ABSTRACTS

LECTURES

LECTURES

L1. CELL REPROGRAMMING: EXTREME CELL PLASTICITY

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Pluripotent stem cells (PSC) are cells endowed with the property of both extensive self-renewal and almost unlimited differentiation capacities. The very recent discovery of a new category of human PSC that can easily be obtained *in vitro*, human induced pluripotent stem cells (hiPS), has profoundly modified the perspectives of regenerative medicine. Indeed, hiPS cells provide a promising source of cells for allo- or autotransplantation and this discovery has clearly shifted the landscape of the field of transplantation.
Based on this model of conversion of a human foreskin fibroblast into PSC, we have established the transcriptional map of this cell conversion using whole genome microarrays. Several major switches are observed, including a mesenchymal to epithelial transition. Though the tissue of origin is of mesodermal lineage, we show that the hiPS cell lines can differentiate in progeny from the ectoderm (neural progenitors) and endoderm (hepatocytes) lineages.

LECTURES

L2. EMBRYONIC AND INDUCED PLURIPOTENT STEM CELL DERIVED HEPATOCYTES: TOWARDS CELL BASED THERAPIES, DISEASE MODELLING, DRUG TESTING AND HUMAN DEVELOPMENTAL MODELS

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Liver disease remains a major leading cause of mortality in the developed world and whole organ transplantation remains the only treatment in the most extreme cases. However the lack of donors combined with high alcohol consumption obesity and hepatitis C epidemic is presenting a major challenge to the health system. Importantly, liver failure results from diverse causes such as massive trauma, cirrhosis, viral infection, hepatic metabolic dysfunction as well as combinations of all these factors. Understanding the mechanisms by which liver disease progresses within each of these potential scenarios will allow for the development of suitable drug based therapies and/or cell replacement therapies that could limit the use of transplantation and its drawbacks. In the Vallier lab, we have developed an in-vitro hepatic differentiation protocol that mimics the known developmental processes of mammalian hepatocyte differentiation in-vivo. Applying this protocol to pluripotent stem cells of embryonic origin (human Embryonic Stem cells, hESCs) or generated from reprogrammed somatic cells (human induced pluripotent stem cells or hIPSCs), we were able to produce hepatocytes following a natural path of development. The resulting cells display characteristics specific to both fetal and adult primary hepatic cells including expression of key developmental genes as well as several functional outputs such as alpha-1-antitrysin secretion, glycogen storage and cytochrome P450 activity. We are currently using this culture system to (i) explore the mechanisms controlling early human embryonic hepatic developmental, (ii) produce patient matched hepatic cells with potential for transplantation purposes, (iii) develop in-vitro models of liver metabolic disease for further studies. Furthermore, we will discuss the potential use of such systems to create suitable drug screening platforms that could be utilised for both drug development for metabolic diseases and/or drug toxicity screening for the pharmaceutical industry.

LECTURES

L3. COMPARATIVE ANALYSIS OF HEPATOCYTE-LIKE CELLS GENERATED FROM LIVER PROGENITOR CELLS AND HUMAN EMBRYONIC STEM CELLS

S Gerbal-Chaloin, N Funakoshi, M Kot, M Terki, A Herrero, C Duret, E Raulet, C Gondeau, P Briolotti, J Ramos, P Blanc, F Navarro, P Maurel and M Daujat-Chavanieu .
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There is a huge need for normal human hepatocytes to generate cellular models for studying liver ontogenesis, physiopathology, molecular pharmacology, virology, etc, and for developing liver biotherapy strategies. The possibility to generate hepatocytes from the differentiation of adult and embryonic stem cells is promising for the future. Moreover investigating the impact of hepatocellular carcinoma etiological agents on adult stem cell phenotype and differentiation may bring new insights in molecular events leading to hepatocarcinogenesis.
We have developed culture conditions to differentiate liver adult progenitor cells and human embryonic stem (hES) cells into hepatocyte and quantitatively compared hepatic gene expression in the resulting cells to that of human hepatocytes in primary culture. Non parenchymal epithelial (NPE) cells are isolated from the liver of patients with no sign of liver failure. They exhibit a marked proliferative potential and harbour a subpopulation suspected to represent LPC (Duret et al, 2007). Interestingly, they are sensitive to HCV infection and permissive to viral genome replication. Transplantation experiments into the hepatic parenchyme of NOD/SCID mice show that NPE cells are indeed able to engraft and differentiate in vivo. When cultured under appropriate conditions, they differentiate into hepatocyte-like cells expressing some hepatocyte enriched transcription factors (HNF4a, HNF1a, C/EBP), and exhibiting a phenotype characterized by both a foetal (CYP3A7 and glutathion-S-transferase π) and a mature expression pattern (glutathion-S-transferase α and CYP3A4). Differentiation of hES is achieved in a sequential protocol that tentatively recapitulates *in vitro* the three major steps that mark liver ontogenesis *in vivo*, that is, definitive endoderm formation, hepatic specification and hepatic maturation. As with NPE cells, and as found by others, a mixed foetal/adult hepatic phenotype was reached in these cells, as demonstrated by a persistent expression of alpha-fetoprotein and CYP3A7 mRNAs and a low level expression of the adult specific xenoreceptor CAR and CYP3A4 mRNAs. These results point to a deficient maturation. Genetic complementation experiments with hepatic transcription factors (in NPE cells) or CAR (in hES) expressing lentiviral vectors allow the improvement of terminal maturation and the acquisition of xenobiotic metabolism in the differentiated cells.

References

Duret C, Gerbal-Chaloin S, Ramos J, Fabre JM, Jacquet E, Navarro F, Blanc P, Sa-Cunha A, Maurel P, Daujat-Chavanieu M. Stem Cells. 2007 Jul;25(7):1779-90.

Key words: stem cell, progenitor, hepatocyte, differentiation, toxicology

LECTURES

L4. HEPATIC MATURATION OF HUMAN FETAL HEPATOCYTES IN FOUR-COMPARTMENT 3D PERFUSION CULTURE

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Bio-artificial liver support systems have been utilized as bridging devices to support acute and chronic liver injury. Prolonged function of adult hepatocytes however, has not been achieved due to compromised proliferation and long-term survival of adult cells in vitro. As an alternative cell source, we investigated the potential of human fetal hepatocytes and human embryonic stem (ES) cell-derived hepatocytes in a four-compartment hollow fiber based three-dimensional (3D) perfusion culture system. Human fetal hepatocytes were isolated from livers, 17-19 gestational weeks, and cultured in the 3D-perfusion bioreactors for 14 days. Human ES cells were differentiated into functional hepatocyte-like cells in the bioreactor by using a defined stepwise differentiation protocol. Metabolism activity, hepatocyte-specific gene expression, protein expression, and hepatic function were investigated. Glucose consumption and lactate production were measured to monitor cell viability and activity in the bioreactor. The ratio of cytochrome P450 3A4 to 3A7 gene expression and the increase of the number of asialoglycoprotein receptor positive cells indicated cell differentiation into mature hepatocytes. Histological and immunohistochemical analysis revealed reorganization of fetal liver cells and tissue like structure formations of ES cell-derived hepatocytes. Hepatic function was further examined for ammonia metabolism and for albumin production using colorimetric assays and ELISA, respectively. In contrast with conventional 2D culture, the 3D-perfusion culture system induced functional maturation to human fetal hepatocytes and human ES cell-derived hepatocyte-like cells; these cells may be useful as alternative cell sources for extracorporeal liver support.

LECTURES

L5. siRNAs TO DELINEATE ROLES OF NUCLEAR RECEPTORS IN INDUCTION OF Oatps IN PRIMARY CULTURES OF HEPATOCYTES

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Organic anion transporting polypeptides (Oatps) are basolateral membrane solute carriers with a broad range of substrates, including bile salts, steroid conjugates, thyroid hormones and numerous drugs. The three predominant Oatps in rat liver are Oatp1a1, Oatp1a4 and Oatp1b2. Nuclear receptors, including the pregnane X receptor (PXR), constitutive androstane receptor (CAR) and farnesoid X receptor (FXR) are implicated in the regulation of both transporters and P450s. Administration of 50mg/kg dexamethasone for 72h to male Sprague Dawley rats caused a 5-8 fold induction of Oatp1a4 expression, and doubled the expression of Oatp1b2, whilst having no significant effect on the expression of Oatp1a1. The mRNA levels were quantified using Quantitative Taqman® real-time PCR. Treatment of sandwich cultured hepatocytes (Matrigel or collagen matrix) with PXR agonists, dexamethasone (10μM) or pregnenolone-16 α-carbonitrile (PCN) (10μM), for 48h increased expression of Oatp1a4 6-8 fold. As was seen *in vivo*, treatment with PXR agonists did not significantly affect expression of Oatp1a1 or Oatp1b2. The FXR agonists, GW-4064 (1-10μM) and chenodeoxycholic acid (10-100μM), caused a dose-dependent decrease in the expression levels of Oatp1a1 and 1a4, but had no significant effect on Oatp1b2 expression.

The role of PXR in the induction of Oatp1a4 by dexamethasone and PCN was confirmed by transfection of cells with PXRsiRNA prior to treatment with PCN. Two types of commercially available PXR-specific siRNAs were used - Silencer® and Silencer Select®. Knockdown of PXR was maintained for 48 h. Silencer® siRNAs had to be used at a significantly higher concentration than Silencer Select® siRNAs (5nM) to achieve a similar level of knockdown. Maximal knockdown achieved with these siRNAs was 40%. When cells were transfected with PXR siRNA and subsequently treated with 10μM PCN for 48h, 40% knockdown of PXR was sufficient to cause a significant decrease in the OATP induction response to PCN, confirming the importance of PXR in this response.

LECTURES

L6. MICRORNA SIGNATURES IN PHENOBARBITAL-INDUCED LIVER TOXICITY

Nigel J Gooderham[1], *Costas Koufaris*[1], *Jayne Wright*[2], *Richard Currie*[2]

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MicroRNAs (miRNA) are ~22 nucleotide long non-coding RNA species that post-transcriptionally downregulate target genes. MicroRNAs are involved in the regulation of cell proliferation, differentiation, and apoptosis. Dysregulation of miRNAs contributes to many diseases, including cancers. Few studies have thus far examined the role of microRNAs in directing the adaptive or adverse responses of the liver following chemical carcinogen exposure. Phenobarbital is a prototype non-genotoxic carcinogen promoting hepatic neoplasia in rodents; the mechanisms whereby phenobarbital drives carcinogenesis are not well understood. This study examined the effect of phenobarbital exposure on liver miRNA expression to investigate whether miRNA profiling can aid identification of carcinogenic potential and help understand the mechanisms of toxicity.

The expression levels of 350 rat miRNAs in the livers of control and phenobarbital-treated, nine week old male Fischer rats, were profiled using microarrays. Carcinogenic and non carcinogenic doses of phenobarbital were administrated in the diet for periods up to 90 days. Analysis of hepatic microRNA expression confirmed that phenobarbital-induced effects were both dose and time dependent. During the first 7 days of treatment, unsupervised clustering based on the miRNA signatures failed to reveal distinct differences between treatments. After 14 days however, exposure to carcinogenic doses of phenobarbital resulted in a more pronounced, dose-dependent deregulation of miRNA expression. Hierarchical clustering analysis grouped the liver samples from treated animals according to the dose of phenobarbital. Significance Analysis of Microarrays (SAM) was used to identify differentially expressed miRNAs, with a False Discovery Rate (FDR) of <5%. A signature of fourteen miRNAs was significantly different between control animals and animals exposed to phenobarbital; these included miR-200 family members. The miR-200 family is known to be involved in epithelial-mesenchymal transition, is deregulated in several types of cancer and is predicted to regulate cancer genes. Targets of miR-200 family members include the mRNAs of E-cadherin, Zeb 1 and 2 transcription factors, proteins of the PI3K and TGFb pathways. Understanding the functional consequences of these signature miRNA changes offers mechanistic insights into drug-induced toxicity.

LECTURES

L7. FROM PLASTIC TO PATIENT: IDENTIFYING THE BARRIERS TO THERAPEUTIC OLIGO DELIVERY

Steve Hood, GOLD team, PTS, GSK R&D, Ware, UK

Oligonucleotide based medicine offers the promise of access to "undruggable targets", where the interaction with the transcription of a single gene product will provide highly specific therapies while minimising "off target" effects. However, while this class of drugs has been investigated for the past 30 years, starting with simple antisense oligos and continuing through aptamers, siRNAs, gapmers and exon shippers, very few have progressed through late stage development and only a couple have reached the market, despite the best efforts of small biotechs and large Pharma alike.

In this presentation we will take a 3D view of therapeutic oligos by reviewing Delivery, Developability and Desirability. We will explore:

- The diverse structures of the oligo types and their chemical modifications,
- What strategies have been employed to optimise delivery to the site of action
- Why there is often a disconnect between in vitro and in vivo efficacy
- What are the in vivo ADME properties of these "Big Small Molecules"
- What are the Regulatory and marketing hurdles to their approval as reimbursable medicines.

By reviewing these topics, we hope to illustrate the challenges inherent in taking a useful research tool and converting it to a novel therapeutic platform.

LECTURES

L8. AAV-MEDIATED KNOCK-DOWN OF APOLIPOPROTEIN B100 *IN VIVO* USING SHRNA

Annemart Koornneef, Piotr Maczuga, Richard van Logtenstein, Florie Borel, Bas Blits, Tita Ritsema, Sander van Deventer, Harald Petry, and Pavlina Konstantinova

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Serum low-density lipoprotein cholesterol (LDL-C) levels are proportionate to the risk of atherosclerotic cardiovascular disease. In order to reduce serum total cholesterol and LDL-C levels, we used RNA interference to inhibit expression of the structural protein of LDL-C, apolipoprotein B100 (ApoB). We developed and screened 19 short hairpin RNAs targeting conserved sequences in human, mouse, and macaque ApoB mRNAs (shApoBs) and subsequently narrowed our focus to one candidate for *in vivo* testing in wild-type mice. Self-complementary adeno-associated virus serotype 8 (scAAV8) was used for long-term transduction of murine liver with shApoB. We observed a strong dose-dependent knock-down of ApoB mRNA and protein levels, which correlated with a reduction in total cholesterol levels, without obvious signs of toxicity. Furthermore, shApoB was found to specifically reduce LDL-C in diet-induced dyslipidemic mice, while high-density lipoprotein cholesterol (HDL-C) remained unaffected. Finally, we demonstrated elevated lipid accumulation in murine liver transduced with shApoB, a known phenotypic side-effect of lowering ApoB levels. These results demonstrate a robust dose-dependent knock-down of ApoB by scAAV8-delivered shRNA in murine liver, thus providing an excellent tool for development of RNAi-based gene therapy.

