Chapter 7

General discussion and perspectives

Marcella Martignoni¹, Ruben de Kanter² and Geny Groothuis³

¹ Preclinical Development, Nerviano Medical Sciences, Viale Pasteur 10, 20014 Nerviano (MI), Italy
² Solvay Pharmaceuticals, C.J. van Houtenlaan 36, 1381 CP, Weesp, the Netherlands
³ University Centre for Pharmacy, Dept. Pharmacokinetics & Drug Delivery, University of Groningen Ant. Deusinglaan 1, 9713 AV, Groningen, the Netherlands
§ 1. Introduction

In this chapter, the results and conclusions of this thesis as well as future perspectives of the use of in vitro models to study species differences in metabolism and induction and to predict the human situation will be discussed.

§ 2. The effect of the cryopreservation of liver slices of different species, including man: comparison of metabolic activities

Precision-cut liver slices are a valuable tool for in vitro metabolism studies. However, human tissue, and even tissues from larger animals like monkey and dog are sparse. Moreover, if relatively large organs become available for research purposes, the number of slices that can be prepared is usually much higher than can be handled at the moment. Therefore a successful cryopreservation method is very much needed. The rapid freezing method as developed by de Kanter et al. [1] was applied for cryopreservation of slices from several species (rat, mouse, monkey, dog and human). Various end-points were used to determine whether cryopreservation of slices was successful and it appeared that, in practice, the judgement "viable" or "not viable" depends on the parameter used for the evaluation. It was shown that ATP and LDH retention were more affected by cryopreservation in all investigated species, whereas MTT reduction and phase I and phase II metabolic activity were less affected. These finding are in agreement with those of other authors who studied the viability of thawed slices [2, 3, 1,4].

§ 2.1. Effect of cryoprotectant concentration on slice cryopreservation

In our work, phase I and phase II metabolism, evaluated by measuring the formation of testosterone metabolites and of 7-HC-glucuronide and 7-HC sulphate, was well preserved in thawed slices of all species tested in accordance with several reports [1,3], even though other parameters, such as ATP and LDH, indicated severe loss of slice integrity. On the contrary, others reported that phase II biotransformation was severely affected by cryopreservation [4,5]. Several explanations were given for this observation, such as loss of the phase II enzyme due to induced membrane leakage, limited cofactor availability and loss of enzyme activity by denaturation. However, we and others [3] showed that the freezing method and the amount of cryoprotectant (18% DMSO) is crucial for the preservation of phase II metabolism in several animal species. In fact, it was demonstrated [6] that the increase of DMSO concentration from 12% to 18% efficiently preserved the rates of 7-hydroxycoumarin glucuronidation and sulphatation in rat liver slices. The improvement by higher DMSO concentration can be explained by improved inhibition of ice crystal formation [7]. In addition, slices cryopreserved in 12% DMSO have a defective respiratory chain and reduced production of ATP, which may explain the observed decreases in UDP-glucuronosyltransferases and sulphotransferase activities [3]. On the contrary, a higher DMSO concentration was demonstrated to be toxic and DMSO concentration up to 30% resulted in potassium content reduction with consequent decrease of slices viability [6].
§ 2.2. Species viability and metabolic function of liver slices after cryopreservation

As expected, the different species demonstrated marked differences in metabolite patterns (see § 4.1) of testosterone and 7-hydroxycoumarin and these qualitative differences were maintained after cryopreservation.

Remarkably, we have found that not only phase I and phase II metabolism was well preserved in thawed slices of all species, but that some metabolic rates were even increased after cryopreservation, such as for testosterone and 7-hydroxycoumarin (7-HC). The reason of this is unknown but it may be due to the increased substrate availability in the damaged slices. In fact, the viability parameters measured, demonstrated that some degree of tissue damage was apparent in cryopreserved liver slices, in particular ATP and LDH content, but not leakage, were affected due to cryopreservation.

On the other hand, the mitochondrial reduction of MTT was not changed after cryopreservation. Therefore, the use of more than one and preferably several viability parameters is important in order to investigate cellular injury including tests on energy status, membrane integrity, and the ability of cells to carry out various biochemical functions. Together, these viability parameters provide a better picture of the overall status of the tissue slice than when only one parameter is investigated. According to the current results, the sensitivities of these viability parameters to detect cellular dysfunction can be ranked as follows: ATP > LDH > MTT [8].

