug metabolizing enzymes, CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 (Figure 1a) and human phase II drug metabolizing enzymes, EPHX1, GST A1 and M1, NAT1, NMO1, SULT1 and UGT 1A6 (Figure 1c) during trans-differentiation of NeoHep cells.

NeoHep cells as well as primary hepatocytes displayed variations in mRNA levels between different donors. To prevent such donor variations, cDNAs from 5 individual experiments were pooled. Overall, expression of drug metabolizing enzymes was present, but much lower in NeoHep cells compared to human hepatocytes. In monocytes and PCMOs, except CYP1A1 and CYP2E1, no other CYP iso-forms were expressed, whereas NeoHep cells showed mRNA and protein of all CYP iso-forms investigated. Compared to human hepatocytes (day 4 of culture) CYP mRNA levels in NeoHep cells were low (1A1 ~26 %, 1A2 ~38 %, 2A6 ~20 %, 2B6 ~32 %, 2C8 ~19 %, 2C9 ~27 %, 2D6 ~41 %, 2E1 ~25 % and 3A4 ~17 % of human hepatocytes). CYP2A6 and CYP3A4 could not be detected in every sample investigated. Noteworthy, PCR for CYP1A2 gave, in addition to the product with the expected size, present in NeoHep cells and human hepatocytes, a smaller not yet identified product (*) for monocytes, PCMOs and NeoHep cells, which was lacking in hepatocytes. Protein expression analysis by Western blot, using pooled samples (N=5) of monocytes, PCMOs, NeoHep cells and human hepatocytes, confirmed the PCR results (Figure 1b).

All phase II drug metabolizing enzymes investigated were expressed (mRNA) in NeoHep cells (Figure 1c). Similar to CYP iso-forms, phase II enzyme expression was generally lower in NeoHep cells (EPHX1 ~68 %, GST A1 ~87 %, GST M1 ~72 %, NAT1 ~66 %, NMO1 ~66

%, SUL1 ~73 %, UGT1A6 ~29 %) compared to human hepatocytes. Most phase II enzymes except GST A1 and UGT 1A6, which were exclusively expressed in NeoHep cells and primary hepatocytes, were also expressed in monocytes and PCMOs.

Incubation with CYP isozyme selective substrates leads to comparable metabolite release over time in NeoHep cells and human hepatocytes

Enzyme kinetic experiments were performed with substrates that are iso-zyne selective for CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2D6, CYP2E1 and CYP3A4 in both NeoHep cells (N=4; n=4) and human hepatocytes (N=4; n=4) (Figure 2a-g). Cells were incubated for 0-120 minutes with the appropriate substrates. Metabolite conjugates formed were hydrolyzed for 2 h at 37.0 ° C with β -Glucuronidase/ Arylsulfatase. The amount of metabolite formed was normalized to total protein content and plotted vs. the incubation time. The correlation coefficients (R^2) from curve fitting are summarized in Table 3. RT-PCR and Western blot indicated lower expression of CYP iso-forms in NeoHep cells compared to human hepatocytes (Figure 1a, b), thus it was not surprising that the plateau values (Table 3) were lower in NeoHep cells. However, the time-point when the plateau is half reached ($t_{1/2\text{Plateau}}$; Table 3) are comparable between both cell types. Based on these results, substrate incubation times for all further experiments were determined to be between $t_{1/2\text{Plateau}}$ and t_{Plateau} (Table 2) not to measure product release during steady state (plateau) conditions.

NeoHep cells and human hepatocytes display similar phase II drug metabolism

Since CYP activities were measured in intact cells, metabolites formed by CYP iso-forms are continuously processed by various phase II drug metabolizing enzymes. Phase II activities in human hepatocytes (N=4; n=4) and NeoHep cells (N=4; n=4) are presented as reduction (conjugation) of metabolites AHMC, CHC, HC, HFC, Fluoresceine and Resorufin (Figure 3a-f). Furthermore, GST activity was measured by conjugation of MCB (Figure 3g).

