Aus dem Berlin-Brandenburg Centrum für
Regenerative Therapien (BCRT)
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

„Untersuchungen zur Optimierung pharmakologischer Studien an hepatischen Zellen in 2D- und 3D-Kulturen“

zur Erlangung des akademischen Grades
Doctorrerummedicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Marc Lübberstedt

aus Zürich

Datum der Promotion: 30.05.2015

TABLE OF CONTENT

ABSTRAKT III

ABSTRACT IV

INTRODUCTION 5

METHODS 6

Cells 6

2D culture of hepatic cells 6

Bioreactor and perfusion system 6

Metabolic parameters 7

Immune histochemistry 7

Gene array analysis 8

Statistics 8

RESULTS 9

Suitability oftheHepaRG cell line as an alternative cell source to primary human hepatocytes 9

Morphology and distribution of HepaRG cells in comparison to pHH 9
Comparison of metabolic activities 9
CYP activity assay in HepaRG cells and primary human hepatocytes 10
CYP activity and induction in pHH and HepaRG, compared to otherhepatic cells 10
Intrinsic clearance of reference drugs 10

Serum-free 3D-culture of primary human hepatocytes in a new miniaturized version of the bioreactor system for pharmacological in vitro studies 10

Downscalingof the bioreactor from clinical scale to laboratory scale 10
Adaptation of perfusion and gas flow rates 11
Optimization of the CYP activity assay and adaptation to the 3D culture system 11
Comparison of metabolic activities in serum-containing and serum-free bioreactor cultures 12
Immune histochemcal characterization of bioreactor cultures 12
Gene expression analysis of bioreactor cultures under serum-containing or serum-free culture conditions 13

DISCUSSION 13

REFERENCES 17

ABSTRAKT

Die Leber ist ein zentrales Organ der Biotransformation und Detoxifizierung endogener und xenobiotischer Substanzen und spielt eine wichtige Rolle in der pharmakologischen und toxikologischen Forschung. Primäre humane Hepatozyten (pHH) representieren ein prädiktives in vitroModell des leberspezifischen Metabolismus, allerdings hat die Verwendung dieser Zellen den Nachteil der limitierten Verfügbarkeit und einer eingeschränkten Lebensdauer der Zellen. Die Verwendung hepatischer Zelllinien und die methodische Optimierung der Kulturbedingungen durch die Verwendung von 3D-Kulturtechnologien gilt als aussichtsreiche Methode, um diese Einschränkungen zu adressieren.

Das Ziel dieses Promotionsvorhabens war die Optimierung eines 3D-Bioreaktor-Kulturmodells für in vitro Studien zum Arzneistoffmetabolismus unter Verwendung von pHH. Hierzu wurden insbesondere der Verzicht der Supplementierung mit fötalem Kälberserum im Kulturmedium und die Verwendung der 3D-Bioreaktorkultur für pharmakologische Studien untersucht. Ferner wurde die hepatische Zelllinie HepaRG als ein möglicher Ersatz für pHH charakterisiert.

Basierend auf der Bestimmung metabolischer Eigenschaften unter Einbeziehung der Aktivität humanrelevanter Cytochrom P450 (CYP)-Enzyme und durch Studien zur Genexpression konnte gezeigt werden, dass miniaturisierte Bioreaktorvarianten die Versorgung von pHH unter serumfreien Bedingungen mit einer verlängerten Stabilität wesentlicher humanrelevanter CYP-Enzyme begünstigen. Immunhistochemische Färbungen von Zellmaterial aus Bioreaktoren zeigten eine Reorganisation parenchymaler und nicht-parenchymaler Zellen zu lebergewebeartigen Strukturen.Studien zum Arzneistoffmetabolismus und zu Wirkstoffaufnahmeraten anHepaRG Zellen und primären humanen Hepatozytenweisen darauf hin, das die HepaRG-Zelllinie optional als Alternative für primäre humane Hepatozyten verwendet werden könnte.

Somit bietet der miniaturisierte Bioreaktor ein geeignetes Instrument für pharmakologische Untersuchungen unter definierten und kontrollierten Bedingungen in Langzeitstudien mit pHHoder geeigneten Zelllinien wie der Zelllinie HepaRG.

ABSTRACT

The liver is a central organ for biotransformation and detoxification of endogenous and xenobiotic substances and plays a major role in research on pharmacokinetics and drug toxicity.Primaryhuman hepatocytes (pHH) represent a predictive in vitro model for liver specific metabolism, but the use of these cells has the disadvantage of a limited availability and lifetime. To overcome these limitations, the use of hepatic cell lines and approaches for improvement of culture conditions using 3D culture technologies have been proposed.

The aim of this dissertation was the optimisation of a 3D bioreactor culture model for in vitro studies on drug metabolism using pHH. Specifically, the possible omission of foetal calf serum supplement in the culture medium and the use of the bioreactor system for pharmacological studies were investigated. In addition the hepatic cell line HepaRG was characterized as a possible surrogate forpHH.

Based on the determination of metabolic performances, including cytochrome P450 (CYP) enzyme activities and gene expression studies, miniaturized bioreactor variants were shown to promote the maintenance of pHH under serum free conditions with a prolonged stability of major human relevant CYP enzymes. Immune histochemical staining of cell material from bioreactors showed a reorganisation of parenchymal and non-parenchymal cells to liver tissue like structures. Studies on drug metabolism and clearence rates using the HepaRG cell line showed that metabolic performances were comparable to those ofpHH, indicating that this cell line has the potential to be used as analternative to pHH.