Summarizing, this rapid freezing method using 18% DMSO as cryoprotectant for liver slice cryopreservation is appropriate to several species and permits the formation of a liver bank from rat, mouse, monkey, dog and human, allowing the easy comparison of qualitative differences in metabolic profiles (both phase I and phase II mediated metabolism) of potential drug candidates among different species, including human. However, the short life span of cryopreserved liver slices (3-4 hours) might limit the application of cryopreserved liver slices in toxicological studies.

§ 3. CYP induction in rodent and non-rodent species

In chapter 3 and chapter 4, CYP induction was investigated in mouse and rat liver and rat intestinal slices and compared to in vivo situation. The aim was to investigate whether slices are a suitable tool to predict drug induction of CYP mediated metabolism and to study species difference therein.

§ 3.1. *In vitro* induction models

There are various *in vitro* models for assessing CYP enzyme induction including precision-cut liver slices [9,10], primary hepatocytes [11], and reporter gene constructs [12]. This latter technique is a cell based assay able to investigate the activation of the nuclear receptor PXR allowing a rapid evaluation of potential human/rodent specific CYP3A inducers during the early phase of drug development [13]. However, this assay is only useful to investigate induction of the CYP3A isoform through PXR activation and no other route of induction of CYP3A4 (CAR, GR, VDR) or other CYP isoforms can be
investigated. Moreover, this method has been used to investigate CYP induction in human and mouse, but not yet in other animal species. Therefore, in our work, we studied in vitro induction using slices in combination with real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), which allows the investigation of mRNA expression of every desired gene, including the CYP genes. Because gene induction of the genes of interest is directly measured, this assay is independent on the mechanism of induction, which can be through PXR, CAR, VDR, AhR and GR activation.

Moreover, the same methodology can be used to investigate CYP mRNA expression both in vitro and in vivo in several animal species.

§ 3.2. Inducers

In chapters 3 and 4, the induction in rat and mouse liver and intestinal slices was reported and compared with in vivo treatment. In addition, we studied the in vitro induction in monkey liver slices and in human cryopreserved hepatocytes, which are reported here (see § 3.3). Several strong inducers were selected taking into account differences of induction among species and included βNF (for mouse, rat and monkey), omeprazole (for monkey and human), PB (for all species), DEX (for mouse, rat and monkey) and rifampicin (for human and monkey). These inducers were selected based on the fact that significant literature information is available and that they cover a range of different CYP isoforms. In particular, we have investigated the expression of four CYPs that are known to be inducible by xenobiotics and that are important for drug metabolism [14]. However, we are aware that only potent inducers were investigated and not weak and moderate inducers that could help to validate this approach. In fact, the investigation of a broader range of compounds from weak/moderate/strong inducers would allow a more quantitative interpretation of the data and thus a better prediction of the in vivo situation.

§ 3.3. Human and monkey CYP induction

Human and monkey primers and probes were designed from cDNA sequences from GenBank and reported in Table 1. Monkey liver slices were prepared as described in Chapter 2 (Materials and Methods). Special plateable cryopreserved human hepatocytes were thawed and plated as described by In vitro Technologies (Inc., Baltimore, MD, USA). Cells were cultured 24 hours before the induction experiment was started (see Materials and Methods of Chapter 3 and Chapter 4 for details regarding the induction experiments).
Table 1. Taqman® primer and probe sequences of human and monkey cyp mRNA