The amount of metabolite degraded was normalized to total protein content and plotted vs. the corresponding incubation times. Correlation coefficients (R^2) and half life times ($t_{1/2}$) obtained from curve fitting were comparable between NeoHep cells and human hepatocytes (Table 4). RT-PCR results indicated that expression of phase II drug metabolizing enzymes is lower in NeoHep cells when compared to freshly isolated human hepatocytes (Figure 1c). This is supported by enhanced degradation of metabolites in human hepatocytes given by lower plateau phase values for CHC, HC and HFC, whereas those for Fluoresceine degradation were comparable between both cell types.

Possible metabolite conjugates formed were hydrolyzed by incubating supernatants with β -Glucuronidase/Arylsulfatase (150 Fishman units/ml and 1200 Roy units/ml) as described (Donato et al., 2004). Kinetics for CHC, HC, HFC and Fluoresceine “back-reaction” were measured and an incubation time of 2 h at 37 °C was sufficient to hydrolyze all formed metabolite conjugates (data not shown).

Basal CYP activities increase during differentiation of NeoHep cells

After having defined reaction conditions for CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2D6, CYP2E1 and CYP3A4, basal CYP activities were measured during trans-differentiation of monocytes to NeoHep cells and compared to freshly isolated human hepatocytes (Figure 4a-g). Monocytes (N=11; n=4) and PCMOs (N=11; n=4) showed no significant activities for the investigated CYP iso-forms. CYP activities of NeoHep cells (N=12; n=4; measured every 5th day in culture) increased during the first 15 days, then reaching a stable level. Compared to human hepatocytes (N=10; n=4), maximal CYP activities of NeoHep cells were significantly lower, which is in line with results from RT-PCR and Western blot (Figure 1a, b).

Model inducers increase CYP1A1/2 and CYP3A4 expression and activity in NeoHep cells and human hepatocytes

Of the six different CYP iso-zymes studied, CYP1A1/2 and CYP3A4 are known to be highly inducible by xenobiotics. 3-MC and RIF were selected as specific inducers for CYP1A1/2 and CYP3A4, respectively. NeoHep cells (N=12; n=3) and human hepatocytes (N=4; n=4) were incubated with 25 μ M 3-MC or RIF. DMSO was used as solvent control. After 72 h the residual stimulation medium was washed off the cells and CYP1A1/2 and CYP3A4 activities were determined. Although basal CYP1A1/2 and CYP3A4 activities were reduced during the 3 days incubation time in primary hepatocytes, values were still slightly, but consistently, higher than in NeoHep cells, which kept the basal CYP1A1/2 and CYP3A4 activity levels. Therefore, induction is given as fold of control. In both cell types CYP1A1/2 activity was significantly increased with 3-MC (Hep 3.78 ± 1.38 ; NeoHep 2.47 ± 0.89), but not with RIF (Figure 5a), whereas CYP3A4 activity was increased with RIF (Hep 3.32 ± 0.97 ; NeoHep 2.89 ± 0.78), but not with 3-MC (Figure 5b). From the same experiment protein lysates were assessed by Western blot, indicating that expression levels of CYP1A1/2 and CYP3A4 were selectively increased by 3-MC and RIF, respectively (Figure 5c), which confirms results from activity measurements.

Effect of model inhibitors on CYP1A1/2 and CYP3A4 activity is comparable in NeoHep cells and human hepatocytes

NIF, VER, KET and QUE are model inhibitors for various CYP iso-forms. Since basal levels of all CYP iso-forms were significantly lower in NeoHep cells compared to freshly isolated human hepatocytes, effects (EC_{50}) of these CYP inhibitors on induced CYP1A1/2 and CYP3A4 activities were investigated in both cell types. Human hepatocytes (N=2; n=2) and NeoHep cells (N=4; n=2) were treated with 25 μ M 3-MC or RIF. DMSO was used as solvent control. After 72 h the stimulation medium was removed and the cells were pre-incubated for

15 min with different concentrations of Nifedipine, Verapamil, Ketoconazol or Quercetin, before measuring CYP1A1/2 and CYP3A4 activities in the presence of the inhibitors. The corresponding correlation coefficients (R^2) and EC_{50} values are summarized in Table 5.

