Pumping new life into preclinical pharmacokinetics: exploring the pharmacokinetic application of ex vivo organ perfusion

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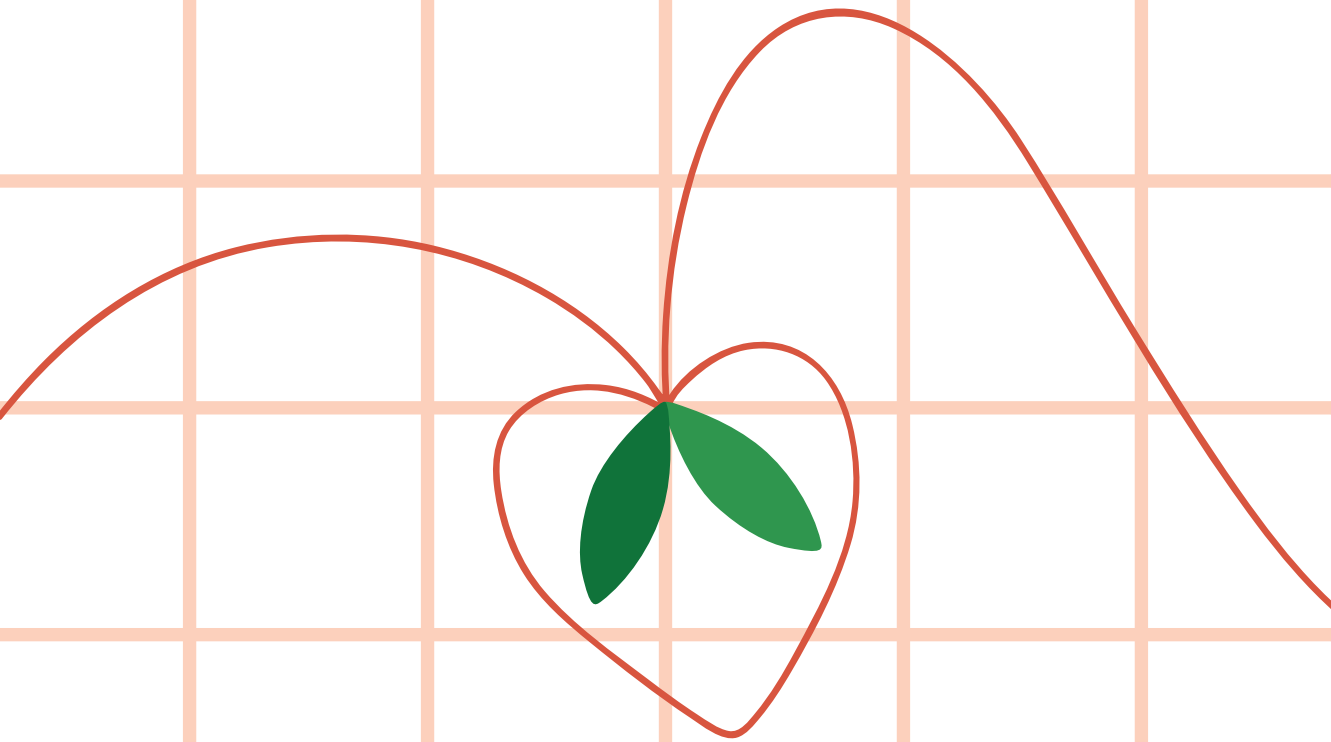
PART I

Exploring pharmacokinetics through ex vivo models



CHAPTER 01

General introduction, scope and outline of the investigation

General introduction

Drug development process

The drug development process is a comprehensive and multi-stage procedure that involves discovery, compound identification, preclinical testing and clinical trials. This is a costly and long process as it takes on average 10-15 years and $1-2 billion for each new drug to be approved for clinical use[1]. It is observed that 90% of drug candidates to not make it do the market[1-3]. A majority of drugs in development tend to fail in phase II of phase III due to the inability to predict toxicity and efficacy *in vivo*[4,5]. The disparate findings during the preclinical phase using animal models and human clinical trials typically manifest during late-stage clinical assessment or during post-market stages[3]. To illustrate, recently the drug fenebrutinib for the treatment of multiple sclerosis resulted in elevated levels of hepatic transaminases and elevated bilirubin levels in some patients in a phase III clinical trial, suggesting a potential risk for drug-induced liver injury[6]. Besides drug induced toxicity, drug-drug interactions (DDI) are also major concerns during the drug development process[7,8]. DDI can occur when patients use multiple drugs which can affect the systemic concentration of the drug. This can potentially result in reduced efficacy, severe adverse reactions or can even result in toxicity leading to withdrawal from the market[7]. It is observed that compounds which are inhibitors or substrates of hepatic transporters are more prone to cause DDI and/or drug induced liver injury, therefore assessment of the hepatic first pass effect and biliary excretion of drugs in development is of major importance. An example is a phase I study where Mita et al.[9] demonstrated that a compound showed non-linear kinetics at the highest dose levels. This could possibly be the effect of saturation in biliary excretion pathway of the compound, as a biphasic plasma profile was observed in the lower dose levels, demonstrating enterohepatic circulation of the compound, however this was not observed in preclinical models and animal studies. Current models that predict biliary excretion often fail due to species differences (rodent/dog) or due to differences in transporter expression in *in vitro* assays (e.g. sandwich cultured hepatocytes)[10-12]. Thus characterization of these complex pharmacokinetic (PK) processes request comprehensive, complete and dynamic preclinical models which can recapitulate the complexity of the human body[13,14], which is crucial to enhance the likelihood of successfully concluding a clinical trial and obtaining approval for a new drug[15]. Developing physiologically relevant models is not only limited to study the DDI potential of newly developed drugs but also to study the outcomes of polypharmacy.

