An alternative approach based on microfluidics to study drug metabolism and toxicity using liver and intestinal tissue
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Summary, Discussion and Perspectives
It is of crucial importance to detect the kinetic, metabolic and toxicity (ADME-Tox) profiles of new drug candidates in an early stage of drug development. The costs and the use of animals can be significantly reduced when the *in vivo* behavior of new chemical entities is accurately predicted by *in vitro* methods earlier on in the development process. Therefore, the role of *in vitro* systems to predict these profiles is essential. Many *in vitro* systems have been developed the last decades,\(^1\) however, a significant number of drugs is still withdrawn in a late stage of drug development.\(^2,\)\(^3\) As mentioned in Chapter 1, the aim of this thesis was to develop a new *in vitro* system which can be used to gain more information about the ADME-Tox properties of new drug candidates in an early stage. The *in vitro* system which has been realized incorporates precision-cut tissue slices in a microfluidic-based biochip. The use of microfluidic technology for the development of *in vitro* systems has many advantages compared to conventional incubation systems.\(^4\) The main advantage is the ability to control the cell’s microenvironment by introducing a continuous flow which enables the establishment of steady-state conditions.

Various microfluidic systems have been developed with liver cells incorporated on chip, with the aim to measure the biotransformation of drugs in the human liver. Chapter 2 reviews the currently available microfluidic-based *in vitro* models for liver metabolism and toxicity profiling and discusses their respective advantages and disadvantages. From this literature review, it became clear that only two of these models, primary hepatocytes and precision-cut liver slices (PCLS), can adequately represent liver metabolism *in vivo*. As PCLS retain an intact architecture with all cell types present in their native configuration and intact cell-cell contacts, they seem eminently suitable for toxicity studies.

The first microfluidic-based system incorporating rat PCLS is presented in Chapter 3. A polydimethylsiloxane device is described, allowing continuous supply of medium by perfusion to rat liver slices for 24-h incubation. Care was taken that the pH, oxygen concentration, and temperature remain constant during the incubation. For this reason aeration windows were integrated into the chip to act as “breathing” membranes. As it was crucial to first verify the performance of the newly developed system by comparison with validated conventional systems, we compared the viability and metabolic function of PCLS in the biochip with PCLS incubated in a conventional well-plate system. The viability and metabolic activity for the tested substrate were not significantly different in both systems, indicating that PCLS remain functionally intact in the biochip for at least 24 h.

An advantage of the continuous flow is that the outflow can be directly and continuously analyzed for metabolite formation. The development of an on-line analysis system by coupling the biochip with an HPLC with ultraviolet detection is demonstrated in Chapter 4. The design of the device results in a low medium-volume-to-slice-volume ratio, thereby resulting in high concentrations of metabolites.
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in the outflow. This automated system makes it possible to repeatedly expose a slice to substrates. For example, PCLS were incubated four times with 7-hydroxycoumarin for 2-h periods, with intervals of 4 hours between incubations. An immediate production of metabolites was observed, and the maintenance of the viability and metabolic activity of the slice was demonstrated. It will be interesting to use this system in the future to try to measure metabolic circadian rhythms. In this chapter, the on-line system was also used to study inhibitory drug-drug interaction. The concentration of inhibitor in this case was increased in a stepwise fashion. Only three slices were used in one experiment, allowing the determination of the dissociation constant for inhibitor binding using a minimal amount of tissue. In the future, the time-dependent induction of metabolism may be studied in this device. The system is also ideal for studies on scarce tissue, like human material. Another great potential of this system is the ability to detect unstable metabolites. This was demonstrated by the measurement of the formation of diclofenac acyl glucuronide, an unstable metabolite formed by the liver from the drug, diclofenac. Acyl glucuronide metabolites are considered to be reactive metabolites and thus cause toxicity. These reactive metabolites are difficult to detect with conventional systems due to their chemical instability. It was shown that the on-line analysis system is able to measure these metabolites directly after their formation at a relatively high concentration, which may be useful for the prediction of the formation of other reactive metabolites as well. In future, it would be interesting to couple this on-line analysis system directly to a mass spectrometer, allowing the simultaneous on-line analysis and identification of metabolites.