Thus the miniaturized bioreactor provides a suitable tool for pharmacological assays under well-defined and controlled conditions in long-term studies with pHH or suitable cell lines, such as the HepaRG cell line.

INTRODUCTION

The liver is a central organ responsible forvarious catabolic and anabolic processes including the biotransformation and detoxification of endogenous and xenobiotic substances.Thereforethe liver plays a major role in research on pharmacokinetics and drug toxicity. Primary hepatocytes provide the complete set of drug-metabolizing enzymes and therefore represent a predictive in vitro model for liver specific metabolism (Brandon et al., 2003). Due to species-specific divergences of the xenobiotic metabolism primary human hepatocytes (pHH) are considered as gold standard for human-relevant pharmacological studies (Donato et al., 1999). However, the availability and lifetime of freshly isolated pHH is limited.To overcome these limitations, the use of hepatic cell lines and approaches for improvement of culture conditions using 3D culture technologies have beenproposed(Abbott, 2003).However the improvement of the phenotypic quality of human hepatic cell lines as well as the optimisation of 3D culture models to achieve prolonged metabolic stability of hepatocytes is still a focus in the field of pharmaceutical research (Gernaey et al., 2012).

The aim of this dissertation was the optimization of a 3D bioreactor culture model for predictive in vitrostudies on metabolism of xenobioticausingpHH or a suitable hepatic cell line. Therefore the HepaRG cell line was investigated as a potential alternative source to the limited available pHH(aim 1). Furthermore the effect of serum free culture on pHH cultured in an miniaturized 3D bioreactor for pharmacological studies was analyzed (aim 2) including the determination of the activity and stability of metabolic functions in order to provide an optimized in vitro model for pharmacological studies on hepatocytes.

METHODS

Cells

Studies on pHH were performed with cells from remaining tissue after partial liver resection. Cells for the study were isolated by Andreas Nüssler and Daniel Knobeloch, Departmentof General, Visceral and Transplantation Surgery, CharitéUniversitätsmedizin Berlin, with agreement of the donors and approval of the local ethical committee.Primary human liver cells were isolated by collagenase digestion as described in detail by Nussler et al. (2009). Pre-differentiated HepaRGcells, cryopreserved human hepatocytes and plated hepatocytes declared as long-term hepatocytes by the provider were purchased from different manufacturers. All commercial available cells were cultured according to the providers´ instructions.

2D culture of hepatic cells

PHH were plated on a collagen layer and cultured in a standard cell incubator. Non-adherent cells were removed by washing the cultures with fresh culture medium after 12 hours of incubation. HepaRG cells were pre-differentiated and investigated within four weeks after completion of differentiation. The comparison with pHH was performed during the 3rd and 4th week after differentiation since the cultures exhibit differentiated functions over at least 28 days according to the manufacturer.Cryopreserved human hepatocytes were used in suspension immediately after thawing and in case of induction studies after 24 hours of cultivation. Long-term hepatocytes were used in culture plates.

Bioreactor and perfusion system

The bioreactors used in the study consist of a cell compartment with three independent interwoven hollow fiber capillary bundles. Two capillary bundles serve for medium supplementation in a counter-current flow and a third capillary bundle is used for decentralized oxygenation of the cell compartment. The cells are located in the inter-capillary space. Medium perfusion in recirculating mode is provided via a tubing system with connections for fresh medium supply and corresponding medium outflow. Bioreactor variants with different volumes were used, including 8ml and 2 ml variants and finally a miniaturized 0.5 ml bioreactor. The miniaturized bioreactor contains only two layers of capillaries with alternating medium and gas capillaries in each layer. The bioreactors are integrated into a perfusion device providing pumps for medium recirculation and medium supply, a gas mixing unit and a heating unit to provide constant conditions for long-term maintenance of the cell culture.

For 3D culture experiments, cells were maintained in the bioreactor at 37 °C, using a gas mix of 95% air and 2-5% CO_2 to keep the pH value between 7.35 and 7.45. Recirculation and feed rates of cell culture medium were adapted for each bioreactor variant. Bioreactors and perfusion devices were manufactured by Stem Cell Systems, Berlin, Germany.

Metabolic parameters

Metabolites were measured in samples from culture supernatants in 2D cultures or from the bioreactor perfusate in 3D bioreactor cultures. Values of metabolic rates were normalized to the inoculated cell number. As a marker for general cell activity the metabolism of glucose and lactate were determined. Albumin synthesis and urea production were measured as hepatocyte specific markers of metabolic activity. The release of the cytosolic enzymes lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and glutamate dehydrogenase (GLDH) were used as indicators of cell damage. The activity of human relevant cytochrome P450 enzymes (CYPs) was tested based on their ability to catalyze the formation of specific products from model drugs. In 2D cultures, single applications of substrates were performed and the formation of the main product was determined in the cultures supernatant.For the investigation of CYP activities in the 3D bioreactor non-interacting substrates were pooled and applied as a substrate cocktail, with the exception of one substance (bufuralol) applied separately, due to possible interactions with other substrates.In order to optimize the application time of the assay, the productconcentrationswere screened over 24 hours and product formation rates were determined based on results from different donors.Theinducibility of CYP enzymes in HepaRGcells,pHH, cryopreserved hepatocytes and long-term hepatocytes was investigated using specific inducers. In addition the intrinsic clearance of 11 model drugs was investigated in HepaRG cells and pHH in 2D cultures by measuring the concentration of each drug in the supernatant following application of the substance. The formation of drug metabolites was analyzed by the cooperation partner Pharmacelsus GmbH, Saarbrücken, using liquid chromatography - mass spectroscopy (LC-MS).