LECTURES

L9. INTERPLAY OF DRUG METABOLISM AND TRANSPORT: A REAL PHENOMENON OR AN ARTIFACT OF THE SITE OF MEASUREMENT?

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The interdependence of both transport and metabolism on the disposition of drugs has recently gained heightened attention in the literature, and has been termed the "interplay of transport and metabolism". Such "interplay" can have a significant impact on interpretation of pharmacokinetic data and in predicting in vivo disposition parameters, including drug-drug interactions (DDI). In this presentation, I will highlight both of these phenomena with theoretical simulations and case studies from the literature.

References

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Acknowledgements

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LECTURES

L10. PROTOTISSUE™ - A NEW FUNCTIONAL HUMAN 3D LIVER TISSUE FOR *IN VITRO* DRUG TESTING

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Prototissue™ are 3D pieces of surrogate human liver generated by cultivating an immortal human hepatocyte cell line using a proprietary rotating bioreactor under well defined conditions.

This ProtoTissue™ exhibits many of the ultrastructural features and physiological functions present in the human liver. These functions are stable for many months and as such ProtoTissue™ offers a novel approach to many of the challenges faced in drug development.

After 20 days in culture, ProtoTissue™ is functionally mature and can import glucose in an insulin sensitive manner (at physiological glucose concentrations only) and synthesise glycogen. ProtoTissue™ can synthesise cholesterol and this synthesis can be blocked by lovastatin. Once the lovastatin is metabolised, ProtoTissue™ synthesises excess cholesterol to recover the homeostatic balance.

Bile acid synthesis and secretion increases substantially during the differentiation process to achieve a relatively steady production after 20-22 days.

ProtoTissue™ responds to several different medicinal compounds. For example ProtoTissue™ can metabolise diclofenac roughly twice as efficiently as commercially available human hepatocytes and to a degree similar to a piece of human liver. This metabolism is very stable over a 30 day period.

The response of ProtoTissue™ when challenged with pharmaceutical compounds is concentration dependent. Thus it is possible to determine the minimum concentration of compounds which kill the ProtoTissue™ after one single dose (the "acute lethal dose"). Lower doses can also be lethal when given repetitively. At even lower doses we observe a threshold ("chronic lethal dose") below which the dose cannot kill the ProtoTissue™, but only induce reversible changes in its cellular activity. Correlation of these values can be correlated to the published values for the chronic or acute lethal plasma concentrations in man for acetaminophen, amiodarone, diclofenac, metformin and valproic acid suggesting that it might be possible to predict the therapeutic window and the lethal toxic dose in vivo from these in vitro studies using ProtoTissue™.

The stability of ProtoTissue™ over many months, combined with its ability to recover following repeated treatment to normal (pre-treatment conditions) suggests that ProtoTissue™ has the potential to be a valuable tool for the investigation of drug-drug interactions and drug induced liver injury.

Drugmode's current plans are to commercialise this system during 2011– possibly in collaboration with an investment partner.

LECTURES

L11. STABILITY OF TRANSPORTERS AND CYTOCHROME P450S IN HEPATOCYTE CULTURE SYSTEMS

Jacob Kramer Jacobsen, PhD fellow
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Drug metabolism in hepatocytes is quantitatively the most important biotransformation pathway in the detoxification of most drugs currently available on the market. Two of the most important players in detoxification are the active uptake- and efflux transporters, and the cytochrome P450 metabolizing enzymes.

When using in vitro hepatocyte cultures for studies of metabolism, clearance and cytotoxicity it is therefore of great importance to understand the level of activity of both uptake- and efflux transporters and cytochrome P450s in the culture system in use.

In a situation where reactive drug metabolites are suspected of being involved in drug toxicity, the absence of efflux transporters might result in higher hepatotoxicity in vitro, while this will be much reduced in vivo. On the other hand, the absence of uptake transporters in vitro may lead to a complete absence of toxicity for molecules that may be highly toxic on intracellular targets or metabolized by intracellular enzymes to toxic agents.

Hence, investigations of drug metabolism and pharmacokinetics, drug-drug interactions and cytotoxicity are all affected by the limitations of the current hepatocyte culture systems and failing to acknowledge these limitations will lead to erroneous interpretations of experimental results.

It is therefore of the utmost importance to characterise the standard hepatocyte culture systems (suspension, monolayer and sandwich culture) in order to guide on the choice of the right system for a specific study. Our current work is focused on assessing the stability of Oatp, Oct, Mrp2 and Mrp3 transport activity, and stability of the CYP1A1/2, 2B1/2, 3A1 and 2D1 activity in cultures of cryopreserved rat hepatocytes.

LECTURES

L12. AN UPDATE ON METABOLISM STUDIES USING HUMAN HEPATOCYTES IN PRIMARY CULTURE

María José Gómez-Lechón, José V. Castell, Agustín Lahoz and María Teresa Donato

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Drug metabolism is one of the key determinants of a drug's disposition in the body, and interindividual pharmacokinetic differences, and an indirect determinant of the clinical efficacy and toxicity of drugs. Drug metabolism includes Phase I and Phase II reactions by which lipophilic xenobiotics are rendered more polar to facilitate their elimination from the body. Similarly to P450 system, each family of phase II enzymes consists of multiple members that differ in terms of substrate specificity and response (inhibition, induction) to xenobiotics. Primary cultured human hepatocytes are differentiated cells expressing the full contingent of drug-metabolizing enzymes and transporter proteins constituting a very predictive model for drug metabolism *in vivo*. Therefore, they are presently considered the "gold-standard" model to anticipate *in vivo* metabolic pathways of drugs or new chemical entities. Liver procurement, hepatocyte isolation procedure and culture anditions (namely, culture configuration, medium composition and plating density) are key issues for the expression of phase I and phase II drug metabolising enzymes. The variability observed in human cultured hepatocytes reflects the existing phenotypic heterogeneity of the P450 expression in human liver. The pharmaceutical perspective is to use hepatic models as predictable of the *in vivo* situation as capable of generating sensible data on drug metabolism, which can be used to make 'go, no-go' decisions. To speed up the selection of drug candidates, the evaluation of metabolic stability, metabolite profiling and identification, and drug-drug interaction potential are key issues in drug development. Advances in the field include: 1) Improvment of cell isolation, culture configuration and definition of media for prolonged survival and maintenance of differentiated functions. 2) The use of cryopreserved hepatocytes for drug metabolism studies has notably increased due to the improvement of cryopreservation procedures to allow cell attachment, thereby extending the use of the cells to assays that requires prolong culturing such as enzyme induction studies. 3) New methodolgies to assess drug metabolising capability and drug-drug interactions. The most recent strategy for the fast analysis of major P450 activities as well as Phase II reactions is the simultaneous screening of enzymatic activities by co-incubation of hepatocytes with a cocktail of several specific probe substrates, and analysis by liquid chromatography tandem mass spectrometry (LC/MS/MS). The high sensitivity and selectivity of this methodology allows traditional assays to be minimized, and has become the method of choice for the fast and routine assessment of P450 activities in early drug discovery and development. Moreover, cocktail strategies and/or miniaturized assay formats will notably helps to optimize the throughput of induction screenings in human hepatocytes. 4) Because of the intrinsic variability and limited accessibility of primary cultured human hepatocytes, the development of alternative 'hepatocyte-like' cell models capable to mimick P450 genetic variability, or P450 induction/inhibition by modulating the expression of individual P450s, without modifying the expression of the others, is being developed. New cell lines co-expressing multiple human enzymes (P450s, conjugating enzymes) mimicking hepatocyte-like cells might represent an alternative to primary hepatocytes and a useful tool for preclinical screening. 5) Finally, a promising tool for the near future will be functional hepatocytes derived from hES and iPS cells which have the potential to combine specific differentiation with an excellent availability for clinical applications and *in vitro* testing.

ABSTRACTS

ORAL COMMUNICATIONS

(presented as posters as well)

ORAL COMMUNICATIONS

OC1/P1. MAINTENANCE OF HEPATIC PHENOTYPE AND FUNCTION IN NOVEL HUMAN 3D TISSUE CULTURES FROM RECONSTITUTED PRIMARY FETAL LIVER TISSUES

Cliff Rowe, Andrew Berry, Jane E. Alder,[1] Lorna Kelly[2] Rosalind E. Jenkins[2], Christopher E. P. Goldring[2], Neil R. Kitteringham[2], Karen Piper Hanley, Lars Sundstrom[3], Neil A. Hanley. University of Manchester, Department of Endocrinology and Diabetes, AV Hill Building, Oxford Road, Manchester, M13 9PT, UK.
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Objectives: The paucity of readily available and functional *in vitro* liver models for hepatotoxicity screening is a major problem to drug development programmes. Primary culture of freshly-isolated adult human hepatocytes is the best available model but shortfalls include unpredictable supply, and de-differentiation during culture. Fetal liver cells, however, are expandable *in vitro* and have the potential to be a useful adjunct to adult cells.

Experimental: Fetal liver tissue was characterised using quantitative proteomics and immuno-histochemical analyses. Fetal liver cells, were cultured for 2 weeks in serum-free Williams' E medium supplemented with ITS and 100nM dexamethasone. Cells were maintained in Matrigel sandwich culture or as high density 3D cultures on semi permeable membranes at the liquid-air interface. Medium was changed after 24h and every 2 days subsequently. Collected medium was assayed for albumin (Bethyl) and urea (Bioassay systems). CYP3A activity was determined by P450glo (Promega) at the same time-points.

Results: Quantitative iTRAQ proteomics, (Fig 1), and immuno-histochemical analyses (Fig 2) show many of the proteins that influence hepatotoxicity, such as major cytochrome P450 enzymes and hepatic transporters are expressed in fetal liver. Albumin secretion (Fig 3a) and urea production (not shown) showed similar trends, increasing throughout the two week culture period in 3D cultures but declining after 5 days in Matrigel culture. CYP3A activity (Fig 3b) increased in the first week of culture and stabilised in 3D culture whereas it consistently declined in Matrigel culture.

Conclusion: In conclusion 3D cultures of fetal liver cells, maintained in culture for two weeks, retain the capacity to deliver a functional liver model for hepatotoxicity studies.

Figures:

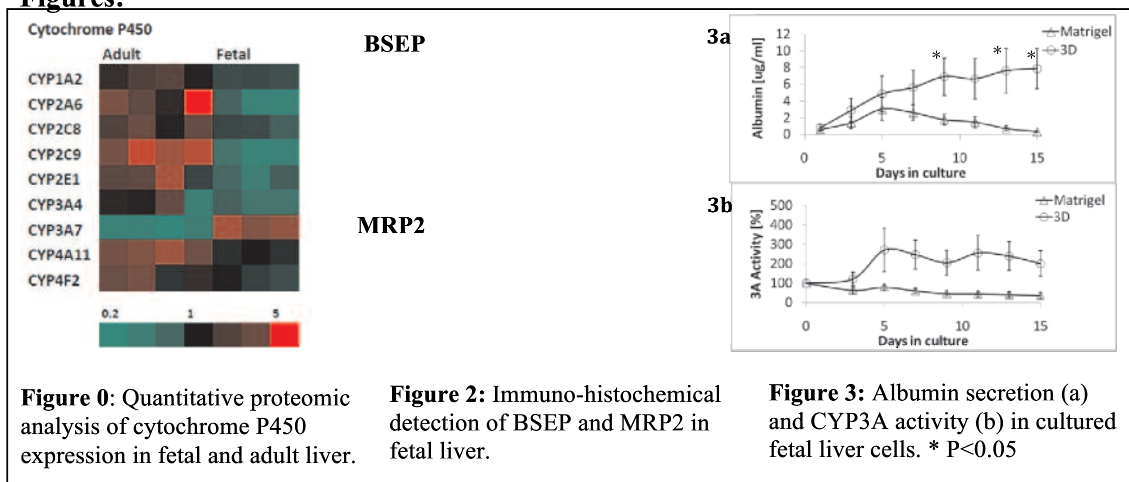

Figure 0: Quantitative proteomic analysis of cytochrome P450 expression in fetal and adult liver.

Figure 2: Immuno-histochemical detection of BSEP and MRP2 in fetal liver.

Figure 3: Albumin secretion (a) and CYP3A activity (b) in cultured fetal liver cells. * P<0.05

ORAL COMMUNICATIONS

OC2/P2. THE HEPATOTROPHIC GROWTH FACTOR AUGMENTER OF LIVER REGENERATION (ALR) PROTECTS HUMAN HEPATOCYTES AGAINST INDUCED APOPTOSIS BY UP-REGULATION OF SURVIVAL PATHWAYS

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Objectives: The liver is a highly metabolic organ with a great potential for regeneration. Besides mitogenic signals via hepatotrophic growth factors like EGF (epidermal growth factor), HGF (hepatocyte growth factor) and ALR, liver regeneration is also triggered by metabolites and anti-apoptotic mechanisms. So far potential protective effects of growth factors like HGF and EGF have been described. The aim of this study was to investigate the hepatoprotective effect of rhALR in vitro.