Discussion

In the present study NeoHep cells were generated from PBMCs donated by healthy volunteers in order to evaluate their potential as an alternative *in vitro* test system for drug screening. Secretion of urea, glucose and production of alanine-amino-transferase (ALT) as well as aspartate-amino-transferase (AST) confirmed earlier observations (Ruhnke et al., 2005a), ensuring the quality of our NeoHep cells. We compared expression and activity of phase I drug-metabolizing enzymes of NeoHep cells and primary human hepatocytes, which comprise over 90 % of drug oxidation in humans, namely CYP1A2 (4-6 %), 2A6 (2 %), 2B6 (25 %), 2C9 (10-11 %), 2D6 (25 %), 2E1 (2-5 %) and 3A4 (52 %) (Shimada et al., 1994). In contrast to PCMOs, which only express CYP2E1, expression of all CYP iso-forms were increased during differentiation of NeoHep cells, however, remained much lower compared to isolated human hepatocytes. Several reports show a gradual decrease in CYP expression over time in primary human hepatocytes (LeCluyse, 2001; Rodriguez-Antona et al., 2002; Gomez-Lechon et al., 2004), limiting their use for long term studies. To overcome this problem hepatocytes have been cultured between two layers of collagen (Kern et al., 1997; Tuschl and Mueller, 2006) or in the presence of appropriate cocktails of inducers (Pichard-Garcia et al., 2002). Therefore, for *in vitro* screening of new substances or to predict their possible toxicology, there are many hepatocyte-like cell lines in use. However, most of these cell lines have major limitations, e.g. reduced expression of xenosensors, leading to differences in drug metabolism (Hewitt et al., 2007), as well as incomplete and very low expression levels of drug metabolizing enzymes (Wilkening et al., 2003, Donato et al., 2008). HepG2 and Hep1c1c7

cells, for example, display strongly down-regulated expression of the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) (Pascussi et al., 2001) strongly involved in the regulation of CYP3A4. Increased CYP3A4 expression in NeoHep cells by RIF indicates a sufficient expression level of both receptors in this cell type (Gerbal-Chaloin et al., 2006). Similar to HepG2 and Hepa1c1c7 cells, the arylhydrocarbon receptor (Ahr), strongly involved in the regulation of CYP1A iso-enzymes, is expected to be expressed in NeoHep cells, since CYP1A1 and CYP1A2 were inducible with 3-MC (Pascussi et al., 2001). A detailed analysis, indicated that CYP expression levels in HepG2 cells varied heavily during culture, leading to strongly variable passage dependent results (Wilkening and Bader, 2003). In contrast, CYP activity levels of NeoHep cells remained constant for several days. Similar to hepatoma cell lines, the basal CYP activities in NeoHep cells were also very low, possibly limiting their use for inhibition experiments. Nevertheless, we were able to show (competitive) inhibition of CYP1A1/2 and CYP3A4 activity induced by 3-MC and RIF, using NIF, VER, KET and QUE. The resulting EC₅₀ values in NeoHep cells were lower than in human hepatocytes. This might be explained by relatively low CYP baseline levels in NeoHep cells or nonspecific binding of substrate to cell membranes and albumin.

Phase II drug metabolizing enzymes were only slightly reduced in NeoHep cells compared to primary human hepatocytes (Ruhnke et al., 2005a). While CYP iso-form activities are reduced in the presence of HGF, phase II enzymes UGT and GST are not regulated by this growth factor (Donato et al., 1998). In contrast to CYP iso-forms, almost all phase II drug-metabolizing enzymes, except GST A1 and UGT 1A6, were already expressed in monocytes and PCMOs and their expression level did not increase significantly during differentiation to NeoHep cells. Thus, it was not surprising that degradation kinetics of AHMC, CHC, HC, HFC, Fluoresceine and Resorufin were comparable between NeoHep cells and primary hepatocytes, with higher plateau values for NeoHep cells. Similar results were observed for GST activity, represented by MCB conjugation. These results are supported by an earlier

observation (Ruhnke et al., 2005b) showing that UGT activity, measured by 4-methylumbelliferone conjugation, is comparable between both NeoHeps cells and primary human hepatocytes.

