Summary, general discussion and future perspectives
Precision cut intestinal slices (PCIS) represent a newly developed _ex vivo_ model to investigate intestinal function including drug disposition. They contain all the intestinal cell types in their natural matrix and maintain high activities of enzymes and transporters involved in drug disposition during culture up to 8-24 hours (van de Kerkhof et al., 2007). Since drug-induced toxicity is usually a result of interaction between multiple cell types, and drug metabolism and transport play a critical role in toxicity, PCIS are a potentially promising model to study drug-induced toxicity _ex vivo_. PCIS can be prepared from each region of the intestine, which allows the study on the gradients of activities of the drug metabolism enzymes (DMEs) and the drug transporters (DTs) along the length of the intestine (van de Kerkhof et al., 2007, Dawson, 2011, Khan et al., 2009). These gradients are of toxicological importance as they determine the exposure of the enterocytes from various regions of the intestine to the ultimate toxicant. The model of PCIS can be applied on various species in a similar manner, which benefits the elucidation of species differences in toxicity, and especially advances the toxicity study in the human intestine (Niu et al., 2014) (**Chapter 1**).

Up to now, the application of PCIS for the evaluation of xenobiotic toxicity, metabolism and transport is still in its infancy. Therefore, **the aim** of the research described in this thesis was to investigate the applicability of PCIS to study drug-induced intestinal toxicity, and we focused on non-steroidal anti-inflammatory drugs (NSAIDs) as model compounds.

First of all, one of the most extensively studied NSAIDs, diclofenac (DCF), was used as a model compound to test whether the rat PCIS system can correctly reflect the mechanism of DCF toxicity in **Chapter 3**. It has been suggested that the topical effects of DCF in the intestine are the result of a multiple hits pathogenesis involving several causal factors. Luminal exposure to DCF is known to induce electrophile stress (induced by biliary or intestinal metabolites), endoplasmic reticulum (ER) stress, mitochondrial injury, and oxidative stress and finally leads to cell death (Mahmud et al., 1996, Boelsterli et al., 2013). The data in **Chapter 3** show that these mechanisms were correctly reflected in PCIS by up-regulated HSP-70 (heat shock protein 70) and BiP (binding immunoglobulin protein) gene expression, caspase 9 activation, GSH (glutathione) depletion and HO-1 (heme oxygenase 1) gene up-regulation respectively. Furthermore, DCF intestinal metabolites, were detected in PCIS, which gave rise to protein adducts and thus electrophile stress, but were not correlated with cell death. In addition, in this chapter, the _ex vivo_ toxicity ranking of five NSAIDs (diflunisal > diclofenac = indomethacin > naproxen >> aspirin) studied in PCIS showed good correlation with published _in vitro_ data, using yeast, hepatocytes and isolated liver mitochondria (van Leeuwen et al., 2012, Somasundaram et al., 1997, Banos and Reyes, 1989, Jurima-Romet et al., 1994). The _ex vivo_ data also showed a good correlation with _in vivo_ data with the exception of diflunisal (Wax et al., 1975, Brune et al., 1987). The correctly reflected multi-hits mechanisms of DCF induced intestinal toxicity in PCIS and the good toxicity ranking prediction of 4 of the 5 NSAIDs using PCIS confirmed the capability of PCIS system to study drug-induced intestinal toxicity _ex vivo_. Although it cannot be excluded that PCIS
Specifically lack some important feature to reflect the diflunisal toxicity, the good correlation with \textit{in vitro} preparations indicate that it is more likely that this lack of correlation is due to some pharmacokinetic or pharmacodynamics aspect of diflunisal that is only represented \textit{in vivo}. For example, it was reported that diflunisal undergoes higher enterohemipatic circulation in rats than diclofenac or indomethacin, which would result in a higher intestinal exposure to diflunisal \textit{in vivo}, and thus an expected higher toxicity (Brune et al., 1987). However, diflunisal is the most potent toxicant \textit{ex vivo} and \textit{in vitro} among five NSIADs tested, but exerts less toxicity than diclofenac and indomethacin \textit{in vivo}. We incubated the PCIS with all 5 NSAIDs for 5 hours to observe the toxic effects. Although 5 hours incubation is long enough to observe the toxicity, and short enough to maintain the functionality of the rat intestinal slices, we are not sure whether the exposure \textit{ex vivo} is representative for the \textit{in vivo} intestinal exposure of these 5 NSAIDs due to their different levels of the enterohemipatic circulation. Further research is needed to elucidate the reason for this \textit{in vivo} versus \textit{ex vivo} difference of diflunisal.