Experimental: Primary human hepatocytes (phH) and hepatic cell lines were incubated in the presence or absence of rhALR and the exposed to different concentrations of apoptosis inducers (ethanol, actinomycin D, TRAIL, TGF-β, Anti-Apo). The influence of the apoptosis inducers on cell proliferation was analyzed by MTT assay. As an indicator of irreversible cell death, the leakage of intracellular LDH into the culture medium was measured as well as the intracellular GSH/GSSG ratio (indicator of cellular oxidative stress). The amount of induced apoptosis was evaluated by FACS analysis with propidium iodide (PI) staining. The proteins involved in signaling pathways leading to cell death or survival (cytochrome c, Akt/PKB) were analyzed by western blot.

Results: Recombinant hALR treatment resulted in increased proliferation of HepG2 cells and primary human hepatocytes. The collected data revealed a concentration-dependent increase in cell damage when cells were incubated with different apoptosis inducers (ethanol, actinomycin D, TRAIL, TGF-β, Anti-Apo) as well as a protective effect by rhALR (FACS analysis, enzyme leakage). This was also confirmed by measuring cytochrome c release into the cytosol. The GSH/GSSG ratio showed that rhALR could improve the redox status of the cells when treated with ethanol. Anti-apoptotic effects of rhALR could be due to increased phosphorylation of Akt, part of an important survival pathway. In comparison to other growth factors showing an anti-apoptotic effects like HGF and EGF, the protective effect of ALR is liver specific.

Conclusion: Unlike HGF and EGF rhALR acts in a liver specific manner and shows not only mitogenic but also protective effects through the PI3K/AKT signalling pathway. This effect implies that rhALR treatment could potentially reduce damage caused by ethanol intoxication or different apoptotic agents. Further *in vivo* tests will be needed to verify its protective effect with other toxicants as well as during ischemia/reperfusion.

ORAL COMMUNICATIONS

OC3/P3. SYSTEM-DEPENDENT INHIBITION OF CYTOCHROME P450 (CYP) ENZYMES

David B. Buckley, Andrew Parkinson, Faraz Kazmi, Phyllis Yerino, Brian W. Ogilvie and Brandy L. Paris
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The ability of a drug to cause clinically significant drug-drug interactions due to direct or metabolism-dependent inhibition of cytochrome P450 (CYP) can generally be predicted from in vitro studies with human liver microsomes (HLM) or recombinant CYP enzymes, as recommended by the FDA and other regulatory agencies. This presentation highlights some examples of system-dependent inhibition of CYP enzymes. In the case of CYP enzymes, examples are presented where in vitro studies with HLM under-predict or over-predict the degree of inhibition observed in the clinic and where the correct prediction comes from studies with human hepatocytes. Studies with HLM under-predict the ability of gemfibrozil and bupropion to cause clinically significant inhibition of CYP2C8 and CYP2D6, respectively, and over-predict the ability of ezetimibe to cause clinically significant inhibition of CYP3A4. Gemfibrozil and bupropion represent examples of glucuronidation-dependent and reduction-dependent activation to metabolites that inhibit CYP2C8 and CYP2D6, respectively, whereas ezetimibe represents an example of glucuronidation-dependent protection against metabolism-dependent inhibition of CYP3A4. This presentation illustrates why, when drug candidates are extensively metabolized by non-CYP enzymes, it would be prudent to use human hepatocytes in addition to HLM or recombinant enzymes to evaluate their ability to inhibit CYP enzymes.

ORAL COMMUNICATIONS

OC4/P4. OPTIMIZATION OF THAWING, CULTURE, TREATMENT AND ENZYME ACTIVITY DETERMINATION OF CRYOPRESERVED HUMAN HEPATOCYTES (CRYOHEPS) FOR THEIR USE IN THE ECVAM INTERNATIONAL VALIDATION STUDY OF CYTOCHROME P450 INDUCTION

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An ECVAM validation study on cytochrome P450 induction was kicked off end 2008. A Validation Management Group, with major expertise in validation of *in vitro* models, toxicokinetics and metabolism, agreed that such a validation study would be very welcome as it will provide a standard assay for human hepatic metabolism and toxicity evaluation. CYP450 induction was chosen as it has a complex underlying mechanism (gene activation followed by *de novo* protein synthesis) and is a good indicator for high quality metabolic competent systems. Human derived HepaRG cell line and Cryopreserved Primary Human Hepatocytes (CryoHeps) were considered as suitable test systems.

As a first preliminary step, the purpose of our group was to optimize and standardize the use of plateable human CryoHeps for induction studies. The results of the present study allowed us to define:

- the thawing protocol reproducible in terms of cell recovery and viability as well as attachment rate,
- the seeding density of viable CryoHeps in collagen home-coated 48-well plates optimal for the response to reference inducers,
- the culture medium optimal for the 72h treatment period with reference inducers and test compounds,
- the substrate cocktail (incubation medium, substrate concentrations, time of incubation) to be used for enzyme activity determinations.

Taking into account the results of this pre-validation step, the final protocol for the ECVAM validation study on CYP450 induction has been defined and is currently in use.

Keywords: cryopreserved human hepatocytes, screening, induction, 48-well culture, cocktail incubation

ORAL COMMUNICATIONS

OC5/P5. COMPARISON OF DIFFERENT PROCESSING TIMES, TEMPERATURES AND STORAGE METHODS ON RNA STABILITY IN HUMAN LIVER

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Tissue bank under the authority of Human Tissue and Cell Research (HTCR) Foundation, Department of Surgery, Grosshadern Hospital, Ludwig Maximilians University, Munich, Germany

Background: The Grosshadern Hospital Tissue Bank is a repository of clinically annotated surgical remnant tissues that can be accessed by researchers for basic and translational research. An important technology for elucidating molecular mechanisms of diseases is reverse transcription polymerase chain reaction (RT-PCR). However, the accuracy of information garnered by RT-PCR is highly dependent on tissue quality. Previous work by other investigators has examined effects of varying surgical durations on RNA quality in various tissues. However, to our knowledge, the effect of processing time, temperature and storage method on human liver RNA quality is not known. Thus, this study aimed to determine if tissues with longer processing times are still suitable for RT-PCR studies and if processing temperatures or storage in RNAlater would help maintain RNA quality.

Methods: Liver samples were collected over a time course; during surgery before blood arrest, after surgery, post sampling by the pathology department (T_{pp}), 3 hours after T_{pp} and 1 day after T_{pp}. For the last 2 time-points, samples were kept at either room temperature or on ice after Tpp for the duration. All samples were then stored with or without RNAlater at -80ºC. Subsequently, these tissues were assessed for RNA quality (by Bioanalyzer, 28S/ 18S ratios) and relative expression levels of 5 genes (*HPRT*, *GUSB*, *MYC*, *GFER*, *HIF1*a).

Results: RNA quality was only significantly decreased by 1.9-fold 1 day after T_{pp} at RT with or without RNAlater. However, normalized relative gene expressions of *HPRT*, *GUSB*, *MYC, HIF1*a and *GFER* were not significantly different when the various time-points were compared against each other ($P > 0.05$). Also, there were generally no significant differences between samples stored with or without RNAlater ($P > 0.05$).

Conclusions: It is recommended that samples be kept on ice during processing and that they be processed as quickly as possible for storage at -80ºC. However, when longer processing times are inevitable, samples can still be used for RT-PCR studies, provided that relative expression of the gene of interest is proven not to be affected. Thus, this study shows that samples with longer processing times can still be banked as they could potentially provide valuable information. This relaxed criterion will increase accrual of valuable samples to the tissue bank.

ORAL COMMUNICATIONS

OC6/P6. NEW IN-VITRO SYSTEMS AS ORGAN MODELS FOR HEPATIC CULTURE

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Introduction

Hepatocytes in-vivo are constantly subject to three types of cues or signals: biochemical cues, including ligands, signaling molecules and other cells, physicochemical cues which comprises gradient-dependent factors such as surface properties, oxygen tension, pH and temperature, and finally mechanical and structural cues. These include the 3-dimensional (3D) architecture and mechanical forces such as stress and strain. Many researchers agree that recapitulating this complex microenvironment in-vitro is the key towards efficient endogenous and xenobiotic metabolism in hepatic cells.

Objectives

Our objective is to reproduce as many features as possible of the in-vitro hepatocyte micro-environment so as to better understand interactions between different cell types in the presence of physical and biochemical stimuli, with particular reference to the liver and the metabolic system. Therefore we have designed a new cell culture system composed of interconnected bioreactor modules which combines different stimuli while conserving metabolic, volumetric and exchange rate relationships between cells.

Experimental

A modular multicompartment bioreactor (MCmB) system with dimensions similar to a 24 well plate was designed using allometric laws which mathematically correlate non linear quantities (organ mass, blood flow, blood retention time, metabolic rate). The bioreactor was seeded with different cell types with and without 3D scaffolds including primary rat and human hepatocytes, human endothelial cells, glioblastoma cells and human adipocytes. The complexity of the system was increased step-wise in order to assess the independent and combined effects of flow, transport, 3D architecture and cell-cell crosstalk on cell function.

Results

Our studies indicate that a 3D architecture is essential for establishing cell-cell interactions and high cell densities, while flow mediates xenobiotic metabolism. On the other hand nutrient turnover and clearance appear to play a key role in maintain equilibrium levels of functional proteins such as albumin.

Conclusions

Overall we demonstrate that for a given set of cell types, the overall system response is a function of 3 variables: cell numbers and cell ratios, passage times or flow rates, and total volume. In order to elicit physiologically meaningful responses a cell culture system requires firstly physiologically scaled cell numbers and ratios, and secondly flow rates which do not cause shear stress related damage to cells and which allow adequate oxygen concentrations and residence times to enable cells to process metabolic signals. The MCmB system, due to its intelligent design and flexibility, can be used to tune each of these parameters to bring in-vitro experiments on drug and molecular metabolism closer to the in-vivo context.

ORAL COMMUNICATIONS

OC7/P7. HEPATIC UPTAKE TRANSPORT IN INDIVIDUAL VERSUS POOLED CRYOPRESERVED HUMAN HEPATOCYTES

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Incubation with human hepatocytes can be considered the best *in vitro* approach to predict *in vivo* hepatic clearance of drug candidates. To maximize the use of available human donor material, cryopreservation protocols have successfully been developed. Due to differences in quality of donor material as well as variable demographical background of donors, large inter-batch variability in uptake transport activities has been demonstrated among different batches of cryopreserved human hepatocytes (CryoHH). While CryoHH can be pooled to overcome this issue, no direct comparison between transport activities of individual and pooled CryoHH has yet been conducted.

The aim of this study was to compare transport activities in individual versus pooled batches of CryoHH. Transporters investigated included: NTCP (Sodium Taurocholate Co-transporting Polypeptide), OATP (Organic Anion Transporting Polypeptide) and OCT (Organic Cation Transporter). The uptake of 5 different substrates (1µM, 1 min) was assessed among 10 different individual and 2 pooled batches of CryoHH (CellzDirect, Inc.). Known inhibitors were used to evaluate the contribution of active processes to overall substrate uptake.

The uptake clearance of taurocholate (NTCP) in individual batches ranged from 13.8 to 66.9 µL/min/mg protein with an average of 30.3 ± 16.8 µL/min/mg protein which was comparable with the uptake clearance in pooled batches of CryoHH (24.0 ± 3.3 and 34.9 ± 2.0 µL/min/mg protein). When extracellular sodium was replaced by choline, the uptake clearance in both individual and pooled batches of CryoHH decreased by 85-90 %.

The mean uptake clearance of digoxin (OATP1B3), estrone-3-sulphate (ES, OATP1B1/1B3) and estradiol-17β-glucuronide (E217βG, OATP1B1) in individual batches of CryoHH amounted to 6.1 ± 2.4 µl/min/million cells, 79.4 ± 24.9 µl/min/million cells and 11.7 ± 9.0 µl/min/million cells, respectively. For all three OATP substrates, mean individual uptake was comparable with the uptake values in pooled batches. The presence of the known OATP inhibitor rifampicin (25 µM) decreased the uptake clearance of ES and E217βG by 50 % and 34 %, respectively in both the individual and pooled batches.

The uptake clearance of the OCT substrate 1-methyl-4-phenylpyridinium (MPP+) in the individual batches ranged from 10.1 to 39.8 µL/min/mg protein with an average of 17.2 ± 9.0 µL/min/mg protein and was lower in pooled batches of CryoHH (9.2 ± 1.1 and 13.3 ± 1.7 µL/min/mg protein). Co-incubation with the OCT inhibitors prazosin (3µM) and corticosterone (1µM) showed an average decrease by 26 % and 18 %, respectively.

In conclusion, this study clearly demonstrates that cryopreserved human hepatocytes can be used to study *in vitro* hepatic uptake. In general, experiments in pooled hepatocytes showed uptake values which were comparable with the mean of the individual batches, thus encouraging the use of pooled cryopreserved hepatocytes for *in vitro* hepatic transport studies.

ORAL COMMUNICATIONS

OC8/P8. MEDIUM FLOW STIMUTATES THE EXPRESSION AND ACTIVITY OF DETOXICATION GENES IN PRIMARY HUMAN HEPATOCYTES CULTURED IN A MULTICOMPARTMENT MODULAR BIOREACTOR

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Objectives
Primary cultures of normal adult human hepatocytes are being considered as the gold standard for investigating drug metabolism and side effects. However, a frequent concern while using these cultures is the early down-regulation of many genes involved in the detoxication function, that is, cytochromes P450 (CYPs), conjugation enzymes and xenobiotic membrane transporters. It has been shown that the expression of several CYP increases in response to high shear stress in vein/artery endothelial cells, although the molecular mechanism of this effect is unknown. The aim of this work was to evaluate the influence of culture medium flow on a panel of human hepatocyte markers.

Experimental
For this purpose, hepatocytes prepared from 12 different liver donors were cultured either in a multicompartmental modular bioreactor system (MCmB, flow rate from 250 to 500 µl/min) or in standard static conditions and the expression of a number of genes, enzyme activities and biological parameters were measured 7 to 21 days later.