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Primers/probe</th>
<th>5'→3' Sequence</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>1A1</td>
<td>Forwards</td>
<td>CCCCACCACCTCCCCCA</td>
<td>NM-00499.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TACAAAGACACAACGCCCTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>AGCACAACAGAGCACACACTTGAGGAGATGGA</td>
<td></td>
</tr>
<tr>
<td>human</td>
<td>1A2</td>
<td>Forwards</td>
<td>ACTCTCTTTGCAATGTCAG</td>
<td>NM-000761.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GATGTAGAAGCCACTTCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>CCCACAGCAACAAAGGGCACAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTATTTCACCAGTGTTCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>CCTCATAGTTGTCAGAGAATCG</td>
<td></td>
</tr>
<tr>
<td>human</td>
<td>3A4</td>
<td>Forwards</td>
<td>GGTGGTGAGATGAAACGCTCAG</td>
<td>NM-017460.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AGTCATCTTTGGTGTCAGCAGTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>CCTCGAGAGGCTGTCTGACAAAAATGATGTTGAGA</td>
<td></td>
</tr>
<tr>
<td>monkey</td>
<td>1A1</td>
<td>Forwards</td>
<td>CCCCACCACCTCCCCCA</td>
<td>AY-635458.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ACAAAGACACAAAGGCCCTT</td>
<td></td>
</tr>
<tr>
<td>monkey</td>
<td>2B30</td>
<td>Forwards</td>
<td>CTCTCTCTACCCACCAACTCC</td>
<td>AY-635461.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTATTTTGCCACACACACTCT</td>
<td></td>
</tr>
<tr>
<td>monkey</td>
<td>3A8</td>
<td>Forwards</td>
<td>GCCTTTATATTGTTGGTCTGAAGAAT</td>
<td>AB124984.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCCGGTTGAGAGAGAAGCAGAAT</td>
<td></td>
</tr>
</tbody>
</table>

In accordance to the data of Roymans et al. [15], a clear induction for human CYP1A1/1A2 after exposure to 50 μM omeprazole (about 18- and 135-fold, respectively), and for human CYP3A4 after exposure to 25 μM rifampicin (about 57-fold) was observed (Figure 1). The 3-fold induction of human CYP2B6 after exposure to phenobarbital (1 mM) is also in accordance with the literature although also higher induction values are reported, depending on the donor. Also, the induction of human CYP3A4 by phenobarbital of about 26 times is in agreement with the data reported in the literature.
Similarly to mouse and rat, βNF significantly induced monkey CYP1A1 (82 fold), whereas omeprazole significantly induced CYP1A1 only 2 fold. A clear induction for monkey CYP2B30 was shown after exposure to 1 mM Phenobarbital (3-fold) and Dex (2-fold). Monkey CYP3A was induced after treatment with 25 µM rifampicin (4-fold), but no induction was found after treatment with dexamethasone. Those results (Figure 2) are in accordance with the data of Nishibe et al. [16] who used monkey hepatocytes. However, the fold induction found in monkey is generally lower than in other species including man. The differences in response between man and monkey in this respect do not support the idea that monkeys are a better model for man than rodents for induction studies.

**Figure 1.** CYP mRNA levels in human hepatocytes after induction. Results are means of three separate experiments, (n=3 wells per experiment each) ± SEM. Values significantly different from control: *p < 0.05.
Figure 2. CYP mRNA levels in monkey liver slices after induction. Results are means of 3 individual slices (one experiment, n=3 slices each) ± SEM. Values significantly different from control: *p < 0.05.

§ 3.4. In vitro evaluation of induction of metabolism

Nowaday, the mRNA levels of P450 enzymes can be quantified accurately by real-time PCR [15]. It was observed that in cases when P450 induction is regulated mainly at the transcription level, determination of the different P450 mRNA levels correlated in at least 82% with the measurement of P450 activity [15]. However, an increased mRNA expression does not necessarily mean the same absolute increase in protein activity, because of various reasons including limited amount of co-factor and/or CYP450 reductase. For example, it has been demonstrated that in rats treated with Aroclor, induction of CYP1A1 gave rise to ~23000-fold induction of mRNA level, but only to 127-fold difference in protein activity [17]. Therefore only activity measurement can give a definitive answer on whether the observed higher mRNA expression level necessary means the same increase of activity.

So far, we demonstrated that the induction profiles (qualitative and quantitative) (chapters 3 and 4) observed in vitro are very similar to in vivo results in mouse and rat, but in human and non-rodent species correlation could not be assessed and should be further investigated.