Phenotypic as well as genotypic differences in the expression of drug-metabolising enzymes are the main causes for high inter-individual metabolic variations (Hewitt et al., 2007), not taking into consideration non-genetic factors like smoking, age, diet, hormonal status, environmental chemicals and disease state (Shah, 2005; Singh, 2006). There are many polymorphisms reported, which may lead to complete inactivation, decreased activity or altered substrate specificity of CYPs (Ingelman-Sundberg, 2005). In line with this, expression and activity of drug-metabolizing enzymes varied strongly among hepatocytes from different donors (Rodriguez-Antona et al., 2001). Similar donor-dependent variations were observed for NeoHep cells. Donor variations in the response to different inducers, further complicates predictions of therapeutic doses, which can lead to severe side-effects. In case of the anti-coagulant Warfarin, certain variants of CYP2C9 and VKORC1 (vitamin K epoxide reductase complex, subunit 1) require lower doses of warfarin than average to obtain the same therapeutic effect, and are more likely to suffer from bleeding complications at standard doses (Jones, 2007). This underlines the need for a “personalized medicine”, that comprises screening of different therapeutic approaches *in vitro* before applying it to the patient (Singh, 2006). Though direct comparison is still missing, NeoHep cells display a similar individual variability in CYP expression and activity as seen in human hepatocytes. Since NeoHep cells can be generated from blood samples, they represent a promising tool for personalized therapeutic screenings in the future.

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Footnotes

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Legends for figures

Figure 1: NeoHep cells express phase I and II drug metabolizing enzymes. RT-PCR and Western blot were performed with pooled samples from 5 individual preparations of monocytes (mono), programmable-cells of monocytic origin (PCMO), NeoHep cells from healthy controls and human hepatocytes (Heps). cDNA template and total protein contents loaded were adjusted not to reach saturation of signal **(a)** RT-PCR for basal mRNA levels of human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1 and CYP3A4. * Unexpected PCR product, which is not present in Heps. **(b)** Western blot showing basal protein levels of CYP1A1/2, CYP2A/B6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4. **(c)** RT-PCR showing basal mRNA levels of the human phase II enzymes, EPHX1, GST A1, GST M1, NAT1, NMO1, SULT1A1 and UGT1A6 **(a-c)** β -actin was used as loading control.

Figure 2: NeoHep cells display similar Cytochrome P450 metabolite release over time as human hepatocytes. NeoHep cells (N=4; n=4; straight line) and human hepatocytes (N=4; n=4; dotted line) were incubated for 0-120 min with the individual substrates. After hydrolysis of metabolite conjugates with β -Glucuronidase/Arylsulfatase, the amount of metabolite formed was normalized to total protein content and plotted vs the corresponding incubation times. Curve fitting for kinetics of **(a)** CYP1A1/2, **(b)** CYP2A6, **(c)** CYP2B6, **(d)** CYP2C8/9, **(e)** CYP2D6, **(f)** CYP2E1 and **(g)** CYP3A4 was done using the GraphPad Prism software. Background signal was determined by incubation of methanol fixed cells and subtracted from each measurement value.

Figure 3: Reduction (conjugation) of metabolites as measure of active phase II drug metabolism. NeoHep cells (N=4; n=4; straight line) and human hepatocytes (N=4; n=4; dotted line) were incubated for 0-120 min with a defined concentration of the metabolites formed during CYP enzyme assays. Reduction (conjugation) of **(a)** AHMC (ex/em = 390/460 nm), **(b)** CHC (ex/em = 390/460 nm), **(c)** HC (ex/em = 355/460 nm), **(d)** HFC (ex/em =

390/520 nm) (e) Fluoresceine (ex/em = 485/538 nm) and (f) Resorufin (ex/em = 530/590 nm) was determined by the decrease in fluorescent signal. The amount of substrate degraded was normalized to total protein content and plotted vs the corresponding incubation times. Background signal was determined by incubation of methanol fixed cells and subtracted from each measurement value. Curve fitting was done using the GraphPad Prism software.