Results
Although some of tested genes were either unaffected or down regulated by medium flow, mRNA expression of AhR- and CAR/PXR-responsive genes involved in xenobiotic/drug metabolism and transport including CYP1A1, 1A2, 2B6, 2C9, 3A4, UGT1A1, GSTα, MDR1 and MRP2 (as well as midazolam and tolbutamide hydroxylation activities) were specifically upregulated in the MCmB as compared with the static controls. In 2 week-old cultures, expression of these genes reached levels that were close to or higher than those measured in freshly isolated hepatocytes. These results suggest that the medium flow interferes with, and upregulates the detoxication function in primary human hepatocytes. Whether medium flow interferes with and stimulates the transcriptional activity of AhR, CAR or PXR xenosensors or regulates directly the expression of detoxication genes is currently unknown and will require further investigations.

Conclusions
Primary human hepatocytes respond to culture medium flow by an increased expression of several genes involved in the detoxication function. The new MCmB system could therefore be used to study drug metabolism and toxicity *in vitro* in long term cultures and for prospective pharmacological or pharmacokinetic studies.

ORAL COMMUNICATIONS

OC9/P9. DEVELOPMENT OF A LIVER MICROFLUIDIC BIOREACTOR FOR TOXICITY AND DRUG METABOLISM INVESTIGATIONS.

Prot J-M, Baudoin R, Videau O., Legallais C., Bénech H.* and Leclerc E.*

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Context: Pharmaceutical and biotechnology industries are in need of improved *in vitro* model systems for predicting potential drug toxicity, efficacy or disposition. For the liver, such models should be able to closely reproduce biotransformation and clearance phenomena.
Experimental: The microbioartificial liver consists in a microstructured PDMS chamber dedicated to living cell cultures. A reservoir extended by silicon tubing is connected to the bioreactor in a close loop circuit. A peristaltic pump controls the flow for dynamic cultures, ensuring continuous feeding of nutrients, and waste removal. Cell cultures were performed for several hours to several days with 2 different cell types such as HepG2/C3A hepatocarcinoma cell line and cryopreserved human hepatocytes (fig.1).
Results: Compared to the monolayer formed in classical static Petri cultures, a 3D tissue organization was observed in the microfluidic bioreactor with HepG2/C3A. Moreover, the dynamic microenvironment enhanced the cell fonctionality in term of glucose and glutamin consumption, albumin and ammonia production. After 96 hours of culture inside the bioreactor, CYP1A activity was confirmed by EROD assay suggesting that cells maintained their functionality.
In a second time, the biotransformation capability of primary human hepatocytes in contact with a 7 molecules cocktail (CIME cocktail) was assessed. In the bioreactor, we observed larger CYP3A4, CYP2C9, CYP2D6, CYP1A2, CYP2C19 and UGT activities after 4 hours of dynamic culture by analysing substrtates and metabolites with a LC-MSMS method (fig.2). These results suggest that dynamic conditions allow for an enhancement in cells functionality.
Conclusion: Dynamic environment is beneficial to human hepatocyte cell cultures. Our microfluidic bioreactor have an important potential for the *in vitro* study of drug metabolism with human cell line or primary human hepatocytes.
Future studies will focus on the validation of the bioreactor use by comparison with *in vivo* data. A parallelized system based on this technology is under development to increase the throughput.

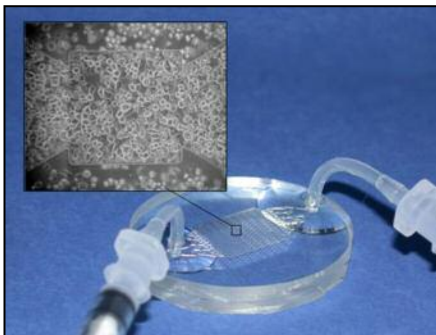

Fig.1: Bioreactor picture and primary human hepatocytes cultivated inside the microstructured chamber.

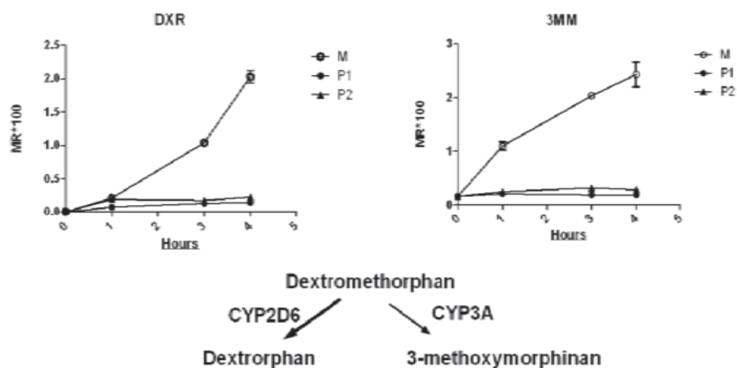

Fig.2: Metabolic ratio of Dextromethorphan in microbioartificial liver (M) and hepatocytes classical static Petri cultures (P1 and P2).

ORAL COMMUNICATIONS

OC10.P10. A NEW MITOCHONDRIAL-BASED APOPTOTIC TEST ADAPTED TO HEPARG HEPATOCYTES APPLIED TO HIGH THROUGHPUT SCREENING OF HEPATOTOXIC COMPOUNDS USING AUTOMATED IMAGING ANALYSIS

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Hepatotoxicity of new drugs and other chemicals must be detected before selection to extensive transfer analyses. HepaRG is an hepatic cell model known to express most of specific liver functions at higher levels than other cell lines, including detoxifying enzymes but also sinusoidal and canalicular drug transporters and have retained the regulatory pathways controlling most of them(1,2). Our objective was to develop a multiparametric test *in situ*, reliable, miniaturized and capable of screening chemical substances inducing cellular toxicity on differentiated HepaRG hepatocytes. The loss of mitochondria integrity in the altered cells, revealed by the JC-1 fluorescent mitotracker dye was chosen and associated with two specific markers for analyses with automated quantitative fluorescence microscopy and imaging analysis. We first, defined optimal culture conditions allowing to get purified HepaRG hepatocyte monolayers with high functional stability, including their detoxication function. Hepatocyte density was found esential to preserve differentiation and the number of 40 000 purified cells per well of 96 wells plates was retained as most efficient in combination with DMSO which contributed to block cell reversion to progenitors. Then, conditions of JC-1 dye loading in HepaRG mitochondria were monitored. In contrast to other cell types, HepaRG cells appeared refractory to dye saturation. We postulated that MDR transporters or detoxification enzymes present in abundance in HepaRG hepatocytes could contribute to JC-1 efflux. Indeed, JC-1 was found to be one main substrate for MDRs and for CYP3A4 to a lesser extend, a process which could be suppressed by saturation incubation with specific target substrates. Finally, reliable mitochondrial-based hepatoxicity test was settled using 2 successive charges. Robustness was demonstrated with a set of referent toxic substances including staurosporin, 4-nitroquinolin, aflatoxin B1, and MMS, and its efficiency was similar to the well established AnnexinV test. In conclusion, the JC-1 test adapted to HepaRG hepatocytes represents a new powerfull apoptotic test competent to automated image-based detection of hepatotoxic compounds.

(1) P. Gripon et al. Proc. Natl. Acad, 2002, 99, 15655; (2) Aninat C.et al., 2006, Drug Metab Dispos. 34(1):75.

ABSTRACTS

POSTERS

- Hepatocytes from stem cells
- RNA interference
- Drug transporters
- Hepatocyte culture improvements
- Toxicology and Metabolism
- Methodologies

POSTERS - HEPATOCYTES FROM STEM CELLS

P11. SCALABILITY AND ROUTINE PRODUCTION OF FUNCTIONAL HEPATOCYTE-LIKE CELLS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS

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Human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) have the capability of self-renewal and pluripotency and therefore they hold an enormous promise of providing an unlimited source of specific cell types, including functional hepatocytes. Hepatocytes derived from human pluripotent stem cells are expected to become exceptionally useful as a human *in vitro* system for studying drug targets, metabolism, and toxicity, and in cell replacement therapies. An additional *in vitro* area of great interest is the use of human hepatocytes in so called extracorporeal livers, or bioartificial liver devices.

We have differentiated hESC and hiPSC into hepatocyte-like cells by using four-step differentiation protocols guiding the cells through discrete stages recapitulating liver development. The resulting cells morphologically closely resemble human hepatocytes and express hepatic markers both on mRNA and protein level, as well as several functional hepatic features. Characterisation will be shown from human pluripotent stem cells differentiated into hepatocyte-like cells. Moreover, we will illustrate how routine production and scaling up the quantities of such cells is performed. Finally, some novel *in vitro* application of these technologies will be described.

In conclusion, the generated hepatocyte-like cells have a great potential as a future unlimited source of functional human cells useful in various drug discovery applications.

POSTERS - HEPATOCYTES FROM STEM CELLS

P12. DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO HEPATOCYTE-LIKE CELLS BY USING 3D CULTURE SYSTEMS

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Pluripotent stem cells like human embryonic stem (hES) cells are some of the most powerful cell types known. Due to their high proliferative capacity and their capability to differentiate into all mature cell types of the human body, hES cells are potentially valuable as tools in drug discovery e.g. for drug metabolism and safety assessment studies. Hepatocytes are considered to be one of the most important cell types for these processes. Cell based in vitro assays with high human relevance are ur gently needed for pre-clinical activities, e.g. target identification, screening of compound efficacy and safety assessment studies. Currently used cell systems e.g. human primary hepatocytes are limited by availability as well as functionality, as primary hepatocytes rapidly lose their functionality when cultured in vitro. Animal tests are neither cost effective nor very clinical relevant or predictive for humans. Hepatocytes derived from hES cells possess a great hope for providing an unlimited supply of improved in vitro models based on human cells, in homogenous assays. hESC-derive hepatocyte-like cells cultured in conventional 2D culture dishes show a morphology that closely resemble primary hepatocytes, and express a number of hepatic markers on mRNA and protein levels. However, for the cells to be useful for e.g. drug safety studies, it is important that the hepatocytes are metabolically competent expressing enzymatic activity and inducibility. We have applied different 3D culture models to our protocols for differentiation of human embryonic stem cells into hepatocytes. By combining 3D culture models with hepatic differentiation we have improved the hepatic functionality and thereby the usefulness of the derived cells. Human stem cells, especially hESC have a tremendous potential to become a large-scale source of specialised human cells and assays for use in predictive pharmaceutical research and development. This is foreseen to significantly contribute to more cost-efficient but also safer new drugs in the future. References: Jensen et al J Cell Physiol. 2009, 219 (3)213-9. Review

POSTERS - RNA INTERFERENCE

P13. A REVERSAL OF THE MULTIDRUG RESISTANCE PHENOTYPE IN HEPATOCELLULAR CARCINOMA USING RNA INTERFERENCE

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ATP-binding cassette, sub-family C transporters ABCC1 and ABCC2, also known as multidru resistance proteins MRP1 and MRP2, are involved in tumor resistance by decreasing th intracellular concentration of toxic compounds. This is a phenotype caused by overexpression o these transporters in tumor cells. Overcoming increased drug extrusion could lead to significan improvement of chemotherapy effectiveness for hepatocellular carcinoma (HCC) patients. RN interference is a suitable approach to knock-down ABCC1/2 genes in HCC, here combined wit an AAV-mediated delivery.

In this study we designed short hairpin RNAs (shRNAs): 6 targeting murine Abcc1 and targeting murine Abcc2. In vitro assays in murine HCC cells showed endogenous mRNA knock down of more than 50% with shAbcc11, 17, and shAbcc22, 23, 27, 28. The knock-down effec was sequence-specific and not due to shRNA off-targeting or toxicity.
In order to determine whether the endogenous Abcc1 and Abcc2 mRNAs can be knocked-dow in vivo, we assessed the liver transduction efficiency of the hydrodynamic tail vein injectio method and self-complementary adeno-associated virus serotype 8 (scAAV8). scAAV8 is clearl superior with 100% hepatocytes transduced against 15% with HTV. scAAV8 expressin shAbcc11/17, shAbcc22/28 were therefore produced and injected in BL/6 mice and knock-dow of Abcc1/2 was determined 2 weeks post-injection.
A multidrug resistant tumor model was developed by subcutaneous injection of murine HCC cell in BL/6 mice. Expression of Abcc1/2 was first induced in cells with doxorubicin and cells wer then injected subcutaneously. We will monitor the knock-down of Abcc1/2 in this multidru resistant tumor model system because of its relevance to HCC. Preliminary data will be presented

We developed promising RNA interference constructs which achieved knock-down of Abcc1/2 i vitro. We are now proceeding with 4 shRNAs in 2 in vivo models in mice: healthy liver an multidrug resistant tumor model. Knocking-down multidrug resistance proteins may improve th effectiveness of the current anti-cancer therapies.

POSTERS - RNA INTERFERENCE

P14. EFFICIENT SMALL-INTERFERING RNA-MEDIATED KNOCKING DOWN OF ARYL HYDROCARBON RECEPTOR IN PRIMARY HUMAN HEPATOCYTES RESULTS IN ALTERATION OF BASAL AND TCDD-INDUCED EXPRESSION OF TARGET GENES

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Objectives: Aryl hydrocarbon receptor (AhR) is a drug-sensing receptor activated by environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and is known to drive regulation of target genes in various human cell types. Its involvement in TCDD-mediated regulation of target genes in human hepatocytes however remains to be formally demonstrated. To gain insights into this point, we have analyzed the effects of AhR silencing on the regulation of various genes targeted by TCDD in primary human hepatocytes.

Methods: Efficient AhR knocking-down was performed through dimethyl sulfoxide-based transfection of small-interfering RNAs targeting AhR (siAhR) in primary culture of human hepatocytes. SiAhR-transfected cells were next treated by TCDD during 8 h and expression levels of various target genes were analyzed by RT-qPCR or enzymatic activity.

Results: SiAhR-transfected human hepatocytes exposed to TCDD, were found to exhibit reduced mRNA expression of various TCDD-responsive genes, i.e. CYP1A1, CYP1A2, CYP1B1, ALDH3A1, IL17RB, FER1L3 and SLC7A5, when compared to TCDD-treated counterparts transfected with non-targeting small-interfering RNAs. AhR silencing was moreover shown to markedly counteract TCDD-mediated induction of CYP1A1/CYP1A2/CYP1B1-related ethoxyresorufin O-deethylase activity in human hepatocytes. It also concomitantly decreased constitutive mRNA expression of some target genes such as CYP1A1, CYP1A2, CYP1B1 and ALDH3A1.