In the case of CYP2E1 the determination of the mRNA expression levels has no predictive value as this isoform is induced by xenobiotics mainly via protein stabilization [18,19], and no induction of its mRNA expression was observed whereas CYP2E1 activity was significantly increased [15].
Induction of CYP mRNA expression has been reported as induction potential (fold induction over control) in most reports on this subject [20, 17, 14, 9]. However, this approach suffers from some limitations: first, the basal levels of some CYPs may be low and difficult to be accurately quantitated, such in the case of CYP1A2 in the intestine or CYP1A1 in liver. Secondly, the basal levels in culture may be highly variable between different preparations especially in human preparations due to large interindividual variability in activity, while the maximum induction levels are more consistent [21, 22]. Therefore when xenobiotics are investigated for potential CYP induction capacity, the potency index (the ratio of induction response of the tested compound compared to that of a standard) rather than induction potential (fold induction over control) may give a more useful value.

It is important to realise that levels of induction are dose and time dependent. For our experiments in slices we selected the concentrations that are not toxic. In addition, the incubation time was kept as short as possible (24h for liver and 6h for intestinal slices), avoiding, as much as possible, a decrease of slice viability, while taking advantage of the sensitivity of the real-time RT-PCR method in comparison to protein activity evaluation for which a longer incubation time was required. Shorter incubation times also avoid artificially high fold induction over control as a result of a drop of the basal levels of the CYPs genes during prolonged in vitro culture. Using RT-PCR we were able to observe mRNA induction after 6 hours incubation in intestinal slices.

However, even though it is relatively easy to assess in vitro drug induction by analyzing gene expression, the correct prediction and extrapolation of the in vitro data to the in vivo situation can be performed only by taking into account the pharmacokinetics of the tested compound and the inducer. Drugs can be classified by whether their hepatic clearance is enzyme-limited or flow-limited. When the intrinsic clearance of the drug is very small relative to the hepatic blood flow, the hepatic clearance is consequently low. Therefore, a change (increase) in the CLint caused by induction will result in almost proportional change in the clearance of "low clearance" drugs. On the other hand, if the intrinsic clearance is high, the hepatic clearance is limited by the hepatic blood flow (CLH=QH). Thus a change (increase) in the intrinsic clearance caused by induction has little or no effect on the hepatic clearance of "high clearance" drugs.

In addition, the effect of induction is related to the time that the inducer is present in the body and after its elimination the induction effect depends on the time that the enzyme remains active before being broken down by the body (turn-over). Therefore, a good in vitro-in vivo correlation should take into account the clearance of the inducer and the turnover rate of the CYP enzymes, which can be organ specific.

In vivo, unlike hepatocytes, that have a long life span and regenerate only when after cell damage occurs, epithelial cells of intestinal mucosa have a programmed limited life span (3-5 days) [23]. Therefore the shortness of the enterocyte life span diminishes the potential of metabolic enzymes inducing agents in the small intestine to produce increased metabolic rates in the enterocytes for an extended length of time and may provide a protective mechanism against carcinogenic toxins [24]. In fact, newly generated cells show normal enzyme level.
In conclusion, despite the problems mentioned, according to our results the use of liver and intestinal slices in combination with real-time PCR is a rapid and suitable procedure to assess changes in CYP mRNA expression in whole organs and organ slices from rats and mice, giving comparable induction profiles both qualitative and quantitative to in vivo situation. In addition, the main advantage is that, in contrast to plateable cryopreserved hepatocytes, which are only available for human, liver slices can be made for many species.

§ 4. Species and strain differences in metabolic rates

§ 4.1. Phase I and phase II metabolism in experimental animals and man

In chapter 2, the metabolism of testosterone and 7-HC was investigated in liver slices of several animal species. As expected, marked differences in metabolite patterns of testosterone and 7-hydroxycoumarin were observed among species and these qualitative differences were maintained after liver slice cryopreservation. 6β-TOH and androstenedione were the main CYP-mediated testosterone metabolites, detected in fresh liver slices in each species, even though the metabolic rates were different among species. In mouse liver slices, 6β-TOH was formed at ~2-fold higher rate than in rat, dog and monkey. 7α-OHT, a CYP2A mediated metabolite, was only detected in fresh slices from mouse and rat, while 2α-OHT, mediated by CYP2C, was only detected in fresh rat liver slices. 2β-OHT was detected in all species studied. 16α-OHT and 16β-OHT were formed only in liver slices from rat, monkey and human. Although less pronounced as observed for testosterone, the formation rate of 7-hydroxycoumarin glucuronide and of 7-hydroxycoumarin sulphate was different among the species. Remarkable was the observation that in human liver slices, the amount of 7-hydroxycoumarin sulphate was minimal.