Immune histochemistry

Immune histochemical staining of samples from cell culture material from different locations within the capillary network was performed after termination of bioreactor cultures. After fixation with 5% paraformaldehyde the material was dehydrated, embedded in paraffin and cut into 5 µm sections. The organization grade of the pHH in the bioreactors was characterized in comparison with that of native human liver tissue. Hepatocytes, endothelial cells, biliary cells,

Kupffer cells and non-parenchymal cells were marked with antibodies specific for epitopes of CK18, and vimentin. Staining was performed with secondary antibodies conjugated with peroxidase or fluorescent markers (Alexa Fluor 488, Alexa Fluor 568).

Gene array analysis

For the analysis of expression of genes related to hepatic functions the genes encoding the following proteins were determined: transcription factors (AHR,CEBPa,CEBPb, DBP and GATA4); nuclear factors (HNF4a,FXR, CAR and RXRb, MDR1); membersof the ATP-binding cassette family (BSEP, MRP2, MRP3,BCRP); membrane transport proteins of the solute carrierfamily (NTCP, PEPT1, OCT1, OAT2, OATP-C, OATP-8 andOACP-B); CYP isoenzymes (CYP1A1, CYP1A2, CYP2B6,CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4,CYP3A7); and the phase 2 metabolism enzymes sulfotransferase2A1 (SUL2A1) and UDP-glucuronosyltransferase 2B7(UGT2B7). In addition, gene expression levels oftheliver-specific proteins transthyretin, albumin (ALB) andglucose-6-phosphatase were determined. Total RNA was isolated from bioreactor cultures maintained with or without serum after culture termination using trizol with phenol/chloroform extraction. RNA was transcribed into cDNA using reverse transcription-polymerase chain reaction (PCR) with randomhexamer primers. Analysis was performed by the cooperation partner (AstraZeneca, Mölndal, Sweden)either with custom arrays of 384-well cards preloaded with TaqMan specific primersand probes or with TaqMan gene expression assay kits in conjunction with ABI PRISM 7900HT Fast Real-Time PCR System.

Statistics

Values were calculated as mean±SD, if not otherwise described. The unpaired t-test was performed to compare metabolic activities of pHH with those of HepaRG or to compare CYP activities of pHH with those of HepaRG cells, long-term hepatocytes or cryopreserved hepatocytes. The one-way ANOVA with Dunnett'smultiplecomparison test was performed to analyze the significance of timedependent changes in activities of pHH or HepaRG cells. Levels of significance were set as$p<0.05$, $p<0.01$ and $p<0.001$. The nonparametric correlation test according to Spearman was performed to determine possible correlations between primary human hepatocytes and HepaRG in the intrinsic clearance of the different substrates.Data from bioreactors run with or without serum were calculated as means ± standard error of the mean (SEM), respectively, if not otherwise indicated. CYP enzyme activities in bioreactors perfused with serum-containing medium and those run without FCS were compared with the two-tailed paired t-test. To analyze the correlation of average Delta Ct values of gene expression, data were

normalized to a housekeeping gene and a non-parametric correlation analysis of the average delta Ct values was performed. Fold changes were calculated with the ΔΔCt method.

RESULTS

Suitability oftheHepaRG cell line as an alternative cell source to primary human hepatocytes

To address the need foranalternative to the limited available pHH the HepaRG cell line was compared to freshly isolated pHH in conventional 2D culturesandto other available human primary cells from different sources.

Morphology and distribution of HepaRG cells in comparison to pHH

The morphology of pHH and HepaRG cells is described in our publication “HepaRG human hepatic cell line utility as a surrogate for pHH in drug metabolism assessment in vitro” by Lübberstedt et al. (2011). Both cell types formed confluent cell layers consisting of polygonal-shaped cells typical for hepatocytes. HepaRG cells developed 3-dimensional cell aggregates surrounded by a single layer of cells, which an epithelial cell like morphology. While pHH cultures revealed some minor detachment of cells and inclusion bodies during the culture time of 14 days, the HepaRG cell aggregates, formed during the differentiation phase under the influence of DMSO supplementation,showed no morphologic signs of decreased viability.

Comparison of metabolic activities

A significant difference between pHH and HepaRG cultures of most metabolic rates of parameters (formation of glucose, lactate, albumin and urea and elimination of galactose and sorbitol)was shown in this study. High initial glucose values, which decreased during the later culture time,wereobservedin cultures of pHH. The course of the glucose formation rates of HepaRG cells developed in a contrary course. Lactate and albumin formation were almost stable over the culture time and both showed higher formation of HepaRG cell cultures as compared to pHH, whereas no urea formation was detected inHepaRG cell cultures. The elimination of galactose and sorbitol showed a small time-dependant decrease in both cell types with higher elimination capacity in HepaRG cultures.

CYP activity assay in HepaRG cells and primary human hepatocytes

The product formation by main human relevant cytochrome P450 enzymes involved in the detoxification of xenobiotics(CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) was comparable in both cell types over the investigated 14 days of culture. However, pHHshowed a slight decrease of most enzyme activities, and the enzyme activity ofCYP2D6 was higher in HepaRG cells compared to that of pHH.

CYP activity and induction in pHH and HepaRG, compared to otherhepatic cells

The comparison of the CYP dependant product formation in pHH, HepaRG and cryopreserved human hepatocytes during the first day of culture showed asignificantlyhigher activity in cryopreserved human hepatocytes than infreshly isolated pHHor HepaRG, with the exception of CYP1A2, which was scarcely expressed in cryopreserved cells, and CYP3A4, which was higher in HepaRG cells than in cryopreserved cellsandpHH.