Conclusion: Taken together, these data indicate that AhR plays a crucial role in both basal and TCDD-induced expression of target genes in human hepatocytes.

POSTERS - DRUG TRANSPORTERS

P15. SODIUM FLUORESCEIN AS SIMPLE SUBSTRATE TO EVALUATE DRUG INTERACTION POTENTIAL WITH THE HEPATIC TRANSPORTERS OATP1B1 AND OATP1B3

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Members of the human organic transporting polypeptide (hOATP/SLC21) family are key transporters in the hepatic uptake of many endogenous (e.g. bilirubin) and exogenous (e.g. statins) compounds; they often constitute the underlying mechanism in clinical drug-drug interactions. Fluorescein-labeled substrates like fluorescein-methotrexate, cholyl-glycylamido-fluorescein and cholyl-L-lysyl-fluorescein are often used to study these hepatic transport-mediated interactions. However, the hepatic uptake characteristics of the fluorescein entity, as such, have never been thoroughly clarified. This study aimed to elucidate the *in vitro* hepatic uptake of sodium fluorescein (NaFluo) and to demonstrate its use in evaluating OATP1B1 and OATP1B3-mediated drug interactions.
The cellular uptake mechanisms and kinetics of NaFluo were evaluated in freshly-isolated rat hepatocytes, cryopreserved human hepatocytes, and in OATP1B1- and OATP1B3-transfected CHO cells. To correct for passive diffusion and non-specific binding, uptake values obtained in experiments conducted at 4°C and in wild-type CHO cells were subtracted from uptake values obtained at 37°C in hepatocytes and transfected CHO cells, respectively.
Concentration-dependent uptake experiments of NaFluo revealed Michaelis-Menten kinetics in hepatocytes as well as in OATP1B1- and OATP1B3-transfected CHO cells. Kinetic parameter values were: Km = 18.7 ± 1.6 µM and Vmax = 91.3 ± 3.8 pmol/million cells/min for rat hepatocytes (n = 4), Km = 14.1 µM and Vmax = 5.8 pmol/million cells/min for human hepatocytes (n = 1, pooled), Km = 12.2 ± 4.9 µM and Vmax = 33.2 ± 3.4 pmol/million cells/mg protein for OATP1B1-transfected cells (n = 4) and Km = 30.1 ± 3.8 µM and Vmax = 195.6 ± 16.6 pmol/million cells/mg protein for OATP1B3-transfected cells (n = 4). In rat hepatocytes, the uptake of NaFluo significantly decreased when NaFluo was incubated with known inhibitors of the Oatp-family, while inhibitors of Oat (organic anion transporter) and Oct (organic cation transporter) had no significant effect. When NaFluo was incubated with HIV protease inhibitors, a concentration-dependent decrease in uptake was shown. Uptake experiments in rNtcp-transfected CHO cells showed that contribution of Ntcp (sodium taurocholate cotransporting polypeptide) to the uptake of sodium fluorescein could be excluded.

In conclusion, this study elucidated the uptake transport characteristics of NaFluo in rat and human hepatocytes and OATP transfected cells. In addition, we showed the use of NaFluo in evaluating OATP-mediated drug-drug interactions, using HIV protease inhibitors as model interacting drugs.

POSTERS - DRUG TRANSPORTERS

P16. IDENTIFICATION AND STRUCTURAL RELATIONSHIPS OF SUBSTRATES OF THE HUMAN ORGANIC CATION TRANSPORTER 1 (hOCT1)

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The organic cation transporter 1 (OCT1/SLC22A1) is one of the major drug transporters in the liver (Hilgendorf et al, 2007). It is expressed on the sinusoidal membrane of hepatocytes where it facilitates uptake of cations into the liver. Together with passive permeability properties, OCT1 influences the intracellular concentration of cationic drugs and consequently it may have an impact on clearance, drug drug interactions and hepatotoxicity of its substrates. Furthermore, since transporter mediated uptake leads to a higher concentration intracellularly, OCT1 may be of importance to target drugs to their site of action as is reported for metformin (Shu et al, 2007). Small organic cations have been reported to be substrates of OCT1 (Koeppsel et al, 2007). Still the number of compounds studied are limited. The objective of this study was to investigate the structure transport relationship of a larger and structurally diverse set of compounds.

Human embryonic kidney, HEK293, cells stably transfected with hOCT1 or empty vector were used. Uptake of compounds into the cells was measured by LC-MS/MS. The transporter mediated uptake was calculated as the difference in uptake between cells transfected by OCT1 and empty vector. In total, 50 marketed drugs and 220 project compounds were assessed in terms of uptake at one selected time point whereas a full concentration dependency curve was performed for a limited set of compounds.

Human OCT1 substrates identified were bases and quartenary amines but also several neutral substrates were found. In terms of the bases the set was found to cover most of the global drug space with exception of small lipophilic bases (which posses often a high passive membrane permeability). Successful QSAR models were generated with both PLS and random forest and important drivers are lipophilicity and charge type. In addition for bases a parabolic relationship between molecular volume and uptake was observed with an optimum at around 300 $Å^3$.

In conclusion, we have studied the relationship of structure to uptake via the human OCT1 for a diverse set of compounds. The information is highly valuable in drug discovery and development to predict transporter mediated involvement in hepatic drug disposition and drug drug interactions. The impact of transporter mediated uptake versus passive permeability will also be discussed.

POSTERS - DRUG TRANSPORTERS

P17. THE EFFECT OF HUMAN SERUM ALBUMIN ON UPTAKE CLEARANCE RATES IN SANDWICH CULTURED HUMAN HEPATOCYTES

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The uptake of six literature compounds has been studied in sandwich cultured human hepatocytes (SCHH)[1]. All the compounds have been shown to interact with members of the organic anion transporter proteins family, known as OATPs. This interaction with OATPs has been further underwritten in SCHH studies in the presence of a pan inhibitor of OATPs, Rifamycin[2]. The aim of these studies was to evaluate the effect of human serum albumin (HSA) on the uptake rate into the hepatocytes. In the presence of 2% HSA the initial uptake rate of each probe substrate was diminished around 20-fold and for some compounds the measured passive uptake into the hepatocyte apparently decreased so increasing the dominance of the active uptake process. Utilising available protein binding data[3-4], intrinsic clearance values were determined in the absence and presence of HSA. These data indicate that the presence of HSA increases the apparent intrinsic clearance of these compounds via active processes.

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Acknowledgments:
Mohammed Ullah, Adrian Walker, Katie Mather

POSTERS - DRUG TRANSPORTERS

P18. REGULATION OF DRUG TRANSPORTER EXPRESSION BY PRO-INFLAMMATORY CYTOKINES IN PRIMARY HUMAN HEPATOCYTES

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Sinusosidal and canalicular hepatic drug transporters, involved in drug uptake into the liver and drug secretion into the bile, respectively, play a major role in liver drug clearance. Inflammation is well-known to impair expression of these transporters in rodents; more limited data about this topic have however been reported in humans. To gain insights about this point, we have analyzed the effects of pro-inflammatory cytokines such as interleukin (IL) -1b, tumor necrosis factor (TNF) -a, IL-6 and interferon (IFN)-g, toward drug and bile salt transporters in primary human hepatocytes. Exposure to IL-1b, TNF-a, IL-6 or IFN-g was found to markedly alter expression profile of human hepatic transporters. Bile salt transporters as well as sinusoidal solute carrier (SLC) transporters are usually repressed, whereas ATP-binding cassette (ABC) drug efflux pumps remain unchanged or are either down-regulated or up-regulated. Changes are observed at mRNA and also, for some of them, at protein and activity levels. Interestingly, the effects of IL-1b on drug transporter mRNA levels were found to be correlated with those of TNF-a, IL-6 and IFN-g. Such alterations of drug transporter expression in human hepatocytes exposed to pro-inflammatory cytokines are likely to contribute to alterations of drug pharmacokinetics, impairment of bile salt secretion and cholestasis caused by inflammation in humans.

P19. ACTIVITY OF THE UPTAKE TRANSPORTER OATP1B1 IN PLATED PRIMARY HUMAN HEPATOCYTES OVER TIME IN CULTURE

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Primary human hepatocytes are widely used as an *in vitro* model for evaluation of drug metabolism and transport, and for hepatotoxicity studies. However, it has been shown that the gene expression of many drug-metabolizing enzymes and transporters in primary hepatocytes may change in culture. The human organic anion transporter protein 1B1 (OATP1B1) is one of the major transport proteins involved in uptake of drugs into human hepatocytes. The aim of the present study was to evaluate the activity of OATP1B1 in plated primary human hepatocytes with increasing time in culture. The kinetics of OATP1B1-mediated transport was evaluated by concentration dependent uptake studies of estradiol-17β-D-glucuronide (^{3}H-E17βG) in plated primary human hepatocytes from five different donors after 2, 6 and 24 hours in culture. CL_{int} values were calculated using V_{max} and K_m parameter estimates from non-linear regression. Moreover, gene expression of *OATP1B1* was determined by RT-PCR, while protein expression and localization of OATP1B1 was studied by confocal microscopy. Our results showed an active uptake of ^{3}H-E17βG in plated primary human hepatocytes at 2 hours, with an average CL_{int} value of 32.3 ± 15.9 mL/min/mg protein. The OATP1B1 activity decreased and/or the passive uptake increased after 6 and 24 hours in culture. In one donor, CL_{int} could only be estimated after 2 hours, while in one additional donor, an active uptake was not possible to measure after 24 hours. In three donors, the average decrease in OATP1B1 activity was 45% after 24 hours in culture compared to the average CL_{int} value at 2 hours. The average gene expression level of *OATP1B1* decreased by 16% ($p<0.05$) and 77% ($p<0.001$) after 6 and 24 hours in culture, respectively, compared to the values after 2 hours. Visual examination of OATP1B1 protein expression by confocal microscopy showed localization to the membrane as expected and supported the functional results by an extensive decrease in expression over time in culture. In conclusion, our results show that plated primary human hepatocytes is a useful *in vitro* model for OATP1B1-mediated uptake studies, but only for a limited period of time (2 hours) after isolation. Longer culturing times induce an extensive variability which may confound the results.

P20. THE EFFECT OF MULTIPLE CRYOPRESERVATION CYCLES ON DRUG-METABOLIZING ENZYMES IN HUMAN HEPATOCYTES.

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Cryopreserved hepatocytes provide a convenient *in vitro* test system to study the phase-1 and phase-2 metabolism of new chemical entities; however cryopreservation (freeze-thaw cycles) can damage the cells. Cryoinjury is associated with rapid dehydration of cells (osmotic effects) and formation of intracellular ice during the freezing process, which in turn leads to a disruption of cellular membranes, changes in protein conformation and nucleic acid damage (single and double DNA strand breaks). This study characterized the effects of multiple cryopreservation cycles on the activities of drug-metabolizing enzymes in individual and pooled samples of human hepatocytes. Hepatocytes isolated from four donors were cryopreserved once, twice or three times according to a stepwise protocol. Pooled hepatocytes (n=5) were prepared by two protocols: one involving the thawing of individual lots of frozen hepatocytes, followed by pooling and re-freezing, and the other by pooling cryopreserved hepatocyte pellets (CryostaX™) without thawing or re-freezing the cells. All cells were stored in the vapor phase of liquid nitrogen and were thawed under the same conditions. Viable hepatocytes were separated from non-viable cells by Percoll® gradient centrifugation. The following enzyme activities were measured *in situ*: CYP3A4 (testosterone 6β-hydroxylation), CYP1A2 (phenacetin *O*-dealkylation), CYP2B6 (bupropion hydroxylation), FAD-containing monooxygenase (FMO, benzydamine *N*-oxidation), UDP-glucuronosyltransferase (UGT), and sulfonotransferase (SULT, 7-hydroxycoumarin sulfonation). Compared with individual lots of hepatocytes that were cryopreserved once, hepatocytes that were cryopreserved twice lost 23% of SULT, 18% of CYP3A4, 7-13% of UGT, and 6% of FMO activity. Hepatocytes that were cryopreserved three times lost even more enzyme activity (at least twice as much as the cells cryopreserved twice). Compared with pooled hepatocytes that were cryopreserved once (CryostaX™), pooled hepatocytes that were cryopreserved twice lost 33% of SULT, 30% of UGT, 24% of CYP1A2, 14% of FMO, 13% of CYP3A4/5, and 10% of CYP2B6 activity. In summary, multiple cryopreservation cycles have a deleterious effect on the activity of drug-metabolizing enzymes in human hepatocytes. In general, the loss of UGT and SULT activity was more extensive than that of CYP and FMO activity.

POSTERS - HEPATOCYTE CULTURE IMPROVEMENTS

P21. HEPG2C3a AND PRIMARY HUMAN HEPATOCYTE ENCAPSULATION FOR FURTHER LIVER IMPLANTATION

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The liver is an organ with a high ability of regeneration, but some factors such as diseases, drugs or alcoholism may affect it and lead to cirrhosis. Up to now, liver transplant is the only treatment available in the most severe cases and many patients die waiting for an organ. Several artificial and bioartificial systems are under study, aiming at replacing either detoxication or whole liver functions in an extracorporeal circuit. Such systems are of extreme interest for the patient's recovery during acute phases. In parallel, a new approach can be proposed to supply liver function on long term basis: hepatocyte encapsulated in porous biomaterials could be directly implanted in the patient's liver. The UTC laboratory has a strong expertise in the area of hepatocyte encapsulation in alginate beads, which led to the design of a fluidized bed bioartificial liver. In the present study, our objectives consist in screening different types of biomaterials to optimize implantation of cells in a cirrhotic tissue. Cell encapsulation will prevent them from immune rejection and act as a niche in the liver. In a first step, several materials such as collagen or fibrinogen, proteins often used in hepatocyte cultures, were combined with alginate or directly composed the gel. Beads were produced using either a co-axial air flow extruder (home made design) In a second step, hepatic cells (human cell line HepG2C3A and primary human hepatocytes) were mixed with the most promising biomaterials. The viability of encapsulated cells and their functionalities were compared to those observed in our "basic" alginate beads. In association with the U632 in Montpellier, several configurations will be implanted in a rodent model, in order to reinforce the feasibility of the approach. Specific experiments will be developed to localize the position of the cells hosting beads.