Figure 4: Basal Cytochrome P450 activities during trans-differentiation of NeoHep cells.

Basal CYP enzyme activities in monocytes (mono; N=11; n=4), PCMOs (N=11; n=4), NeoHep cells (N=12; n=4) at days 5, 10, 15 and 20 of differentiation and human hepatocytes (Hep; N=10; n=4). Cytochrome P450 iso-forms measured were (a) CYP1A1/2, (b) CYP2A6, (c) CYP2B6, (d) CYP2C8/9, (e) CYP2D6, (f) CYP2E1 and (g) CYP3A4. Activity was given as pmol of metabolite/min/mg protein. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ of values being significantly greater than 0.

Figure 5: Induction of CYP1A1/2 and CYP3A4 in NeoHep cells and human hepatocytes with rifampicin and 3-methylcholanthrene NeoHep cells (N=12; n=3; filled bars) and human hepatocytes (N=4; n=4; empty bars) were incubated for 72 h with 25 μ M 3-MC or 25 μ M rifampicin (RIF). DMSO (0.1 %) was used as solvent control. (a) CYP1A1/2 activity and (b) CYP3A4 activity was determined and fold induction by 3-MC and RIF was calculated. (c) Protein lysates were taken to confirm increased expression of CYP1A1/2 and CYP3A4 by Western blot in both cell types. 30 μ g total protein lysate was loaded per lane. β -actin was used as loading control. ** $p < 0.01$; *** $p < 0.001$.

Tables

Table 1: PCR conditions

	Accession [NM_]	Sequence 5' to 3'		Amplicon bp	T _m [°C]
		Forward Primer	Reverse Primer		
CYP1A1	000499	TTC GTC CCC TTC ACC ATC	CTG AAT TCC ACC CGT TGC	302	55
CYP1A2	000761	TCG TAA ACC AGT GGC AGG T	GGT CAG GTC GAC TTT CAC G	254	60
CYP2A6	000762	CAA CCA GCG CAC GCT GGA TC	CCA GCA TAG GGT ACA CTT CG	423	60
CYP2B6	000767	ATG GGG CAC TGA AAA AGA CTG A	AGA GGC GGG GAC ACT GAA TGA C	283	62
CYP2C8	000770	CAT TAC TGA CTT CCG TGC TAC AT	CTC CTG CAC AAA TTC GTT TTC C	147	60
CYP2C9	000771	CTG GAT GAA GGT GGC AAT TT	AGA TGG ATA ATG CCC CAG AG	308	59
CYP2D6	000106	CTT TCG CCC CAA CGG TCT C	TTT TGG AAG CGT AGG ACC TTG	222	59
CYP2E1	000773	GAC TGT GGC CGA CCT GTT	ACT ACG ACT GTG CCC TTG G	297	59
CYP3A4	017460	ATT CAG CAA GAA GAA CAA GGA CA	TGG TGT TCT CAG GCA CAG AT	314	64
UGT1A6	001072	TGG TGC CTG AAG TTA ATT TGC T	GGC TCT GGC AGT TGA TGA AGT A	210	60
EPHX1	000120	GGC TTC AAC TCC AAC TAC CTG	GCA AGG GCT TCG GGG TAT G	184	60
GST A1	145740	TCT GCC CGT ATG TCC ACC T	GCT CCT CGA CGT AGT AGA GAA GT	185	59
GST M1	000561	TCT GCC CTA CTT GAT TGA TGG G	TCC ACA CGA ATC TTC TCC TCT	117	60
SULT1A1	177534	CGG CAC TAC CTG GGT AAG C	CAC CCG CAT GAA GAT GGG AG	91	62
NAT1	000662	GGG AGG GTA TGT TTA CAG CAC	ACA TCT GGT ATG AGC GTC CAA	128	60
NMO1	000903	TGA AGG ACC CTG CGA ACT TTC	GAA CAC TCG CTC AAA CCA GC	185	61
β-actin	007393	CAC CCA CAC TGT GCC CAT C	CTC CTG CTT GCT GAT CCA C	607	60