Exposure with CYP inducers performed with β-naphtoflavone(CYP1A2) and rifampicin (CYP2C9, CYP2C19 and CYP2A4) over 72 hoursinduced an increase in all cell types investigated. The most prominent increase in CYP activity was observed in HepaRG cells and cryopreserved cells as a result of β-naphtoflavoneexposure.Uponrifampicinexposure,plated (long-term) hepatocytes showed the most prominent increase in CYP2C19 activity.

Intrinsic clearance of reference drugs

The intrinsic clearance of eleven different model drugs determined in pHH and HepaRG cells showed a significant correlation between the two cell types. Most of the substances were cleared slightly faster by primary cells,howevervalues varied between individual donors.

Serum-free 3D-culture of primary human hepatocytes in a new miniaturized version of the bioreactor system for pharmacological in vitro studies

Downscalingof the bioreactor from clinical scale to laboratory scale

The bioreactor culture technique investigated in this study, originally developed by JörgGerlach (1997), was designed to support a high-density cell culture in a closed cell compartment made of hollow fibre perfusion capillaries for nutrition and oxygenation. The comparison of different scales of the technology showed a comparable performance of primary human liver cells and a good stability of metabolic functions over at least 14 days in bioreactorvariants of 800 ml, 8 and2

ml,as shown in our publication “Scaling down of a clinical three-dimensional perfusion multi compartment hollow-fiber liver bioreactor developed for extracorporeal liver support to an analyticalscaledevice useful for hepatic pharmacological in vitro studies” by Zeilinger et al. (2011). These studies show a high reorganization grade of the cells with tissue-like arrangement of parenchymal and non-parenchymal cells in the bioreactorandextended activity of human relevant CYPs for up to 23 days.

In order to enable parallel experiments in 3D bioreactors with primary hepatocytes isolated from the same donor a further down-scaled miniaturized version with a 0.5 ml cell-compartment volume was designed.

Adaptation of perfusion and gas flow rates

The adaptation of the miniaturized bioreactor to the existing perfusion system resulted in a reduced tubing system of 12 ml total volume recirculating through the bioreactor with a flow rate of 3 ml/min. A rate of 0.5 ml/h for the addition of fresh culture medium respectively removal of used culture medium was chosen in order to exchange one total system volume per day. For the oxygenation of the system, without the formation of air bubbles in thecell compartment a maximum flow rate of 5 ml/min. was applied.

Optimization of the CYP activity assay and adaptation to the 3D culture system.

To determine the CYP activity of pHH in the 3D culture within the bioreactor theCYPactivity assay established for 2D culture systems was modified. The investigation of interactions between the CYP substrates used in this study was performed by the cooperation partner Pharmacelsus GmbH, Saarbrücken. As a result of these studies, a substrate cocktail of four non-interacting substrates (phenacetin, midazolam, diclofenac, bufuralol and bupoprion) for the measurement of CYP1A2, CYP2C9, CYP4A4/5 and CYP2B6 was applied. In order to prevent interactions with other substrates the application of bufuralolhad to be performed separately to determine the activity of CYP2D6.. The modified CYP activity assay allowed the characterization of product formation kinetics of main human relevant CYPs. Linear formation rates were detected during the first three hours of application in all investigated cultures and allowed the calculationof product formation rates as an indicator of enzymatic activity. The successfully established CYP assay, optimized for 3D bioreactor cultures was used as a tool for quality control to determine the stability of xenobiotic metabolism in our publications “Serum-free culture of pHH in a miniaturized hollow-fibre membrane bioreactor for pharmacological in vitro studies” and

“Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies”published by Lübberstedt et al. (2012) and Hoffmannet al.(2012).

Comparison of metabolic activities in serum-containing and serum-free bioreactor cultures

In this study, 3D bioreactor cultures were investigated in the presence of 2.5 % fetal calf serum (FCS) and under serum-free conditions over a culture period of 10 days. The results are described in our publication “Serum-free culture of primary human hepatocytes in a miniaturized hollow-fibre membrane bioreactor for pharmacological in vitro studies” by Lübberstedt, et al. (2012).

No significant metabolic differences between serum-containing and serum-free cultures were observed in terms of glucose, lactate, urea production and albumin synthesis. Moreover release rates of the cytosolic enzymes AST, ALT and GLDH as indicators of cell injury remained similar in serum-containing compared to serum-free cultures. The activity of main human relevant CYP enzymes (CYP1A2, CYP3A4/5, CYP2C9, CYP2D6, CYP2B6) was comparable over 10 days in both groups investigated, and showed a similar time course of preservation with a good stability of CYP1A2 and CYP3A4.

The serum-free culture was used as a standard method in further studies such as the investigation of drug metabolism in our study “Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies” published by Hoffmann et al.(2012).

Immunehistochemcalcharacterizationof bioreactor cultures

Among the different fixation and embedding methods tested forpreparation of cell material thefixation of cell in thebioreactorfollowed by an embedding in Paraffin showed the best results.Therefore this preparation method was used as a standard method for immune histochemicalstudies.

Immune histochemical staining of material extracted from serum-free bioreactorsshoweda similar cell distributionasobserved in native liver tissue. In our publication “Serum-free culture of primary human hepatocytes in a miniaturized hollow-fibre membrane bioreactor for pharmacological in vitro studies” by Lübberstedt et al. (2012), we showed that most cells were stained CK18 positive,indicatingthat the majority of cells were hepatocytes Non-parenchymal cells stained positive for vimentinweresporadically observed in the periphery of cell clusters. The distribution of CK19 positivecells showed comparable biliary structures in bioreactors and native

liver tissues. These findings were confirmedin our publication "Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies" published by Hoffmann et al. (2012).