§ 4.2. Investigation of strain-related differences in drug metabolism and efflux transporters characteristics

In chapter 5, CD-1 mouse and nude mouse (athymic mouse) were compared for drug disposition of new chemical entities. We concluded that CD-1 and athymic nude mice demonstrate remarkable similarities for hepatic phase I and phase II metabolism and for efflux transporters (phase III), and therefore the potential error in extrapolating pharmacokinetic data obtained from CD-1 to nude mice, or vice versa, is expected to be minimal. Several transgenic mice models are used to study human diseases, such as diabetes, obesity, tumors, cardiovascular and renal diseases. These models were often shown to be predictable, reproducible and easy to standardize. However, animals, such as rat and mouse, belonging to different strains are similar in size and shape, yet they may exhibit some differences in physiological and pharmacokinetic characteristics. Strain differences in the pharmacodynamics of diazepam have been reported between Sprague-Dawley (SD) and Dark Agouti (DA) rat strains or among outbred Wistar (W) rats [25]. Interestingly, it has been reported that DA rats have poor CYP2D activities compared with SD and Wistar rats [26-28] and especially, CYP2D activities in female DA rats was
lower than those in male rats [28,29]. Also the effect of several antidepressants (desmethylimipramine, amitryptiline and bupropion) varies across strains of mice [30]. Therefore differences in drug metabolism, in which major metabolites differ depending on the strain of the animal, may cause significant differences not only in the result of the pharmacokinetics of the drug, but also in pharmacological or toxicological studies, especially when the metabolites of the test compound have a pharmacological or toxicological effect.

In addition, it is well known that the inductive response to polycyclic aromatic hydrocarbons is variable among different mice strains. It was reported that inbred mice (B6C3F1, C57B1/6 and C57B1/10ScN) having a lower response in comparison to outbred mice (CD-1 mouse). This phenomenon can be explained by variability of the Ah receptor expression [31,32]. Similar to mice, also rat strain variation in hepatic CYP1A1 gene induction was observed caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and it was suggested that differential expression of both AhR (4 times more expressed in high responders and ARNT is correlated to different induction level).

Another important topic is the effect of inhibitors of drug metabolism in different strains of animals. Different response to inhibitors may help to interpret variation in pharmacokinetic and pharmacodynamic drug characteristics among animal strains. So far, no inhibition differences related to strain have been reported, but dissimilarities among species are well known (see chapter 1).

§ 5. Metabolism in liver and intestine

§ 5.1. Intestinal metabolism

One of the aims of this work was to evaluate the role of the intestine in the metabolism of compounds. The small intestine can be divided into three parts: duodenum, jejunum, and ileum. These regions are anatomically somewhat distinct, and there are differences in their absorptive and secretory capabilities. We mainly focused on the very first part (20 cm) of the intestine (mainly duodenum and jejunum in the rat), because it is known that the distribution of P450 enzymes is not uniform along the length of the small intestine [33] and that CYP enzymes have the highest concentration in this proximal part of the intestine and most drugs are absorbed here. Also in humans, both the content and activity of P450 was reported to be higher in the proximal than in the distal small intestine [34].