P22. ADAPTATION OF THE RAT HEPATOCYTES LONG-TERM CULTURE FOR HIGH CONTENT IMAGING TO PREDICT CHRONIC LIVER TOXICITY *IN VITRO*

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The conventional Collagen-sandwich culture with the soft/wet gel proved not to applicable to the high-content cellular imaging method due to interferences of the collagen with fluorescence dyes. In addition the Cellomics™Arrayscan® technological platform requires cells to be evenly distributed and orientated on the same plane, since it does not work in confocal mode. To overcome these hurdles Matrigel™ was used instead of Collagen as the top layer. Multiple Matrigel™ administrations were found to be essential to maintain the hepatocyte integrity for 14 days in culture. The optimal conditions achieved were three times application of Matrigel™ on day 1, 6 and 9 to cells seeded on Collagen I-coated plates. Markers of cellular integrity investigated were morphology, cellular ATP content, LDH release and the mRNA expression of several canalicular/basolateral transporters, cytochrome enzymes and nuclear receptors. The secretion of the fluorescent bile acid Choly-lysyl-fluorescein (CLF) by canalicular bile salt excretion pump (BSEP) was used as functional marker. CLF secretion rates were stable and similar during 14 days. Inhibition experiments with the BSEP inhibitor Cyclosporine A (CsA) at day 5, 9 or 14 of the culture indicated no quantitative differences in terms of the inhibitory CsA concentrations. These results demonstrated that the BSEP kept its function for 14 days under the current culture conditions. In conclusion, the further development of the rat hepatocyte long term culture allows the application of high content microscopy imaging, which may serve as powerful tool to investigate chronic liver toxic drugs under *in vitro* long-term incubation conditions.

POSTERS - HEPATOCYTE CULTURE IMPROVEMENTS

P23. SURETRAN - A METHOD TO TRANSPORT HIGH QUALITY HEPATOCYTES

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Abcellute tissue bank is utilising the Suretran Hepatocyte transport system, with its ability to maintain fresh hepatocyte characteristics for several days, to help researchers on a global basis work with fresh human hepatocytes. Isolated Human Hepatocytes are extensively employed in the pre-clinical discovery, development and evaluation of drugs in the biopharmaceutical industry. Although fresh human hepatocytes are considered the "gold standard" model, their lack of regular availability means that many researchers are often driven to utlise Cryopreserved hepatocytes with the obvious convenience, immediate availability. Many papers support the use of the cryopreserved hepatocytes. Here we present data, generated with the help of Pfizer (Sandwich, Kent) that shows that Cryopreserved hepatocytes could be mis-representing some aspects of *in vivo* liver metabolism. Pfizer compared data generated using cryopreserved and Suretan Rat and Human Hepatocytes. The Suretran rat hepatocytes maintained viability in excess of 70% for over 4h in suspension culture. In contrast, cryoprserved rat hepatocytes showed rapid decrease to 13%. In addition, the rate at which parent substrate (Diclofenac) disappeared and metabolite (Dic-OH) formed was far greater in Suretran than in Cryopreserved hepatocytes. This suggests that the rates may be misrepresented and the formation of metabolites, including reactive metabolites may not be well represented in Cryopreserved hepatocytes. Finally, LCMS data generated using Suretran showed great clairty (i.e. minimal background). In contrast the use of cryopreserved human hepatocytes resulted in LCMS data with very high background (interference). The clear background obtained when employing hepatocytes from the Suretran system makes identification of metabolites far easier.

POSTERS - HEPATOCYTE CULTURE IMPROVEMENTS

P24. STANDARDIZED EXPERIMENTAL PROCEDURES FOR THE ISOLATION AND CULTURE OF PRIMARY HUMAN HEPATOCYTES FOR USE AS A MODEL IN TRANSLATIONAL RESEARCH

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Background: Isolation and culture of primary human hepatocytes from partial hepatectomies serves as an established model for the analysis of hepatic pathophysiology. Due to ethical issues, only remnant tissue from liver segment resections can be used for hepatocyte isolation. Human hepatic cells remain an important model as they can be potentially examined for protein expression, proliferation or apoptosis. The aim of this study is to obtain a standardized procedure for the isolation of liver cells and to determine the characteristics of these isolated cells.

Materials and Methods: Donor and procedure specific data were collected. Hepatocytes were then isolated from human liver resections by a multi-step EGTA/collagenase high pressure perfusion. The quality of the isolated cells were defined by viability (trypan blue) and cell yield, apoptosis (FACS), enzyme-leakage (LDH, GOT, GPT) as well as metabolic activities like albumin synthesis, cytochrome P450 (CYP) activity and the proliferation rate (MTT).

Results: Results of the liver cell isolation were analyzed by characteristics like perfusable vessels, size of specimen and cutting area as well as pathologic variations (fatty, cirrhotic or fibrotic liver). In order to improve the process of isolation, collagenase digestion time and EGTA or $CaCl_2$-perfusion time depending on tissue consistency were optimized. Since 2003, 627 isolations have been performed in Munich and Regensburg, with an average viability of $77 \pm 11\%$ and a cell yield of 15 ± 18 million cells per gram perfused liver tissue. Donor specific parameters (tissue damage, enzyme induction by medication, ability for regeneration) were measured during cell culture.

Conclusion: Experiences gained from 627 cell isolations since 2003 has led to a standardized procedure for isolation and culture of liver cells, that successfully isolates hepatocytes regardless of the patient background or tissue pathologic variations of the tissue. Hence, this study makes an *in vitro* human hepatocyte model available, which can be potentially an important tool in translational research.

P25. FUNCTIONAL CHARACTERIZATION OF ANIMAL HEPATOCYTES POOLED AFTER ISOLATION AND THEN CRYOPRESERVED

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BACKGROUND: Cryopreserved hepatocytes are extensively used in drug industry today. However, comparing with cryopreserved human hepatocytes the single animal hepatocytes particularly rodent hepatocytes are limited on small size of batch regarding what is needed for setting up large scale study. In addition, animal hepatocytes are less resistant to isolation and cryopreservation process, with a significant alteration both at viability and plateability. Hence, the aim of the present study was designed to (i) to prepare freshly pooled hepatocytes from several small animals for making large size batch of pooled and cryopreserved rodent hepatocytes; (ii) to validate the pooled and cryopreserved rodent hepatocytes by comparing with fresh and cryopreserved hepatocytes from single animal. (iii) to characterize the post-thawing cell quality and pre-qualification of cryopreserved cells

STUDY DESIGN AND METHODS: For producing large size batch, rat and mouse hepatocyte were isolated from three to eight animals and pooled and then cryopreserved by using an optimized process in BPI. The post-thaw viability, yield and plateability as well as the functionality of cryopreserved hepatocytes were checked and compared with both fresh and small size batch of cryopreserved hepatocyte from single animal donor. The pooled cryopreserved hepatocytes were also pre-qualified according to application including prediction of metabolic clearance, evaluation of CYP induction and hepatocyte transporter uptake assays.

RESULTS: We have developed an optimized technique for preparing and freezing of large size lot of pooled hepatocytes from multiple animal donors like rat and mice. They retain their fresh hepatocytes ability to attach to a collagen I coated matrix (post-thaw plateability), thereby permitting their use for long-term plated assay, such as induction and sandwich cultures. The comparison study shows that metabolism activity is comparable between the fresh and pooled cryopreserved hepatocytes. As well, a good lot-to-lot reproducibility was observed. Furthermore, some pre-qualified applications like induction or transport on cryopreserved pooled cells shown an acceptable inducibility of cytochrome P450 and efflux activity with sandwich-cultured hepatocytes.

CONCLUSIONS: Our pooled cryopreserved hepatocytes from multi-animal donors and multi-animal species represent a good alternative for use of freshly isolated hepatocytes for drug studies.

POSTERS - HEPATOCYTE CULTURE IMPROVEMENTS

P26. A MODIFIED METHOD FOR SIMULTANEOUS ISOLATION OF HUMAN HEPATOCYTES AND KUPFFER CELLS FOR BASIC AND TRANSLATIONAL RESEARCH *

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Objectives: The vast majority of biomedical research is carried out using transformed cell lines and animal models. However, the limitations of these models are the transformation of primary cells into a permanently growing cell line and differences due to species-specificity. Therefore, primary human cells remain an important tool for validation of translational research, such as development of new drugs or molecular characterization of disease pathways. The aim of this study was to establish, evaluate and standardize a procedure for simultaneous isolation of human hepatocytes and Kupffer cells for further investigations.

Experimental: Isolation of hepatocytes and Kupffer cells in parallel were carried out based on a publication by Smedsrød and Pertoft (1985) using a modified method. Briefly, a liver cell suspension was obtained from human liver resections by a multi-step EGTA/ collagenase perfusion. Hepatocytes and a non-parenchymal cell fraction were then isolated by differential centrifugation. Kupffer cells were isolated by performing a percoll gradient followed by selective adhesion. Following plating, culture conditions were optimized to maintain cell-specific functions.

Results: Viability of isolated hepatocytes and Kupffer cells was determined by trypan blue exclusion assay (>80 % and >95%, respectively). In culture, hepatocytes were found to express specific markers of hepatocyte function whereas Kupffer cells were found to express CD68 and showed phagocytotic activity (marker of Kupffer cell function).

Conclusion: Primary human liver cells, such as hepatocytes or Kupffer cells, are used for human *in vitro* validation studies to support drug discovery and drug safety studies based on permanent cell lines or animal models. Therefore this study describes an important procedure for isolation of primary human hepatocytes and Kupffer cells suitable for homo and hetero-typic liver cell studies resembling physiological and pathophysiological conditions in humans.

P27. OPTIMIZATION OF A METHOD FOR CRYOPRESERVATION OF PRIMARY HUMAN HEPATOCYTES AND SUBSEQUENT THAWING FOR FURTHER EXPERIMENTS

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Objectives: Hepatocytes are used extensively in pharmacological and toxicological research. The gold standard for validation of translational research done with animal models and transformed cell lines is to replicate the research using isolated primary human hepatocytes. Presently, the use of cryopreserved hepatocytes as an alternative is increasing in popularity due to advantages, such as allowing re-testing or pooling of samples for analysis. Thus, this study aimed to optimize a method for cryopreservation and subsequent thawing of the primary hepatocytes for further experiments.

Experimental: After isolation, primary hepatocytes were cryopreserved according to a modified method based on publications by Alexandre *et al.* (2002) and Lyod *et al.* (2003). After thawing, the effects of storage over liquid nitrogen on the hepatocytes were evaluated by measuring viability, morphology, adherence and functional characteristics such as the activity of cytochrome P450 and phase II enzymes.

Results: Viability of cryopreserved hepatocytes from 72 donors was determined after thawing by trypan blue-exclusion assay. The results demonstrated that the hepatocytes from 4 out of 10 donors show viability greater than 70% and hepatocytes from 1 out of 10 donors can be plated with an adherence greater than 70%. The morphology of the cryopreserved hepatocytes was found to be similar to the freshly isolated cells in suspension as well as in culture.

Conclusion: In conclusion, this analysis showed that long-term storage of primary hepatocytes and subsequent thawing with recovery of differentiated properties is possible. This allows for creation of a large inventory, which can provide a ready supply of hepatocytes for another investigations. A further advantage is that isolated hepatocytes are characterized after cryopreservation, for example the activity of cytochrome P450 and etc is measured. This gives researchers an option to pool hepatocyte batches with similar activities if required. Additionally, since the hepatocytes are derived from donors with clinically annotated data, investigators can choose batches of hepatocytes most suitable for their research.

POSTERS - HEPATOCYTE CULTURE IMPROVEMENTS

P28. PROTEOMIC PROFILING OF RAT HEPATOCYTES; WHOLE CELL AND NUCLEAR DATA SETS FOR ISOLATED AND CULTURED CELLS

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Objectives: Freshly-isolated hepatocytes are considered the best *in vitro* model system available for drug safety assessments. However, rat hepatocytes in primary culture rapidly lose their metabolic phenotype. A more detailed knowledge of the changes that occur throughout the hepatocyte proteome is required to aid research into developing better *in vitro* liver models. As cellular phenotype is orchestrated by the nucleus we also sought to increase the coverage of this organelle through purification and sub-cellular iTRAQ proteomics.
Experimental: Quantitative iTRAQ proteomics experiments were performed on whole cell proteins and purified nuclear proteins from freshly-isolated rat hepatocytes and from hepatocytes that were cultured for 48h as monolayers on collagen I coated plates. The molecular functions of the identified proteins were assigned using **P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships (PANTHER http://www.pantherdb.org/).
Results: 453 proteins were identified in whole-cell experiments and 307 in nuclear fractions (figure 1). 140 proteins were common to whole cell and nuclear samples. Changes in the abundance of the quantified proteins after 48h of culture, and their associated P values are represented as volcano plots (figure 1). Comparison of the molecular functions represented in the experimental data sets confirms an increased coverage of the nuclear proteome with nuclear fractionation with significant over-representation of functions associated with the nucleus, such as nucleic acid binding, transcription and RNA processing. Similarly, other functions, such as metabolic processes (oxidoreductase, dehydrogenase, transferase) are significantly under-represented in the nuclear dataset. In nuclear preparations, more than 100 proteins were significantly changed in abundance after 48h in primary culture. Of these, more than 60 proteins were not identified in whole-cell preparations.
Conclusion: These data provide novel datasets for the changing nuclear proteome of cultured rat hepatocytes, provide insight into the mechanisms underlying the loss of function of hepatocytes and will aid in the development of better *in vitro* hepatic models.