Gene expression analysis of bioreactor cultures under serum-containing or serum-free culture conditions

The investigation of the expression of genes encoding for proteins relevant for hepatic metabolism (transcription factors, nuclear factors, members of the ATP-binding cassette family, membrane transport proteins of the solute carrier family, CYP isoenzymes, phase 2 metabolism enzymes and gene expression levels for the liver-specific proteins transthyretin, albumin (ALB) and glucose-6-phosphatase), were analyzed from samples gained at the end of the bioreactor cultures. The majority of genes showed a tendency towards higher expression in the group without FCS in the culture medium. However, the correlation coefficient showed no significant differences between the investigated groups.

DISCUSSION

In this study, a 3D bioreactor culture model was optimized for predictive in vitro studies on metabolism of xenobiotics using pHH or a suitable hepatic cell line.
In the first part of the study the HepaRG cell line was investigated as a potential alternative source to the limited available pHH.
Although pharmacological and toxicity studiesin whole-animal experiments are used to predict human health effects, their advantages and disadvantages as well as the need to reduce refine and replace (3R principle) animal test systems are controversially discussed (Hartung, 2008). Due to species-dependant differences in xenobiotic metabolism human liver cells are considered the source of choice for predictive studies (Hewitt et al. 2001). Human primary hepatocytes provide a complete picture of metabolic fate of xenobioticsin vitro. However disadvantages like restricted availability, elaborate isolation procedures and limited survival time could be addressed by the use of a human derived hepatic cell line. Most cell lines generally have limited or fractional CYP expression (Gómez-Lechón et al., 2001). The human derived hepatic cell line we investigated in our publication, "HepaRG human hepatic cell line utility as a surrogate for pHH in drug metabolism assessment in vitro" (Lübberstedt et al., 2011), was first described by Gripon et al. (2002) as a cell line derived from a human hepatocellular carcinoma which has the ability to differentiate into biliary and hepatic cells when treated with dimethyl

sulfoxide(DMSO). Compared to the monolayer culture of the pHH, the morphological appearance of 2D HepaRG culturesobserved in this study indicate a differentiation into three-dimensional cell aggregates surrounded by a single layer of epithelial-like cells. These findings are consistent with the findings of Aninat et al. (2006). The results of metabolic parameters showed thatin contrast to pHH, which showed a decreasing glucose production, glucose consumption was observed in the first week of the study and switched to glucose release until day 14. Both cell types showed comparable time courses of lactate production. However the absolute production rates were much higher in HepaRG cultures. The high production of lactate in the HepaRG cells could be an effect of cell aggregation, which often leads to anaerobic metabolism due to limited oxygenationin the center of cell aggregates.HepaRG cells showed a comparable time course of albumin secretion and elimination of galactose and sorbitol over the assayed time. However those hepatocyte-specific metabolism parameters were significantly higher in the cell line.However in cultures of HepaRG cells no urea production was detectable, indicating a low or disturbed nitrogen elimination via the urea cycle. The HepaRG cell line exibited hepatocyte-specific albumin production and showed a high stability of CYP dependent drug metabolism in a differentiated state for at least four weeks.
Commercially available cryopreserved human hepatocytes showed a distinctly higher CYP activity of CYP 2C9, CYP 2C19 and CYP2D6 while CYP 1A2 and CYP3A4 showed lower activities than HepaRG cells. However cryopreserved hepatocytes are not suitable for long-term cultures. Furthermore the cell line showed a positive response to enzyme inducers and the determination of the intrinsic clearance of model drugs was comparable to those of pHH.
As an advantage over pHH the HepaRG cell line could also allow a screening of compounds in induction studies providing direct comparable results and a constant availability. This study indicates that the HepaRG cell line could be a suitable surrogate for the use of pHH in pharmacological studies in the 3D -bioreactor system. These findings are supported by studies on CYP dependent metabolism over four weeksusingHepaRG cells in the 3D bioreactor culture system(Darnell et al., 2012).However the cell line represents cells from one donor and does not provide information of inter-individual variances of liver functions in different donors.

In the second part of the study the effect of serum-free culture on pHH cultured in a miniaturized 3D bioreactor for pharmacological studies was analyzed including the determination of the activity and stability of metabolic functions in order to provide an optimized in vitro model for pharmacological studies on hepatocytes.

The investigation of a new miniaturized bioreactor maintained under serum free or serum-supplemented cultures showed similar results in both groups in terms of general metabolic parameters and cell integrity markers. The higher release of transaminases and glutamate dehydrogenase at the early cultures time (day 3) of both serum-containing and serum free-cultures can be ascribed to initial cell damage resulting from stress during the isolation procedure. The production rates of glucose, lactate, albumin and urea decreased over time. However none of the parameters showed differences between the two groups investigated.

The performed activity assays of major human-relevant CYPs in cultures of pHH isolated from four different donors showed comparable results in serum–free and serum–containing bioreactor cultures. Especially the rates of CYP1A2 mediated acetaminophen formation and CYP3A4/5 mediated OH-midazolam formation were preserved to a large extent in the miniaturized bioreactor for up to 10 days in bothgroups. The observed decreasing activity over time in CYP2C9, CYP2D6 and CYP2B6 mediated metabolite formation shows the same comparable decline, independent from serum supplementation. This indicates that serum free culture conditions do not influence the activity of the drug metabolism related enzymes investigated.