Phase II enzymes, such as GST and UGT have been demonstrated to be markedly lower expressed in the human colon relative to the small intestine [23]. Therefore it will be interesting to extent our research to the remaining parts of the intestine, including colon in order to better predict the total contribution of the intestine. Immunoblotting studies have shown that CYP3A4 is the dominant cytochrome P450 isof orm in the human small intestine where it accounts for the majority of total microsomal P450 found in the mucosal epithelium [35]. A recent study [36] demonstrates that CYP3A4 and CYP2C9 are the highest expressed CYP enzymes accounting for 82% and 14% respectively, whereas CYP2C19, CYP2D6 and CYP2J2 are expressed lower. Median values of 31, 23 and 17 pmol/mg microsomal protein of CYP3A4 were measured in duodenum, jejunum
and ileum, respectively. A similar differential distribution of intestinal P450 was observed in animals. In rats, CYP2B1/2 and CYP1A were detected in the intestine in addition to CYP3A with the highest level in duodenum [37]. In the mouse, CYP3A is the predominant form, even though CYP1A and CYP2B were detected at least at the mRNA levels [38]. Therefore in addition to CYP3A, more enzymes need to be investigated to further characterize the role of the intestine in first-pass metabolism using a broader range of marker substrates covering more CYP isoenzymes, such as CYP1A and CYP2B isoforms. Also the effect of different inhibitors could be used for this purpose. In our work, only the contribution of ketoconazole was evaluated. However, ketoconazole has been demonstrated to be a non-selective inhibitor of CYP3A in rats because it also inhibits the activities of other P450 isoforms, such as CYP1A2 and CYP2C6. Therefore apart from ketoconazole, other CYP3A inhibitors, such as the protease inhibitor ritonavir or the mechanism-based inhibitor troleandomycin, could be used to further investigate the role of CYP3A in the intestine.

§ 5.2. Quantification of CYP3A isoforms in liver and intestine

CYP3A isoforms are expressed differently between liver and intestine. An absolute quantification of CYP3A1, CYP3A2, CYP3A9, CYP3A18 and CYP3A62 in rat liver and intestine has been described [39] (see discussion in chapter 6). We showed that similar to rats (chapter 6), mouse CYP3A isoenzymes are expressed differently in liver and intestine: CYP3A11, CYP3A25 and CYP3A41 are predominantly expressed in the liver, whereas CYP3A13 is more prominent in the intestine. However, only a relative quantitative determination of mRNA expression rather than an absolute expression of each single isoform (e.g. attomole of CYP3A mRNA /µg total RNA) was performed, allowing only a comparative expression of the investigated CYPs among tissues (liver versus intestine), but not among the different isoforms. The absolute quantification of CYP3A isoforms in mouse, monkey and dog has not been reported yet.

§ 5.3. Factors affecting intestinal metabolism

The intestine is a perfused organ: in man, rat and mouse the respective blood flow are 500 ml/min, 14.5 ml/min and 0.9 ml/min. The blood that perfuses the small intestine flows immediately into the liver by way of the portal vein. Blood entering the intestine via the artery is distributed to the various layers of the small intestine. In dogs, approximately three-fourths of total resting intestinal blood flow is distributed to the mucosa and the remainder to the submucosa, muscularis, and serosa [40]. In rats, 60-70% of the intestinal blood flow is distributed to the epithelial mucosal cells [41]. Therefore, the metabolism of the compounds in the intestine that are reaching the intestine via the blood may be limited by the blood flow. For compounds reaching the intestinal cells via the lumen, the time of intestinal passage may be of higher influence. We show that intestine and liver slices are a very useful in vitro tool because of the maintenance of the tissue integrity and the relatively simple and straightforward preparation technique suitable to different species and to compare different organs. However, slices are not a "perfused" model similar to the in vivo situation where the
intestine is constantly perfused with blood flow that removes the metabolites from intestinal enzymes. In addition to that, the rate and the extent of metabolism may also be regulated by other factors, such as by efflux proteins and by phase II enzymes, which have not been investigated in our work. Efflux of drugs from the blood by the intestinal mucosa into the intestinal lumen has been acknowledged as a clearance mode for drugs [42], although this is often neglected. The activity of P-glycoprotein, the major membrane transporter involved in intestinal luminal efflux of drugs, may have an impact upon the rate and extent of intestinal drug metabolism by recycling drugs between enterocytes and lumen with the consequently higher drug exposure to intestinal metabolic enzymes. This effect of transporters may be difficult to evaluate using intestine slices. However the co-incubation of drugs with PgP inhibitors, such as verapamil and quinidine, would be a valuable approach to cover these aspects.