In the first chapters, the maximum slice incubation time was 24 hours. In Chapter 5 this incubation time was extended to 72 hours for rat PCLS by embedding slices in a hydrogel matrix. When slices were suspended in solution in the biochip, the viability was well maintained for 72 hours; however, the metabolic capacity of the slices both for phase I and phase II metabolism decreased over time. It was found that PCLS had attached themselves to the upper filter of the microchamber after prolonged incubations (>24 hours). The embedding of PCLS in a hydrogel significantly improved the maintenance of metabolism. Matrigel was used as hydrogel, which is a basement membrane matrix. The embedding prevented the attachment of the PCLS onto the polycarbonate filter at the outflow of the chamber. In addition, by supplementing the WME medium with bovine serum, dexamethasone and insulin, the capacity for phase II metabolism was fully maintained at the level of fresh slices for 72 hours. However, phase I metabolism of 7-ethoxycoumarin still decreased over time in a manner analogous to that in well plates, as was also found by others. This is probably due to an unoptimized medium composition and the lack of endogenous stimuli to activate transcription factors involved in the expression of the phase I metabolizing enzymes, rather than damage incurred in the incubation system. For
further research, the addition of a low concentration of cytochrome P450 inducers might result in a more stable phase I metabolism. It is now generally accepted that for proper prediction of drug metabolism in man, human *in vitro* preparations are preferred, because of the well-known species differences in drug metabolism. PCLS can be prepared from surgical liver waste tissue obtained after liver transplantation, or after partial hepatectomy (resection of liver parts to remove tumors). In addition, it is also possible to prepare slices from diseased tissue, thereby enabling the study of pathophysiological processes. In Chapter 6, the successful incorporation of healthy human liver slices in the microchip was demonstrated. The viability was determined by morphological evaluation and enzyme leakage tests. The metabolic activity was assessed by incubating the slices with four different substrates, testosterone, lidocaine, 7-ethoxycoumarin and 7-hydroxycoumarin, covering a wide range of phase I and phase II metabolism pathways. The results showed that human PCLS function similarly to those incubated in the conventional well plate system, which also for human PCLS correlates well with the *in vivo* situation. The flow introduced in the microfluidic device did not result in different metabolic activities or lower viability. We conclude, therefore, that human liver slices can also be incubated in the biochip for metabolism and toxicity studies.

The incorporation of precision-cut rat intestinal slices was also established, as shown in Chapter 7. This is the first example of intestinal slices incubated in a microfluidic device. The slices were incubated with three different substrates, and high metabolic activity was observed for 3 hours of incubation, comparable to the activity in well plates. In contrast to liver slices, which remain viable for 72 h, intestinal slices partially lose their metabolic function and ATP content after 24 h of incubation in well plates. Therefore, in future studies, it would be interesting to test if intestinal slices retain their viability during longer perfusion in the biochip. However, it should be noted that the apical and basolateral sides of the intestinal tissue were not perfused separately in our system. It would also be interesting to develop a device which does separate the two sides of the intestine, to be able to perform absorption studies. Conventional system exists in which the two sides can be separated, so-called Ussing chamber. It would be interesting to develop an Ussing chamber on chip to investigate the transport and metabolism of drugs in very specific regions of the intestine.

Due to the continuous flow, the outflow of a chamber containing the metabolites and products formed by a slice can be directed to the inlet of another chamber which also contains a slice. It thus becomes possible to measure interorgan effects, something which is not possible in well plates. Two slices from different organs can be placed in one well; however, mutual interaction can take place, and it is impossible to conclude which organ was responsible for an observed effect. To verify whether
such a sequential perifusion of slices would affect the functioning of the slices, two slices from the same organ were first sequentially perifused with model substrates. No significant differences in the specific metabolic rates resulted when one or two slices were perifused. Thereafter, sequential perifusion of an intestinal slice and a liver slice with model substrates showed that liver slices functioned normally. Apparently, the excreted products from the intestine did not affect the liver metabolism, indicating no interorgan effects for these substrates. Interestingly, interplay between intestine and liver could be demonstrated for the regulation of bile acid synthesis by primary bile acids. After exposure to the bile acid chenodeoxycholic acid, a significant down regulation of CYP7A1 in the liver slice occurred. This could be ascribed to the bile acid-induced release of fibroblast growth factor 15 (FGF15) by the intestine, which was directed by the medium flow towards the liver slice. The enzyme, CYP7A1, is responsible for the synthesis of bile acids in the liver, and is known to be regulated by FGF15. This bile acid regulation pathway showed the potential of the biochip system to investigate interorgan effects.