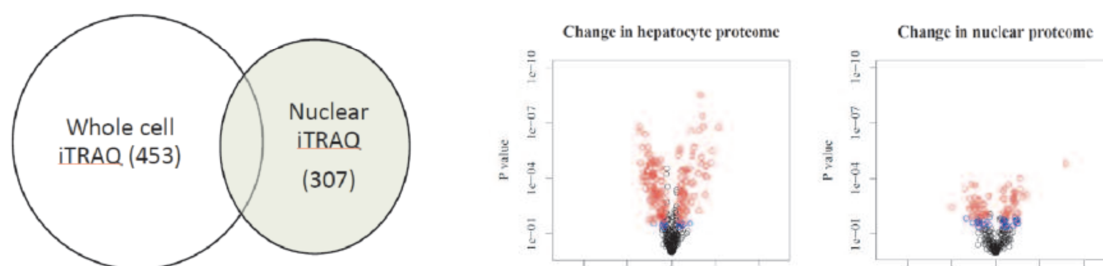

Figure 1. Venn diagram of the common and differential proteome constituents identified in whole cells and nuclear proteins enriched from primary rat hepatocytes. Volcano plots depicting the quantitative changes in the whole hepatocyte and nuclear proteomes after 48h in primary culture.

POSTERS - TOXICOLOGY AND METABOLISM

P29. EC FP7 HEALTH - 2007- GRANT PREDICTIV: PROFILING THE TOXICITY OF NEW DRUGS: A NON-ANIMAL-BASED APPROACH INTEGRATING TOXICODYNAMICS AND BIOKINETICS – FIRST YEAR RESULTS WITH HUMAN HEPATOCYTES

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The overall aim of PredictIV is to develop strategies to improve the assessment of drug safety in the early stage of development and late discovery phase. A major goal of PredictIV is the adoption and optimization of the existing *in vitro* models for the assessment of potential adverse effects of therapeutic candidate compounds. The liver being the organ most frequently affected by drugs in repeat dose toxicity studies and during clinical drug development, the aim of PredictIV Liver Sub Group is to improve and optimize hepatocellular culture models (rat hepatocytes, HepaRG cells, human hepatocytes) which allow repeat treatment, and to evaluate the repeat treatment effect of 11 chosen reference test compounds (TCs) on both cell components by using the "omics" approach and cell metabolic capacity of the TC itself.

The specific task of our group is to optimize the long-term sandwich culture (sw) for human hepatocytes allowing repeat treatments and perform culture, treatment and harvest of samples for these assessments.

As a first step of the project:

1. different configurations of sw were tested in 96-, 24-, and 6-well plates: Collagen gel/collagen gel (C/C), home coating (thin layer)/collagen gel (HC/C), home coating (thin layer) or Biocoat plates/Matrigel (HC/M). The best sw configuration in terms of stability over 14 days and in confluency of the cell monolayer appeared to be HC/M for all plate formats,

2. cytotoxicity assays were performed using human hepatocyte sw cultures in 96-well plates at different time points (d1, d3, d7 and d14 of daily treatment) but also with human hepatocytes in suspension after 4h of treatment, in order to determine low, mid and high concentrations for the repeat toxicity study, the highest concentration being defined as displaying 10% cell death over a 14 day treatment period. Suspension of human hepatocytes were found as suitable as d1 and d3 cultures for acute cytotoxicity determination and concentration range-finding, repeat 14d sw cultures were needed for refinement of long-term treatment concentrations.

P30. ALTERATION OF BILE ACID SYNTHESIS BY DEXAMETHASONE IN PRIMARY HUMAN HEPATOCYTES

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Introduction: *De novo* synthesis of bile acids (BAs) is a liver specific function that is difficult to maintain in cultured cells. There are significant species differences in both types of BAs formed and in the regulation of BA homeostasis. This highlights the need for a good human *in vitro* model. Isolated primary human hepatocytes have the ability to synthesize normal conjugated BAs.
Aim: To investigate how different culture conditions alter the expression of enzymes in BA production and the subsequent synthesis and secretion of BAs.
Study design: Primary human hepatocytes were isolated from liver tissue obtained from patients (n=12) undergoing surgical liver resection or from donor livers that could not be used for transplantation. Hepatocytes were cultured in William's E medium under standard conditions. Two different cellmedia were used; 'Standard media' which were supplemented with 120nM insulin and 100nM dexamethasone (dex) and 'Swedish media' which were supplemented with 12nM insulin only. On day 5, cells were harvested in Trizol for quantification of specific mRNAs, and cell culture medium was analyzed for BAs.
Results: Cells cultured on matrigel produced more BAs than cells cultured on collagen. mRNA expression of the rate limiting enzyme in BA synthesis, CYP7A1, was higher in Swe on matrigel compared to either media on collagen. mRNA expression of CYP8B1 was higher in cells cultured in Std media compared to cells cultured in Swe media regardless of substrate. Since the difference between Swe and Std media is concentration of both insulin and dex we did another set of experiments were the combination of dex and insulin were tested. Analysis of BAs in the media showed that addition and removal of dex significantly altered the production of both cholic acid (CA) and chenodeoxycholic acid (CDCA) and that insulin at these concentrations did not affect BA synthesis. In agreement with an increased synthesis of CA the mRNA expression of CYP8B1 increased when dex were present. The expression of CYP7A1 and CYP27A1 were not changed.
Surprisingly, the increase of CYP8B1 was not paralleled by an increased expression of CYP7A1, indicating a difference in the regulation of these enzymes. The effects of dex can be mediated by either PXR or the glucocorticoid receptor (GR). In order to investigate which pathway is involved in the regulation of CYP8B1 cells were treated with RU486, a GR antagonist. Treatment with RU486 indicates that dex stimulates the expression of CYP8B1 through a GR independent pathway.
Conclusion: In these experiments we investigated the importance of different culture conditions such as choice of substrate and supplements on the total BA production as wells as the BA composition. Primary human hepatocytes synthesize more BAs when cultured on matrigel compared to collagen, a finding confirmed by an increased mRNA expression of CYP7A1.
Addition of dex stimulates the expression of CYP8B1, leading to an increased formation of CA and a decreased CDCA formation. The effect is most likely mediated through a GR independent pathway.

POSTERS - TOXICOLOGY AND METABOLISM

P31. HUMAN HEPATOCYTE ENZYME INDUCTION STUDIES: HISTORICAL DATA TREND ANALYSIS

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Xenobiotic-mediated up-regulation of drug metabolising enzymes, particularly cytochromes P450 (CYP), can have significant pharmacological and toxicological consequences. For example, induction of enzymes which metabolise compounds to inactive products can attenuate the desired clinical effect, whereas increased production of active products or intermediates can lead to a potentiated pharmacological effect and/or toxicity. Over the decade or so, the use of monolayer cultures of human hepatocytes have come to be regarded as the definitive model for the prediction of enzyme induction *in vitro*. Covance has been performing *in vitro* enzyme induction studies for many years and, as such, a significant amount of data on the effects of positive control inducers in hepatocyte cultures has been generated. In this presentation we report some of the noteworthy trends these data have revealed, including the effect of donor demographics and basal enzyme activities on the extent of induction elicited. In addition, the use of this model for the evaluation of the induction of UGT-mediated enzyme activity is discussed.

POSTERS - TOXICOLOGY AND METABOLISM

P32. HPLC/MS-BASED METABOLIC STUDY OF THE BENZO[*C*]PHENANTHRIDINES IN HUMAN HEPATOCYTES

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Sanguinarine (SG) and chelerythrine (CHE) are quaternary benzo[*c*]phenanthridine alkaloic (QBA) synthesized in some plants especially of the Papaveraceae family. SG and CHE have bee intensively studied for their anti-microbial, anti-inflammatory, anti-fungal and anti-parasiti activities. As a result, both are currently used in human and veterinary medicine as anti-microbi and anti-plaque preparations and feed additives.
Their metabolism in mammals has not been well-explained. Recently, Deroussent et al. [1] studie sanguinarine transformation in non-induced/BNF-induced rat liver microsomal fractio transfected Ad293 cells expressing selected recombinant cytochromes P450 and in rat urine aft oral administration. The authors proposed the structural characterization of metabolites. The ai of our study was to elucidate the biotransformation of SG, CHE and their dihydro derivative (dihydrosanguinarine, DHSG and dihydrochelerythrine, DHCHE) by using a human hepatocy suspension model.
The suspension was incubated with 5 µM SG and CHE, 50 µM DHSG and DHCHE respectivel for 1 and 2 h. The culture medium and cells were analyzed by an optimized HPLC/ESI-M method employing an ion trap MS analyzer.
Dihydro derivatives were found as metabolites for SG and CHE, as we have already reported f SG in experiments on rats [2]. Additional metabolites, formed mainly from CHE and DHCH were determined. The main metabolite was found to be compound with m/z 336, corresponding t a putative structure of dihydro derivative with one methoxy and one hydroxy group in the place methylenedioxy (for SG) or two methoxy groups (for CHE). It seems from the data that the ne step in QBA transformation, after dihydro derivative production, is the cleavage of th methylenedioxy/methoxy group leading to formation of hydroxy metabolites. More detaile characterization of QBA metabolites is needed.

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P33. INFLUENCE OF GENDER AND ETHNICITY ON DRUG METABOLIZING ENZYME ACTIVITY IN HUMAN HEPATOCYTES

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Purpose: Interindividual variability in the pharmacokinetic profile of most drugs is largely the result of differences in liver function caused by factors such as disease state, genetic differences in metabolizing enzymes and drug interactions, including enzyme inhibition and induction. Most of the currently used therapeutics are effective in less than 60% of patients, and metabolism of 90% of these drugs is predominantly by CYP1A2, CYP3A4 CYP2C9, CYP2C19, CYP2D6 and CYP2E1. To better understand variable liver function and in particular, drug metabolism, we have developed a data base with 258 human hepatic tissues and characterized these for hepatocyte viability, activity and genotype of the major drug metabolizing enzymes.

Methods: Human hepatic tissues used in this study were from transplant reject livers and liver resections that were harvested for healthy tissue, mainly from patients with hepatic cancers. Within 18 hours of retrieval hepatocytes were isolated and subsequently cryopreserved. Cryopreserved hepatocytes were thawed and characterized for Phase I cytochrome P450 enzyme activities of CYP 1A2, 2C9, 2D6, 3A and Phase II (sulfation and conjugation) activity. Some preparations of hepatocytes were placed in primary culture and enzyme regulation of CYP1A1/2 and CYP3A studied after 3 days of treatment in primary sandwich culture. Genotyping assessment was conducted for the following SNPs: CYP2C19*2, CYP2C9*2 and *3, CYP2D6*4, *6 and *9 and CYP3A5*3.

Results: Our data indicates differences in expression of drug metabolizing enzymes between resections and whole livers, as reflected by higher CYP1A2 and CYP3A4 activity in hepatocytes harvested from resections with CYP1A2: 96.3 vs. 52.7 pmol/min/mg protein and CYP3A4: 631 vs. 444 pmol/min/mg protein. BMI of the donor does not affect drug metabolizing enzyme activity. Another interesting observation is that CYP3A4 and 7-HCG activities (sulfation and glucuronidation) are higher in females vs. males. CYP3A4: 581 vs. 430; 7-HCG 698 vs. 557 pmol/min/mg protein. We also observe up to 3- fold, significant ethnic differences e.g. 7-HCG activity (pmol/min/mg protein) in hepatocytes from Hispanic (1011), Caucasian (640), Asian (604), and African American tissues (356). Highest 2C9 and 1A2 activities are found in hepatocytes from Asian donors. CYP2C9 (pmol/min/mg protein) in particular demonstrates race-dependant activity: Asian (125) >Caucasian (112) >African American (76.8) >Hispanic (68.9). This correlates with CYP2C9 polymorphisms, as reflected by our genotyping data with the *2 and *3 SNPs.

Conclusions: Our data highlights that the large interindividual variability in drug clearance and responses in the clinic are likely due to gender and ethnic differences in activities of the major human hepatic drug-metabolizing enzymes. Personalized medicine where these enzymes are evaluated prior to medication will likely result in much higher success and safety of therapeutics.

P34. *IN VITRO* ASSAY STRATEGY BASED ON RAT HEPATOCYTE SANDWICH CULTURE FOR EARLY EVALUATION OF CYP3A INDUCTION POTENTIAL

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The pharmaceutical industry demands for fast and reliable *in vitro* assay strategies implementable during early drug discovery. Fresh and lately also cryopreserved human hepatocytes are regarded as the “gold standard” for regulatory agencies and pharmaceutical industry to assess cytochrome P450 (CYP) induction potential *in vitro*. However, performing studies with human hepatocytes are still limited by availability and/or costs. The main goal of the present study was to evaluate the applicability of fresh rat hepatocytes incorporating a non-lytic bioluminescent CYP3A enzyme activity assay and *CYP3A* gene expression for the investigation and prediction of CYP3A induction potential of compounds.
Rat hepatocytes isolated via *in situ* two-step collagenase perfusion, fresh human hepatocytes in suspension obtained from surgical surplus tissues, as well as cryopreserved human hepatocytes were plated and maintained as sandwich cultures under serum-free conditions. The cells were treated for 48 hrs with prototypical CYP3A inducer Rifampicin (RIF) and Dexamethasone (DEX), non-CYP3A inducer Omeprazol and ß-Naphthoflavone, as well as with five MRZ-compounds, one known CYP3A inducer (MRZ-A). CYP activity and viability were measured by a duplex bioluminescent assay, *CYP3A* expression was analyzed applying quantitative Real Time RT-PCR. The results obtained from rat were in a good accordance to human CYP3A activity. Herein the ability to use luciferin isoprolyl acetal (Luciferin IPA, Promega Corp.) as sensitive CYP3A substrate facilitating the investigation of CYP3A induction potential in rat hepatocytes could be shown. Relative to the vehicle control Dexamethasone and Rifampicin enhanced CYP3A activity by 51.2± 6.4-fd in rat and 51.2± 8.8-fd in human, respectively. MRZ-A showed to up-regulate *CYP3A* mRNA 17.3-fd (5.1% of DEX) at 10 µM and 117-fd (37.5% of DEX) at 50 µM. Inducible effects were equal or slightly more pronounced based on enzyme activity comparing the %-positive control values (10.2% of DEX at 10 µM in rat and 12.5% in human).
The results suggest the application of a Luciferin IPA cell-based assay as an appropriate alternative for early CYP3A activity screening over time-consuming HPLC/MS/MS experiments. The rat hepatocyte-based approach shown here has been integrated into the Hit-to-Lead phase to speed up decision-making processes and to guide medical chemistry by facilitating an early identification and evaluation of potential risks arising from interaction of compounds with metabolizing enzymes.