Table 2: Reaction conditions for CYP iso-enzyme measurement

	Substrate	Assay conc.	Incubation time	Metabolite	microtiter plate	Quenching Solution	Ex / Em [nm]
CYP1A1/2	CEC	30 μ M	90 min	CHC	black	DPBS	390 / 460
CYP2A6	Coumarin	50 μ M	60 min	HC	black	0.1 M TRIS (pH 9.0)	355 / 460
CYP2B6	EFC	30 μ M	120 min	HFC	white	0.1 M TRIS (pH 9.0)	390 / 520
CYP2C8/9	DBF	10 μ M	90 min	Fluoresceine	black	10 mM NaOH	485 / 538
CYP2D6	AMMC	10 μ M	120 min	AHMC	white	0.1 M Tris (pH 9.0)	390 / 460
CYP2E1	MFC	10 μ M	120 min	HFC	white	0.1 M TRIS (pH 9.0)	390 / 520
CYP3A4	BFC	100 μ M	120 min	HFC	white	0.1 M TRIS (pH 9.0)	390 / 520

Table 3: CYP metabolite release time-course parameter

	human hepatocytes			NeoHep cells		
	Plateau	$t_{1/2\text{Plateau}}$	R^2	Plateau	$t_{1/2\text{Plateau}}$	R^2
	[pmol metabolite/ mg protein]	[min]		[pmol metabolite/ mg protein]	[min]	
CYP1A1/2	3022 ± 810	109.1 ± 30.0	0.7429	2683 ± 199	64.4 ± 9.9	0.8677
CYP2A6	430.1 ± 116.3	85.6 ± 34.3	0.6810	107.0 ± 24.3	77.9 ± 27.9	0.7256
CYP2B6	4199 ± 769	71.7 ± 20.0	0.7790	4055 ± 1025	102.0 ± 60.5	0.8264
CYP2C8/9	310.7 ± 73.8	100.4 ± 33.7	0.7889	277.9 ± 47.9	91.1 ± 28.5	0.6651
CYP2D6	1272 ± 257	111.7 ± 55.7	0.8231	586.3 ± 118.7	94.5 ± 27.6	0.8226
CYP2E1	3524 ± 770	89.6 ± 28.6	0.7872	1359 ± 223	67.0 ± 22.5	0.5933
CYP3A4	1262 ± 785	119.4 ± 85.5	0.8019	234.3 ± 37.0	98.5 ± 27.4	0.7242

Table 4: Summary for time-course of metabolite conjugation data

Substrate	human hepatocytes			NeoHep cells		
	R2	Plateau	half life	R2	Plateau	half life
		[nmol substrate/ min/mg protein]	[min]		[nmol substrate/ min/mg protein]	[min]
AHMC	0.7506	13.47 ± 0.18	8.17	0.6584	12.32 ± 0.36	16.16
CHC	0.8303	19.73 ± 0.61	7.20	0.7921	31.21 ± 1.33	13.34
HC	0.6853	2.39 ± 0.28	12.75	0.7952	7.66 ± 0.25	16.51
HFC	0.8575	13.54 ± 1.00	18.41	0.8057	38.15 ± 1.56	28.83
Fluorescein	0.7546	8.79 ± 0.41	4.61	0.6606	10.45 ± 0.58	6.23
Resorufin	0.9026	14.13 ± 0.54	7.90	0.6246	34.19 ± 1.07	17.74
MCB	0.8868	45.93 ± 7.34	214.69	0.8630	49.10 ± 5.08	264.20