The quality of serum free cultivation was also evaluated by histological stainingof cell material from bioreactors maintained with serum free culture medium. Analyses showed a tissue-like aggregation of parenchymal and non-parenchymal cells with biliary structures. These findings were also confirmed in our publication "Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies"Hoffmann et al., 2012 ". In that study staining of serum-free cultures showedliver-like distribution of drug transporter proteins and CYPs in the miniaturized bioreactor in a comparable manner as in larger bioreactor versions as published by Zeilinger et al., 2011.

Results from gene expression analysis showed that the majority of hepatocyte–specific genes, in particular those encoding for transport proteins and CYP enzymes were even higher in serum-free cultures compared to cultures supplemented with serum. The differences observed between the groups amounted to a factor of 2 to 3,which is in accordance with the suppression of gene expression by serum observed in primary rat hepatocytes (Tushl and Mueller, 2006, Tuschl et al. 2009).

A major advantage of the miniaturized bioreactor can be seen in the reduced cell number of 20 million cells, required per bioreactor culture. Thus, experiments with freshly isolated cells from the same donor can be performed under various conditions in parallel cultures. In addition, due to the adaptation of medium recirculation and medium feed rates maintenance costs are reduced.

A further advantage of the technique is the suitability for serum free culture of primary human hepatocytes in the bioreactor system. The serum-free culture allows a more precise downstream analysis of metabolites released to the culture media and provides improved standardization of experimental conditions. Furthermore the established CYP activity assay allows a simultaneous determination of up to four enzyme activities in a single culture.

Various bioreactor designs from cell carriers in oxygenated medium, cell free matrices as fixed bed bioreactors and hollow fiber perfusion bioreactors are currently in use (Zeilinger et al., 2011, Miranda et al., 2010, Linke et al., 2007). One of the main aims in the approaches of 3D hepatocytes cultures is the formation of a highly physiological environment to support cell-cell contacts and signal exchange. The design of the perfused bioreactors including the miniaturized bioreactor model used in this study, provide favorable conditions for high mass exchange and integral decentralized oxygenation. This enables intercellular communication and physiological exchange of autocrine and paracrine factors, which play an important role in the maintenance of differentiation of pHH (Schmelzer et al., 2009, LeCluyse, 2001). In addition self-conditioned medium in the bioreactor cultures by parenchymal and non-parenchymal cell types could also explain the high grade of phenotypic preservation of hepatocytes in the absence of serum. This effect is also shown in our study "Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies" published by Hoffmann et al., (2012), showing that metabolic activities of pHH including the expression and activity of CYP enzymes were clearly more stable in the bioreactor system than in conventional 2D cultures.

In conclusion, HepaRG cells provide a suitable model of humanhepatocytes for studies of xenobiotic metabolism and could be used as a surrogate for pHHin the miniaturized bioreactor system. The HepaRG cell line allows the generation of reproducibleexperimental data andmetabolic stability for up to four weeks. For induction studies,HepaRG could provide an advantage over pHH, since the use of a cell line allows the screening of compound libraries and directcomparison of results.

These studies performed in the miniaturized bioreactor show that the culture method could be used asatool for pharmacological assays under well-defined and controlled conditions.Although a bioreactor culture cannot represent the full complex organ, this bioreactor type enables pHH culture, e.g. for investigations ofdrug toxicityand metabolism studies of xenobiotics as an alternative to animal experiments.

REFERENCES

1. Brandon EF, Raap CD, Meijerman I, Beijnen JH, Schellens JH. An update on in vitro test methods in human hepatic drug biotransformation research: Pros and cons. ToxicolApplPharmacol. 2003; 189: 233-46.

2. Donato MT, Castell JV, Gómez-Lechón MJ. Characterization of drug metabolizing activities in pig hepatocytes for use in bioartificial liver devices: comparison with other hepatic cellular models. J Hepatol. 1999; 31:542-9.

3. Abbott A. Cell culture: biology's new dimension. Nature. 2003; 424: 870-2.

4. Gernaey KV, Baganz F, Franco-Lara E, Kensy F, Krühne U, Luebberstedt, M, et al. Monitoring and control of microbioreactors: an expert opinion on development needs. Biotechnol J. 2012; 7: 1308–14.

5. Nussler AK, Nussler NC, Merk V, Brulport M, Schormann W, Hengstler JG.The holy grail of hepatocyte culturing and therapeutic use. In: SantinM, editor, Regenerative Medicine Today. USA: Springer; 2009. p. 84-122.

6. Lübberstedt M, Müller-Vieira U, Mayer M, Biemel KM, Knöspel F, Knobeloch D, et al. HepaRG human hepatic cell line utility as a surrogate for primary human hepatotcytes in drug metabolism assessment in vitro. J PharmacolToxicol Methods. 2011; 63: 59–68.

7. Gerlach JC. Long-term liver cell cultures in bioreactors and possible application for liver support. Cell BiolToxicol. 1997; 13: 349-55.

8. Zeilinger K, Schreiter T, Darnell M, Söderdahl T, Lübberstedt M, Dillner B, et al. Scaling down of a clinical three-dimensional perfusion multicompartment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological in vitro studies. Tissue Eng Part C Methods. 2011; 17: 549-56.