P37. APPLICATION OF UPCYTE® TECHNOLOGY TO PRIMARY CELLS FOR DEVELOPING ALTERNATIVES TO CURRENT *IN VITRO* METABOLISM MODELS

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Primary human primary hepatocytes are used routinely for *in vitro* drug metabolism and induction studies; however, the supply of these cells is sporadic and the quality may vary. In addition, once cultured, primary hepatocytes tend to rapidly lose CYP activities, making them less desirable for chronic studies or assays which require high metabolic activities. We have applied upcyte® technology to primary human hepatocytes so that they are able to proliferate to over 30 population doublings (PDs). In these studies we have measured CYP activities of these cells over time in culture using prototypical substrates or P450-Glo substrates.

CYP activities (CYP1A2, CYP 2B6, CYP 2C8, CYP 2C9, CYP 3A4) in upcyte® hepatocytes were dependent on the seeding density and, with the exception of CYP2B6, the time in culture. When upcyte® hepatocytes were seeded at confluence (150,000 cells/cm^2), CYP1A2, CYP 2C8, CYP2C9 and CYP 3A4 were all increased after 96 h compared to their initial levels by between 5- and 80-fold. By contrast, CYP1A2, CYP2C9 and CYP 3A4 activities in primary hepatocytes from two (different from the upcyte® hepatocytes) donors were all decreased to less than 20% (CYP1A2 and CYP2C9) and 50% (CYP3A4) of their initial levels over the same time. Although the primary and upcyte® hepatocytes were from different donors, initial CYP3A4-Glo activities were comparable (12.1 and 12.5 pmol luciferin/h/mg protein in 2 Lots of primary hepatocytes and 7.7 and 3.7 pmol luciferin/h/mg protein in 2 Lots of upcyte® hepatocytes). After 96 h in culture, CYP3A4 activities in primary hepatocytes were 3.6 and 6.2 pmol luciferin/h/mg protein and 93 and 18 pmol luciferin/h/mg protein in upcyte® hepatocytes.

CYP2B6, CYP 2C8, CYP 3A4 activities remained stable up to 24 PDs. CYP3A4 induction responses of upcyte® hepatocytes to 20 μM rifampin were similar in different PDs from the same donor and were in accordance with the pass criterion for CYP3A4 induction in human hepatocytes recommended by the FDA (i.e. greater than 4-fold).

In summary, upcyte® hepatocytes can be seeded at sub-confluent densities to grow to confluence, whilst maintaining certain phase 1 metabolising enzyme activities. As the upcyte® hepatocytes establish a confluent culture, most CYP activities increase; whereas CYP2B6 activities remain stable. Upcyte® hepatocytes are responsive to rifampin induction of CYP3A4. Since upcyte® technology allows for the generation of hepatocytes with differentiated function, they offer a unique advantage of combining the phenotype of primary hepatocytes with the virtually unlimited availability and ease of handling human tumour cells and cell lines.

P38. APPLICATION OF UPCYTE® TECHNOLOGY TO PRIMARY CELLS FOR DEVELOPING ALTERNATIVES TO CURRENT *IN VITRO* ADME-TOX MODELS

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We have developed a new technology to allow the proliferation of differentiated primary cells without inducing permanent immortalization, uncontrolled cell growth, or loss of phenotype. The Upcyte® ("upregulated") technology involves a viral gene transfer system to introduce a unique combination of genes that induce and maintain cell proliferation until the cells reach confluence. This allows the primary cells to be passaged many times and the generation of billions of cells. Upcyte® technology has been applied to different cell types, including keratinocytes, endothelial cells and hepatocytes. Here, we summarise some of the comparisons we have made between primary cells and their upcyte® equivalents.

Gene expression: The gene expression of native human skin keratinocytes was compared with upcyte® keratinocytes from the same donor using the Illumina human Sentrix-8 V3 array. Of a total of 24,626 genes, less than 1% were differed between the primary and upcyte® keratinocytes (of population doubling (PD) 15, 25 and 35).

Karyotype stability: Using the Illumina HumanHap370CNV copy number variants analysis, we demonstrated that no genetic rearrangement occurred in upcyte® keratinocytes, even after 30PDs.

Signalling pathways: Activation of VEGFR-2 signalling (VEGF-stimulated phosphorylation of the VEGF-2-receptor) was comparable in primary and upcyte® (PD35) HUVECs from the same donor.

Adult-specific markers: Upcyte® hepatocytes to over 40PDs retain primary cell characteristics over the course of the expansion. The adult liver specific markers, human serum albumin, α-1-antitrypsin, cytokeratin 8, cytokeratin 18, were clearly expressed in upcyte® hepatocytes from all PDs tested and they were able to store glycogen, indicating a functional and differentiated adult phenotype.

Metabolic enzymes and transporter function: Efflux (MRP1, MRP2, MDR1) and uptake (OATP1A2, OATP1B3 and OCT1) transporters are expressed in upcyte® hepatocytes. Likewise, CYP activities e.g. CYP3A4, CYP2C8 and CYP2B6, are also present in significant abundance and, moreover, can be induced by prototypical inducers. Upcyte® hepatocytes will also help to solve the current problems with traditional genotoxicity tests which are prone to "false positives", since cultures of these cells possess both inherent metabolism and proliferative capacity.

Application of upcyte® cells as optimal alternatives to current in vitro screening assays: Upcyte® technology allows for the mass production of primary cells from different organs and donors for use in multiple *in vitro* ADME-Tox assays. The flexibility of the application of upcyte® cells to different cellular-based assays, together their abundant availability from different donors for routine testing means models are now available with sustained quality and sufficient quantities to allow for reproducible and reliable *in vitro* ADMET studies.

P39. COCKTAIL VERSUS INDIVIDUAL INCUBATION FOR DETERMINING CYP1A2, 2B6 AND 3A4 INDUCTION: COMPARISON BETWEEN HEPARG® CELLS AND HUMAN HEPATOCYTES

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Introduction: Assessment of CYP induction in drug discovery is essential to enable potential induction-mediated drug-drug interactions to be minimized. CYP induction data can be generated using primary human hepatocytes, but limited availability and inter-individual variability somewhat limits routine application within a drug discovery setting. The recently developed HepaRG® human hepatoma cell line therefore has been evaluated as an alternative *in vitro* system[1, 2, 3].

Method: To further evaluate the suitability of this cell line compared to human hepatocytes, we compared the induction factors measured with the FDA-preferred positive controls omeprazol, (CYP1A2), Phenobarbital (CYP2B6) and rifampicin (CYP3A4/5) with three marker substrate namely phenacetin, bupropion and midazolam either as a cocktail (in the Vmax condition), or individualaly after a 48h incubation period .

Results: Regarding CYP2B6 and 3A4, the induction factors measured by incubating a cocktail of CYP1A2, 2B6 and 3A4 specific substrates were not different to those measured by incubating the same substrates alone. Regarding CYP1A2, the induction factors measured with the cocktail was lower than those measured in individual incubations. These results were found either with the HepaRG® hepatoma cell line (induction factor of 6.6 with individual incubation versus 3.7 with cocktail incubation, for example) and primary human hepatocytes (induction factor of 11.3 with individual incubation versus 4.3 with cocktail incubation, for example).

Conclusions: We demonstrated that HepaRG® is a good alternative *in vitro* system and could be used for *in vitro* DDI predictions in the respect of the FDA recommendations.

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P40. INTER-SITE EVALUATION OF HepaRG CELLS FOR DETERMINING CYP INDUCTION

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Introduction: Induction of human cytochrome P450 (CYP) enzymes can result in significant clinical consequences via reduced exposure leading to suboptimal efficacy of co-administered drugs and/or enhanced bioactivation. Therefore, assessment of CYP induction in drug discovery is essential to enable potential induction-mediated drug-drug interactions to be minimized. "Gold standard" CYP induction data can be generated using primary human hepatocytes, but limited availability of quality cells and inter-individual variability somewhat limits routine application within a drug discovery setting. The recently developed HepaRG human hepatoma cell line therefore has been evaluated as an alternative *in vitro* system.

Methods: HepaRG cells were cultured and incubated with 3 prototypical inducers and 9 AstraZeneca compounds at two sites (AstraZeneca, UK, and Xenoblis, France) to investigate inter-site variability and reproducibility. CYP induction was assessed following a 24h incubation, using a mRNA endpoint. mRNA levels were determined using single and multiplex quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Results: The data obtained at the two sites was in good agreement with <2-3-fold variation for both the prototypical inducers and the AstraZeneca compounds. For example, rifampicin induced CYP3A4 mRNA 47-fold and 66-fold when incubated at concentrations >10x EC_{50} at Xenoblis and AstraZeneca, respectively, and omeprazole (50µM) induced CYP1A2 mRNA 92-fold and 129-fold at Xenoblis and AstraZeneca, respectively.

Conclusion: The data presented shows that HepaRG cells are an excellent surrogate for analysis of CYP1A, CYP2B6 and CYP3A4 induction in primary human hepatocytes and that robust, reproducible data can be obtained using different batches of cells at different sites.

POSTERS - METHODOLOGIES

P35. REAL TIME CELLULAR IMPEDANCE: A NEW TOOL FOR ASSESSING CHEMICAL'S BIOACTIVATION TO HEPATOTOXIC COMPOUNDS

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Objective: The extent of hepatocyte's death *(i.e. apoptosis, necrosis or autophagy)* in response to xenobiotics is required for any regulatory pharmacotoxicological assessment. Classical cytotoxicity analysis are based on specifically dedicated experiments and performed at specific times. In this context, we assessed the potential of an alternative cell analysis system to monitor, in real time, the biological and functional state of adherent hepatocytes. Based on electric impedance, without prior marking or staining, measurements are performed in a non-invasive context. This technology allows monitoring cyto-morphological changes as early as late, and gets a wealth of information unmatched in endpoint measures.

Experimental: Cell Index *(CI)* was derived from the electric impedance measured on electronic sensor plates containing adherent cells *(hepatocytes, hepatoma and 3T3 fibroblasts)*. In these plates, wells are covered by interdigitated gold microelectrodes. Impedance across the electrodes is measured in Real Time *(RT),* providing sensitive & immediate detection of the cellular conditions and kinetic responses, from low cell number to confluency. CI variations hence reflect changes in cell morphological parameters *(mortality, motility, membrane receptor activation….)*.

Results: In the work presented, we show the usefulness of this tool of investigation in different areas of toxicology. The first application deals with the relationships between bioactivation and cytotoxicity. Indeed, metabolism is a bottleneck in *in vitro* toxicological test development. The absence of such determinant of toxicity could give rise to false-positive *(lack of detoxication)* or negative data *(lack of bio-activation)*. On this matter, we followed the ECVAM recommendations by comparing cytotoxicities obtained on 8 reference compounds on 3 different cell types namely, 3T3 fibroblasts, FAO rat hepatoma cell and primary cultures of rat hepatocytes. An inter-validation test between classical cytotoxicity methods *(MTT, Neutral Red, ATP)* and RT cell data has also been performed.

Conclusion: This innovative method of analysis was shown to generate particularly relevant and informative kinetic data and to increase the sensitivity of tests. Moreover, this tool makes possible the identification of multiparameter biological signatures that would classify the compounds into families according to their impedance profile and mode of action. Real Time Cellular Impedance thus represents a promising technology, in the arsenal of modern tools of predictive toxicology.

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POSTERS - METHODOLOGIES

P36. FACILITATING TRANSLATIONAL RESEARCH ON HUMAN LIVER TISSUE SPECIMENS – REPORT ON TISSUE BANKING EXPERIENCE OVER THE LAST 10 YEARS

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Objectives: Research with human tissue is constantly extending. As part of translational research, tissue banks are linking scientists and clinicians. This leads to a variety of related questions and problems concerning legal aspects and the technical procedure of collection, processing, storage and transfer of tissue specimens. Therefore, a tissue bank with specific guidelines and a prospective data collection is of vital importance.

Experimental: In 1997 the organization of a surgical tissue bank for translational research started in Regensburg. Over the last ten years a surgical tissue bank focussing on liver pathology was developed with a regulatory framework to cover the necessity of ethical, legal and quality guidelines. In consequence, the Human Tissue and Cell Research foundation (www.htcr.de) was established in 2000 and is acting as an „honest broker", monitoring tissue collection, processing and transfer. To apply appropriate procedures of tissue collection pre-, intra- and postoperative parameters (e.g. medication, ischemia time, storage conditions) were observed.

Results: So far from 2034 donors with *informed consent* who underwent liver surgery, 1551 tissue specimens were collected together with the corresponding blood samples and relevant clinical data. Beside liver tissue more and more kidney-, gut-, pancreas-, stomach-, skin-, thyroid gland- and lung tissue were collected on request, generally as combination of normal- and tumour tissue. The liver tissue bank, certified according to ISO 9001:2000 in 2007 by TÜV Süd, has built up a research platform which offers multiple approaches for translational research in future. Up to now, 2516 tissue aliquots were allocated, 71% for researchers and 29% for industrial partner. Currently there are 6 university and community hospitals, delivering samples for the surgical tissue bank.

Conclusion: The built-on structure of the surgical tissue bank and its role as „honest broker“ serves as a model throughout Germany and in Europe, demonstrating a new perspective in tissue-based translational research within the field of surgery.