PCIS can be prepared from each region of the intestine, gradients of activities of DMEs and DTs along the length of the intestine were reported previously (van de Kerkhof et al., 2007, Dawson, 2011, Khan et al., 2009). In the present study, DCF induced toxicity along the rat intestine was studied. The results showed that DCF 200\(\mu\)M induced more ATP decrease in rat jejunum slices (46\% decrease) than that in duodenum (23\% decrease), ileum (25\% decrease) and colon (10\% decrease), which indicates that rat jejunum is more sensitive to DCF toxicity than the other three regions, and colon was the most resistant region to the DCF toxicity (figure 1).

\begin{figure}
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\includegraphics[width=\textwidth]{DCF_toxicity_graph.png}
\caption{DCF induced toxicity along the rat intestine. PCIS were obtained from duodenum, jejunum, ileum and colon, and incubated with 200\(\mu\)M DCF for 5 hours. The ATP level is presented as the percentage of control (ATP level of control slices from each region incubated without DCF for 5 hours). Data represent the average ± SEM (n=3 rats). * significantly different from control (100\%), \(p<0.05\).}
\end{figure}

It is well known that the concordance of xenobiotic metabolism and toxicity in humans and animals is alarmingly low (O’Brien et al., 2006). PCIS provide the opportunity to study these processes in human slices, and elucidate potential species differences between human and experimental animals. DCF-induced toxicity was studied in human intestine in Chapter 4. PCIS from human small intestine (derived from patients undergoing Pylorus-Preserving
Pancreaticoduodenectomy surgery) were incubated with a concentration range of DCF. A TC50 value of 371 µM was found, which was substantially higher than the TC50 found in rat PCIS (229 µM, calculated by the ATP level, Chapter 3), indicating that the rat intestine is more sensitive to DCF toxicity than the human intestine (figure 2). Three main DCF metabolites 4'-hydroxy DCF, 5-hydroxy DCF and DCF acyl glucuronide were detected in both rat and human PCIS, however human PCIS produced much more metabolites than rat PCIS after incubation with 200 µM DCF (figure 3).

Reactive metabolites-related protein adduct formation is a traditional concept to explain DCF-induced toxicity. Previous studies in rats suggested that diclofenac-acylglucuronide (DAG) produced by the liver and excreted into the intestine via the bile, was responsible for the intestinal toxicity by covalently binding to enterocyte proteins (Atchison et al., 2000, Seitz and Boelsterli, 1998). Data supporting this mechanism in man are not available due to the lack of adequate in vitro methods. Drug metabolism is relatively well preserved in human PCIS during culture up to 24 hours (van de Kerkhof et al., 2007b). For this reason, human PCIS are an excellent ex vivo model to study metabolism-related drug toxicity, and, more importantly, provides the opportunity to investigate this process not only in animals, but also...
in human tissue. Moreover, using this model we could evaluate whether the intestinal toxicity is induced by DCF itself or by its intestinal metabolites without the influence of liver-derived metabolites. In Chapter 4, PCIS prepared from the jejunum of 18 human donors were used. DCF (≥ 400µM) caused direct toxicity to the human PCIS as demonstrated by ATP depletion, morphological damage, caspase 3 activation and lactate dehydrogenase (LDH) leakage. Three main metabolites and their protein adducts were detected in PCIS. Several observations done in this study led to the conclusion that DCF itself rather than its metabolites causes the observed toxicity: first of all, DCF induced similar toxicity to all samples regardless of the high variation in metabolism among them and no correlation was observed between the extent of metabolism and the observed toxicity. Furthermore, less metabolites were produced by slices incubated with 400µM DCF than by those incubated with 100µM DCF, whereas 100µM did not induce toxicity. Finally, the addition of inhibitors of the most prominent metabolic pathways decreased the metabolite formation but increased the toxicity.

Another advantage of using an ex vivo model to study drug-induced intestinal toxicity is that the influence of the liver (especially the liver metabolites) can be excluded. It has been reported that Mrp2 deficient rats (Mrp2−) were resistant to DCF-induced intestinal toxicity due to the impaired Mrp2-mediated transport of DCF metabolites formed in the liver to the intestine in vivo (Seitz and Boelsterli, 1998). In Chapter 5, this mechanism was tested using two ex vivo setups: PCIS and Ussing Chamber using the intestine of wild type (WT) and Mrp2− rats. It was observed that Mrp2− rat intestine was intrinsically more resistant to DCF toxicity than its wild type counterparts ex vivo. This lower sensitivity could be explained by the lower uptake of DCF in the Mrp2− rat intestine, but was not related to other consequences induced by Mrp2 deficiency in the intestine such as Mrp3 and BCRP transporter gene expression, reduced glutathione content and DCF metabolism. Previously it has been concluded that the impaired biliary transport of DCF metabolites (substrate of Mrp2) into the intestine can fully explain the lower DCF toxicity in the Mrp2− rat intestine. The experimental set up presented in Chapter 5 allowed us to exclude the influence of the biliary metabolites, and the results indicate that the reduced uptake of DCF by the Mrp2− rat intestine contributes to the reduced DCF toxicity ex vivo and therefore is at least partly responsible for the reduced DCF toxicity in vivo. Moreover, the data obtained in Chapter 5 showed consistency with the results in Chapter 4 that DCF instead of its reactive metabolites is responsible to its intestinal toxicity.