9. Lübberstedt M, Mueller-Vieira U, Biemel K, Darnell M, Hoffmann SA, Knöspel F, et al. Serum-free culture of primary human hepatocytes in a miniaturized hollow-fiber membrane bioreactor for pharmacological in vitro studies, J Tissue EngRegen Med. 2012; Nov. 20 [Epub ahead of print]

10. Hoffmann SA, Müller-Vieira U, Biemel K, Knobeloch D, Heydel S, Lübberstedt M, et al. Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies. BiotechnolBioeng. 2012; 109: 3172-81.

11. Hartung T. Food for thought... on animal tests. Altex, 2008; 25: 3–16.

12. Hewitt NJ, Bühring KU, Dasenbrock J, Haunschild J, Ladstetter B, Utesch D. Studies comparing in vivo: In vitro metabolism of three pharmaceutical compounds in rat, dog, monkey, and human using cryopreserved hepatocytes, microsomes, and collagen gel immobilized hepatocyte cultures. Drug MetabDispos. 2001; 29: 1042-50.

13. Gómez-Lechón MJ, Donato T, Jover R, Rodriguez C, Ponsoda X, Glaise D, et al. Expression and induction of a large set of drug- metabolizing enzymes by the highly differentiated human hepatoma cell line BC2. Eur J Biochem. 2001; 268, 1448-59.

14. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. ProcNatlAcad Sci USA. 2002; 99: 15655-60.

15. Aninat C, Piton A, Glaise D, Le Charpentier T, Langouët S, Morel F, et al. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug MetabDispos. 2006; 34: 75-83.

16. Darnell M, Ulvestad M, Ellis E, Weidolf L, Andersson TB. In vitro evaluation of major in vivo drug metabolic pathways using primary human hepatocytes and HepaRG cells in suspension and a dynamic three-dimensional bioreactor system. J PharmacolExpTher. 2012; 343:134-44.

17. Tuschl G, Mueller SO. Effects of cell culture conditions on primary rat hepatocytes - Cell morphology and differential gene expression. Toxicology. 2006; 218: 205-15.

18. Tuschl G, Hrach J, Walter Y, Hewitt PG, Mueller SO. Serum-free collagen sandwich cultures of adult rat hepatocytes maintain liver-like properties long term: a valuable model for in vitro toxicity and drug-drug interaction studies. ChemBiol Interact. 2009; 181: 124-37.

19. Miranda JP, Rodrigues A, Tostoes RM, Leite S, Zimmerman H, Carrondo MJ, et al. Extending hepatocyte functionality for drug-testing applications using high-viscosity alginate encapsulated three-dimensional cultures in bioreactors. Tissue Eng Part C Methods.2010; 16:1223-32.

20. Linke K, Schanz J, Hansmann J, Walles T, Brunner H, Mertsching, H. Engineered Liver-Like Tissue on a Capillarized Matrix for Applied Research. Tissue Eng. 2007; 13: 2699-707.

21. Schmelzer E, Mutig K, Schrade P, Bachmann S, Gerlach JC, Zeilinger K. Effect of human patient plasma ex vivo treatment on gene expression and progenitor cell activation of primary human liver cells in multi-compartment 3D perfusion bioreactors for extra-corporeal liver support. BiotechnolBioeng. 2009; 103: 817–27.

22. LeCluyse EL. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. Eur J Pharm Sci. 2001; 13: 343-68

Eidesstattliche Versicherung

„Ich, Marc Lübberstedt, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Untersuchungen zur Optimierung pharmakologischer Studien an hepatischen Zellen in 2D- und 3D-Kulturen“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -www.**icmje**.org) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.
Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.
Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Marc Lübberstedt hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: **Lübberstedt M**, Müller-Vieira U, Mayer M, Biemel KM, Knöspel F, Knobeloch D, Nüssler AK, Gerlach JC, Zeilinger K. HepaRG human hepatic cell line utility as a surrogate for primary human hepatotcytes in drug metabolism assessment in vitro. J Pharmacol Toxicol Methods. 2011; 63: 59–68.

Beitrag im Einzelnen: Konzeption der Versuchsreihen, Durchführung der Versuche zur Untersuchung der morphologie, der metabolischen Parameter, der aktivitäts- und Stabilitätsbestimmung der human relevanten Cytochrom P450-Enzyme und zur Induzierbarkeit von CYP-Enzymen. Die statistische Auswertung der Rohdaten sowie die Zusammenfassung der Ergebnisse.

Publikation 2: **Lübberstedt M**, Mueller-Vieira U, Biemel K, Darnell M, Hoffmann S, Knöspel F, Wönne E, Knobeloch D, Nüssler A, Gerlach CJ, Andersson TB, Zeilinger K. Serum-free culture of primary human hepatocytes in a miniaturized hollow-fiber membrane bioreactor for pharmacological in vitro studies. J Tissue Eng Regen Med. 2012; Nov. 20 [Epub ahead of print]

Beitrag im Einzelnen: Konzeption der Versuchsreihen, Aufbau der Bioreaktorkultursysteme, Optimierung von Perfusionsraten, Adaption und Optimierung und Durchführung eines für die 2D Kultur entwickelten CYP-Aktivitätstests. Betreuung und Versorgung der 3D Bioreaktorkulturen sowie die Optimierung zur Entnahme von Probematerial für

Genexpressions- und histologische Studien. Die statistische Auswertung der Rohdaten sowie die Zusammenfassung der Ergebnisse.

Publikation 3: Hoffmann SA, Müller-Vieira U, Biemel K, Knobeloch D, Heydel S, **Lübberstedt M**, Nüssler AK, Andersson TB, Gerlach JC, Zeilinger K. Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies. Biotechnol Bioeng. 2012; 109: 3172-81

Beitrag im Einzelnen: Vorbereitende Planung der Versuchsreihen. Vorversuche zur Arzneimitteldosierung im 3D Kultursystem. Beteiligung am Aufbau und Betreuung der 3D-Bioreaktorkultursysteme.