Until now, only liver and intestinal slices have been incorporated in the biochip. As it is also possible to produce precision-cut slices from other organs such as lung and kidney, this biochip technology offers the potential to study the effect of liver metabolites on these tissues as well, or vice versa. The influence of metabolites excreted into the bile of the liver on the intestine can also be studied with the biochip. By integrating multiple organ slices on chip, the in vivo situation is more closely approached than when cell monocultures are employed.

All the experiments were carried out with a biochip made from polydimethylsiloxane (PDMS). This is an ideal elastomer for rapid prototyping during the development of the biochip; however, the adsorption/absorption of hydrophobic compounds is an important disadvantage. This problem was observed with the incubation of PCLS with testosterone in Chapter 6. In Chapter 8, we set out to investigate whether other materials, such as plastics, could replace PDMS as fabrication material. In this study, the thermoplastic polymers, polystyrene (PS), polycarbonate (PC), polymethylmethacrylate (PMMA), and cyclic olefin copolymer (COC), were tested. These polymers were selected because of their reported biocompatibility and because chips can be fabricated using a hot embosser, which is superior to PDMS casting for producing large numbers of biochips in a shorter period of time. In their native form, these plastic substrates are also hydrophobic. However, the surface could be modified by oxidation using UV ozone or oxygen plasma treatment, resulting in more hydrophilic surfaces which remain stable for up to 4 weeks. PC, PS and COC chips showed no significant adsorption of testosterone and 7-ethoxycoumarin and their metabolites. As a model to test the biocompatibility of these plastics after treatment, a liver cell line (HepG2) was cultured on the UV ozone treated plastics. The cells were able to grow on all treated substrate surfaces after
coating with collagen. Hardly any dead cells were found (~2%) on PC, PS, and COC. This indicates that the plastics are not toxic after treatment. In contrast, 25% of the cells were non-viable on PMMA, indicating a potential effect of the plastic or the UV ozone treatment on cell viability. We concluded that PC, PS and COC are all suitable to fabricate biochips with low adsorptive characteristics and high biocompatibility. Since PS is less resistant to the organic solvents used, for example, for on-line introduction of liquid chromatography on chip, the use of PC or COC is recommended. Of these two, COC has the additional advantage of exhibiting a lower autofluorescence compared to PC, which is beneficial for optical imaging.

A biochip made from COC with aeration windows to control the pH and oxygen concentration in medium would be the best option for further studies. This low-cost device could be produced in batch, would not adsorb hydrophobic compounds if surface-oxidized, and could be used in the on-line analysis system. Multiple chambers containing different slices could be sequentially perfused in a manner similar to the PDMS device. Another interesting aspect for further studies would be the integration of components for the generation of concentration gradients and other components such as a pump for medium flow or detectors for on-chip monitoring of pH or metabolite formation.

The successful development of a microfluidic-based in vitro system with perifusion of liver and intestinal slices to study drug metabolism has been realized. A stable incubation environment, e.g. pH, oxygen concentration and nutrients, was achieved by introducing a low flow rate. Liver slices could be maintained viable for 72 hours and intestinal slices for at least 3 hours. Although it was previously suggested that the introduction of medium flow in cell or tissue culture would promote longer viability, this has not yet been confirmed in our system. Possible causes for this and their resolution remain to be elucidated. A limitation of the system is the possible adsorption/absorption of hydrophobic compounds onto the PDMS material; however, by producing the chip in COC, this can be prevented. This newly developed in vitro system is very promising for gaining better insight into possible interorgan effects. Moreover, earlier detection of unstable metabolites is made feasible with the on-line HPLC system. As an alternative to well plates, this system will contribute to a better prediction of metabolism and toxicity in man with a concomitant reduction in the use of experimental animals. The microfluidic biochip thus has the potential to significantly impact drug metabolism and toxicology studies of novel chemical entities.

References