Table 5: EC₅₀ values for CYP1A1/2 and CYP3A4

Inhibitor	CYP	human hepatocytes		NeoHep cells	
		R2	EC50	R2	EC50
		[μM]		[μM]	
Nifedipine	1A1/2	0.9965	213.2 ± 1.36	0.9838	106.3 ± 1.28
	3A4	0.9960	87.22 ± 1.13	0.9986	51.23 ± 1.05
Verapamil	1A1/2	0.9893	92.60 ± 1.32	0.9999	54.73 ± 1.01
	3A4	0.9969	149.0 ± 1.17	0.9994	87.20 ± 1.05
Ketoconazol	1A1/2	0.9971	28.14 ± 1.62	0.9947	15.16 ± 1.18
	3A4	0.9683	6.039 ± 1.25	0.9995	3.954 ± 1.06
Quercetin	1A1/2	0.9974	171.2 ± 1.12	0.9977	84.04 ± 1.07
	3A4	0.9954	62.33 ± 1.10	0.9999	41.86 ± 1.01

Figure 1

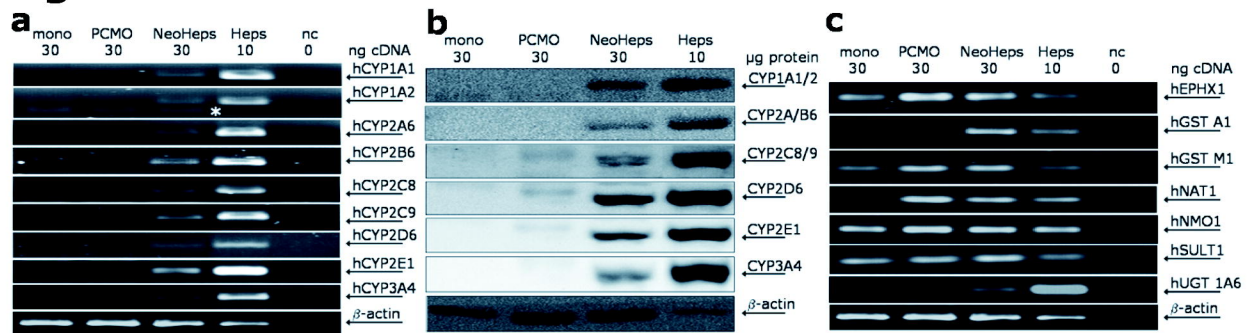


Figure 2

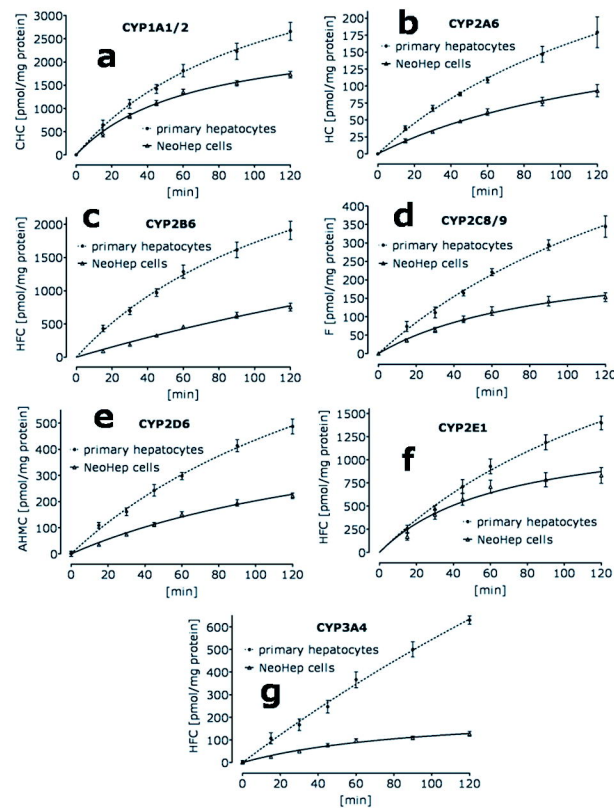


Figure 3

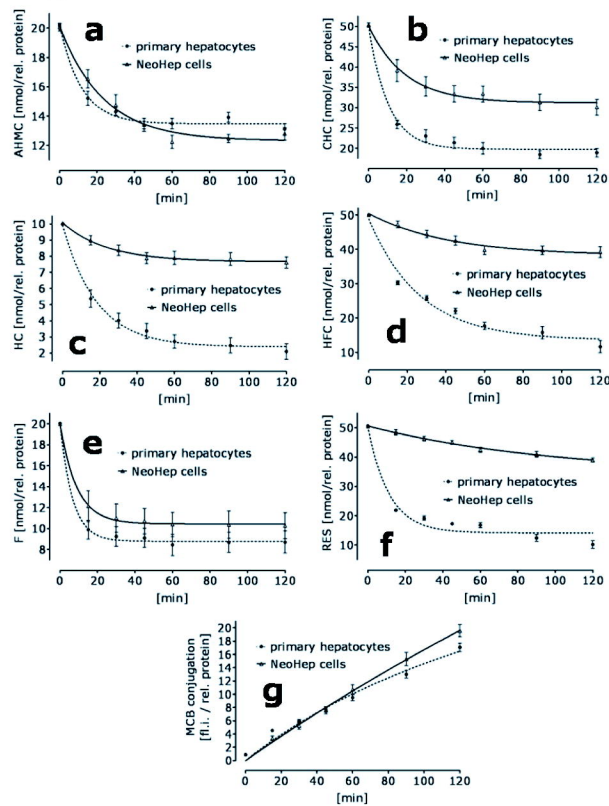


Figure 4

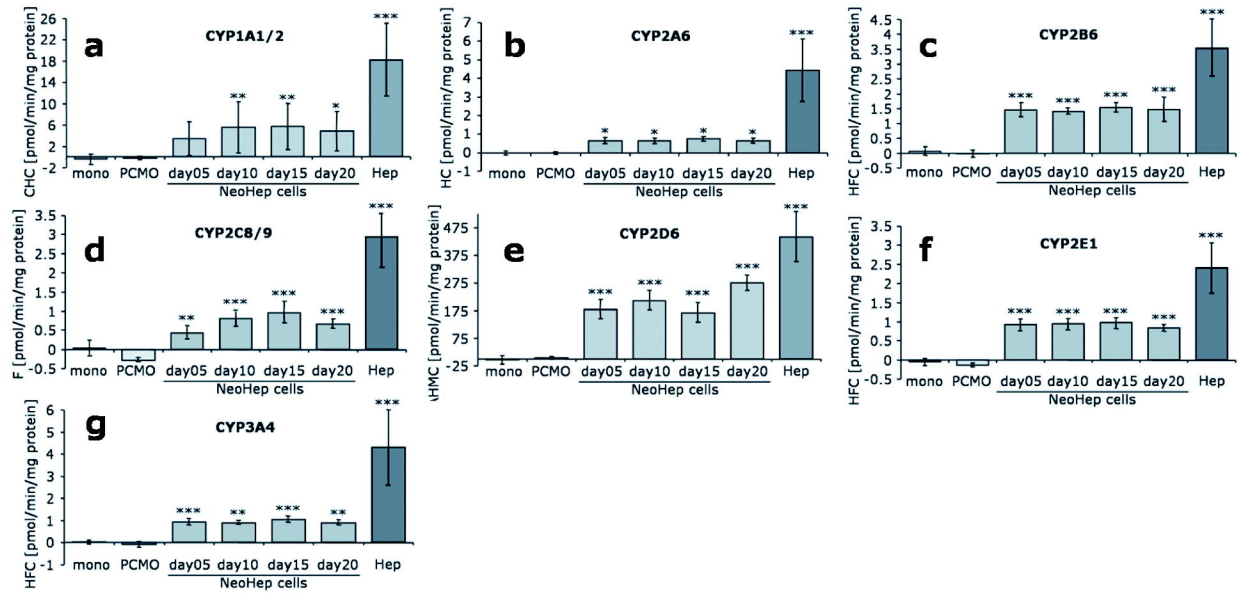


Figure 5