Publikation 4: Zeilinger K, Schreiter T, Darnell M, Söderdahl T, **Lübberstedt M**, Dillner B, Knobeloch D, Nüssler AK, Gerlach JC, Andersson TB. Scaling down of a clinical three-dimensional perfusion multicompartment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological in vitro studies. Tissue Eng Part C Methods. 2011; 17: 549-56.

Beitrag im Einzelnen: Vorbereitende Planung der Versuchsreihen. Beteiligung am Aufbau und Betreuung der 3D-Bioreaktorkultursysteme. Durchführung und Auswertung von Enzymaktivitätstest. Beteiligung an der Isolation von primären humanen Hepatozyten.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

____

Unterschrift des Doktoranden/der Doktorandin

____

Liste der ausgewählten Publikationen

Lübberstedt M, Müller-Vieira U, Mayer M, Biemel KM, Knöspel F, Knobeloch D, Nüssler AK, Gerlach JC, Zeilinger K. HepaRG human hepatic cell line utility as a surrogate for primary human hepatotcytes in drug metabolism assessment in vitro. J Pharmacol Toxicol Methods. 2011; 63: 59–68.
IF: 2,32

Lübberstedt M, Mueller-Vieira U, Biemel K, Darnell M, Hoffmann S, Knöspel F, Wönne E, Knobeloch D, Nüssler A, Gerlach CJ, Andersson TB, Zeilinger K. Serum-free culture of primary human hepatocytes in a miniaturized hollow-fiber membrane bioreactor for pharmacological in vitro studies. J Tissue Eng Regen Med. 2012; Nov. 20 [Epub ahead of print]
IF: 2,83

Hoffmann SA, Müller-Vieira U, Biemel K, Knobeloch D, Heydel S, **Lübberstedt M**, Nüssler AK, Andersson TB, Gerlach JC, Zeilinger K. Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies. Biotechnol Bioeng. 2012; 109: 3172-81
IF: 3,65

Zeilinger K, Schreiter T, Darnell M, Söderdahl T, **Lübberstedt M**, Dillner B, Knobeloch D, Nüssler AK, Gerlach JC, Andersson TB. Scaling down of a clinical three-dimensional perfusion multicompartment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological in vitro studies. Tissue Eng Part C Methods. 2011; 17: 549-56.
IF: 4,64

Lübberstedt M, Müller-Vieira U, Mayer M, Biemel KM, Knöspel F, Knobeloch D, Nüssler AK, Gerlach JC, Zeilinger K. HepaRG human hepatic cell line utility as a surrogate for primary human hepatotcytes in drug metabolism assessment in vitro. J PharmacolToxicolMethods. 2011; 63: 59–68.

http://dx.doi.org/10.1016/j.vascn.2010.04.013

Lübberstedt M, Mueller-Vieira U, Biemel K, Darnell M, Hoffmann S, Knöspel F, Wönne E, Knobeloch D, Nüssler A, Gerlach CJ, Andersson TB, Zeilinger K. Serum-free culture of primary human hepatocytes in a miniaturized hollow-fiber membrane bioreactor for pharmacological in vitro studies. J Tissue EngRegen Med. 2012; Nov. 20 [Epub ahead of print]

http://dx.doi.org/10.1002/term.1652

Hoffmann SA, Müller-Vieira U, Biemel K, Knobeloch D, Heydel S, **Lübberstedt M**, Nüssler AK, Andersson TB, Gerlach JC, Zeilinger K. Analysis of drug metabolism activities in a miniaturized liver cell bioreactor for use in pharmacological studies. BiotechnolBioeng. 2012; 109: 3172-81

http://dx.doi.org/10.1002/bit.24573

Zeilinger K, Schreiter T, Darnell M, Söderdahl T, **Lübberstedt M**, Dillner B, Knobeloch D, Nüssler AK, Gerlach JC, Andersson TB. Scaling down of a clinical three-dimensional perfusion multicompartment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological in vitro studies. Tissue Eng Part C Methods. 2011; 17: 549-56.

http://dx.doi.org/10.1089/ten.tec.2010.0580.

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

Danksagung

Da wissenschaftliches Arbeiten nie ausschließlich von einer Person allein durchführbar ist, möchte ich an dieser Stelle all jenen danken ohne die die Anfertigung dieser Arbeit in dieser Form nicht möglich gewesen wäre.
Ein großes Dankean Frau Dr. Katrin Zeilinger, Herrn Prof. Dr. Jörg Gerlach und Prof. Dr. Michael Sittinger für die konstruktive Kritik, die vielfältige Unterstützung. Dank auch an Prof. Tommy B Andersson für seine Unterstützung und lehreiche Beratung.Des Weiteren möchte ich allen Mitarbeitern von StemCell Systems für die gute und schnelle Umsetzung von neuen Ideen Danken.Dank auch an alle aktuellen und ehemaligen Mitgliedern der BioreactorGroup (BCRT, Charite), insbesondere an Frau Dr. Ing. Fanny Knöspel, Steffan Hoffman und Christin Schneider für die exzellente Zusammenarbeit.
Am Ende möchte ich ganz besonders meiner Familie Danken, für deren Liebe, Geduld danken, meiner Frau und allen Freunden und Freundinnen für Ihre liebevolle Unterstützung.