Although the rat PCIS remain viable until 5 hours incubation with respect to the maintenance of an intact epithelial lining, metabolism and transport capacity (van de Kerkhof et al., 2007), the life span of the intestinal slices (up to 24 hour) is much shorter compared to other organ slices (up to 96 hours) (Vickers and Fisher, 2004). In Chapter 6, decreased ATP, damaged morphology, increased ROS formation, induced pro-inflammatory gene expression (IL-6 and TNF), and induced oxidative stress (HO-1) were shown in rat PCIS after 5-24 hours incubation. These findings indicate that an ischemia-reperfusion (IR) insult may occur during
the preparation and culturing of the PCIS, since the intestine is known to be very sensitive to IR injury (Mallick et al., 2004). In this chapter, ischemic preconditioning and H$_2$S preconditioning were used as an attempt to improve the reduced viability in PCIS. These two methods appeared promising in protecting the intestine against IR injury in vivo (Blackstone et al., 2005, Liu et al., 2009, Liu et al., 2010). However, although our results show that they seemed to reduce the pro-inflammatory response and oxidative stress in PCIS, the viability of the PCIS was not improved.

Although the PCIS provide the opportunity for mechanistic studies of drug-induced intestinal toxicity as shown in Chapter 3 and 5, the highly up-regulated pro-inflammatory gene expression and oxidative stress after culture could be a limitation for the application of this model. It was reported that the addition of lipopolysaccharide (LPS) together with toxicants could enhance their toxicity in liver slices probably by inducing a pro-inflammatory response, (Hadi et al., 2012). We also found that adding TNFα to liver slices increased caspase 3 activity, indicating increased apoptosis (figure 4a). However, the addition of TNFα in intestinal slices did not further increase the DCF induced caspase 3 activation (figure 4b). This could be explained by the higher level of the caspase 3 in the intestinal slices than the liver slices, indicating a higher apoptotic status in the intestinal slices after 5 hours incubation, and leaving no space for further induction. Another explanation could be that the expression of the TNF receptor in the intestine is lower compared to the liver (Roulis et al., 2011). In contrast, a pilot experiment in the present study with rat PCIS showed that a combination of ischemic pre-conditioning and dexamethasone treatment reduced the pro-inflammatory response and attenuated the toxicity of DCF in (data not shown). Further investigations are needed to explore the mechanisms behind it. At present, research is ongoing to see whether anti-inflammatory compounds such as dexamethasone or aspirin can reduce the inflammatory stress in the rat PCIS during culture, and thereby could influence the sensitivity of the PCIS to DCF-induced toxicity. Furthermore, the innate immune system often plays an important role in the later stage of the drug-induced toxicity. The presence of macrophages in the PCIS is suggested by the up-regulated IL-6 and TNFα during culturing. Further research is needed to investigate in more detail the role of macrophages and neutrophils in the PCIS.

In conclusion, the studies described in this thesis have shown that PCIS are a suitable model to study the mechanism of drug-induced intestinal toxicity ex vivo. Using human PCIS not only allows to obtain human-specific data, but also to obtain insight in potential species differences and thereby helps to select the best representative animal model for in vivo studies. It is considered as a higher throughput model compared to in vivo animal models and can reduce and refine the use of experimental animals. At the same time, it is more representative than other in vitro intestinal models as all the cell types are present in their natural environment in PCIS, its metabolism and transport functions are maintained during culture, and it can represent the differences in response in different regions of the small and large intestine. Taken together, using PCIS obtained from both humans and animals will...
result in a better prediction of drug-induced toxicity during drug development, reducing drug failures due to species differences.

Fig. 4. The effect of TNFα on viability or DCF toxicity in rat liver slices (a) or intestinal slices (b). Data represent the mean ± SEM, n=4 rats. * significantly different from the vehicle controls (slices incubated for 5 hours with DMSO), p< 0.05.

References