§ 5.4. Metabolite formation rate in intestine compared to liver

The metabolite formation rates of the investigated substrates were in general higher in liver than in intestine expressed on a per protein basis. Those differences may be related to the different expression level of CYP3A isoforms between liver and intestine in both rat and mouse (see chapter 6). For example, if the higher expression of CYP3A62 in rat intestine is responsible of the observed higher 3-OH-quinidine and 16β-OHT formation in rat can only be speculated at this moment. Therefore in the future, to investigate which CYP3A isoform may be involved in the metabolism of a particular drug, incubation with specific isoenzymes expressed in cell systems is necessary. However unfortunately, only supersomes containing baculovirus-expressed rat CYP1A1 and CYP1A2 are commercially available, whereas rat CYP3A9, CYP3A18, CYP3A62 and mouse CYP3A11, CYP3A13, CYP3A41 and CYP3A25 are not available yet. But even when possible activity by a specific isoform has been determined, this will not necessarily mean that this isoform is solely involved in metabolism in the intestinal cell. In addition, liver and intestinal slices differ in their cell composition and this may explain at least partly the differences in the metabolic rate between the two organs. In fact, liver slices are mainly composed by hepatocytes (80%), whereas only 20% of intestinal slices is enterocytes.

§ 5.5. Viability of intestinal slices

After an initial increase a drop of ATP content was measured after the first 30 min of incubation in intestinal slices, whereas in liver slices the level was maintained during the incubation time. We have also observed this phenomenon in earlier studies [43], where we showed that ATP levels in vivo (about 2 nmol ATP/mg protein) and the ATP content in intestinal slices during incubation were comparable. Therefore, additional studies are necessary to further investigate whether this difference in ATP content may be ascribed to ATP synthesis at 4°C in the oxygenated media [43]. In addition to that, further viability parameters, such as LDH content or the mitochondrial reduction of MTT could be very useful. Furthermore, some viability
parameters specific for enterocytes such as the determination of the alkaline phosphatase related to the brush border of the epithelial cells or the morphologic investigation of epithelial cells and villi may provide a better picture of the overall state of the tissue slice.

§ 5.6. Linearity with time

Functional viability was measured by analysing linearity of metabolite formation in time, by incubating intestine and liver slices with several human CYP3A substrates. It was noticed that in intestinal slices linearity was maintained for 3 hours only for testosterone, whereas for the other substrates linearity was kept for the first 20-30 min, only (Fig.3). On the contrary, in liver slices for most substrates, including triazolam, midazolam, verapamil and carbamazepine, metabolite formation was linear with time for 3 hours, expect for quinidine and lidocaine for which the linearity was maintained during the first 20-30 min only. It is quite difficult to interpret those findings. Both intestine and liver slices were viable as demonstrated by ATP content during time incubation. We can speculate that the co-factors should be maintained at the same levels in both systems. The substrate concentrations were similar in both liver and intestinal slices (100 µM), and were non-toxic concentrations. Therefore, this difference may be explained assuming the involvement of different isoforms with different stability in the liver and intestine. Also, the involvement of transporters that take up or excrete the compounds in a different rate into and out of the cells may explain those differences. Another possible explanation is that some metabolites may have an inhibitory effect on the enzymes with the consequent decrease of the metabolite rate.

§ 6. Conclusions

Suitable in vitro methods have been developed in combination with sensitive analytical techniques and with analysis of gene expression for the investigation of interspecies differences in metabolism and its regulation. Organ slices, such as liver slices and intestinal slices may be used to enhance our understanding of the metabolism and mechanism of interactions of drugs. In addition, they are a valuable tool for the prediction of human-specific phenomena. In vivo testing in man is often difficult if not impossible while in the case of animal models, in vivo models are complicated by multiple regulatory mechanisms. The pharmaceutical industry now recognizes the value of developing in vitro models systems to assess the safety and efficacy of drugs during both the discovery and the development phase.
In conclusion, the data reported in this thesis demonstrate that the use of slices from different species is a powerful tool that can be used to help to predict metabolic rate and induction in the *in vivo* situation. The availability of suitable *in vitro* tool in animals and in man together to *in vivo* animal data allows a better prediction of the human situation. In addition, the knowledge of species differences in drug metabolism, as detected in slices in combination with sensitive analytical techniques and with analysis of gene expression may be applied to select the best animal species as a model for man in toxicological studies. This will also prevent toxicity studies in inadequate animal models and may result in a safer first administration to humans and in a reduction in the use of experimental animals.
References


General discussion and perspectives