Patients and elderly patients in particular, often receive more than one drug at the same time as they can suffer from multiple conditions. As a result, the uptake or excretion can be mediated by the same transporters and thereby interfering which each other's clearance[16-18].

Drug ADME processes and models involved

Intestine

Oral delivery of a drug is the most preferred route of administration in terms of costs and medication adherence[13]. After oral absorption, the gastrointestinal tract serves as the first barrier for the entry of drugs into the bloodstream. After passive or active (transporter-mediated) absorption, drugs can be metabolized by cytochrome P450 (CYP450) enzymes located within the gut-wall[19]. These drug transporters and CYP450 enzymes are broadly and heterogeneously expressed along the gastrointestinal tract and they can have a major impact on the drug absorption into the portal vein[20]. The intestinal tract expresses a broad range of efflux transporters which belong to the ATP-binding cassette (ABC) family, using ATP as energy source to efflux drugs and endogenous compounds out of cells[21,22]. Main transporters in the intestine are multidrug resistance protein 1 (MDR1), also known as P-glycoprotein (Pgp), breast cancer resistance protein (BCRP) and multidrug resistance protein 2 (MRP2) and they belong to the ABC transporter family and efflux compounds from the apical membrane back into the lumen, thereby limiting the absorption of substrates for these transporters such as rosuvastatin and digoxin[23]. Additionally, CYP450 drug metabolism by the gut wall, which is known as the intestinal extraction (E_G), contributes to the first-pass effect and thereby limits the oral bioavailability[24,25]. Midazolam for instance, a CYP3A4 substrate, undergoes partial metabolism in the gut wall before reaching the liver[24,26,27]. Multiple intestinal preclinical models have been established to measure the absorption from the lumen (apical side) to the portal venous blood (basolateral side). The Caco-2 transwell model is often used to study intestinal permeability[28]. However a major drawback of this cell-based model is the limited expression of CYP450 enzymes and altered transporter protein expression levels compared to human intestinal tissue[29]. The use of *ex vivo* intestinal tissue models is therefore preferred since the morphological structure is intact as well as the presence of uptake and efflux transporters and CYP450 enzymes thereby properly reflecting *in vivo* conditions[30]. In preclinical intestinal models, the intestinal transport is reflected as the apparent

permeability (P_{app}) which represents the apical to basolateral permeability per centimeter per second[30,31]. Subsequently, the P_{app} can be incorporated in physiologically based pharmacokinetic modelling (PBPK) modeling to predict the intestinal absorption and clearance[32]. Current intestinal models are predominantly static, while in vivo, the intestinal luminal flow as well as the superior mesenteric artery flow affect the absorption and metabolism of drugs[33,34]. This shows the importance of incorporation of flow in preclinical intestinal models to properly predict absorption and metabolism. The developments in the field or organ on-a-chip have therefore the capacity of better reflecting the *in vivo* intestinal transport[35-37].

Liver

After intestinal absorption, the drug reaches the liver via the portal vein. Similar to the intestine, transporter proteins and CYP450 enzymes play an important role in the uptake, efflux and metabolism of drugs and endogenous compounds. The organic anion transporting peptides (OATPs) belong to the superfamily of the solute carrier class of organic anion transporters and are key uptake transporters expressed on the basolateral membrane of hepatocytes[38]. This is exemplified by guidelines from the FDA for drugs in development, emphasizing the significance of testing whether a drug is a substrate for OATP1B1/1B3 when biliary secretion of hepatic metabolism constitutes ≥25% of the total drug clearance[39]. Moreover, OATP1B1/1B3 play also an important role in the uptake of endogenous compounds and toxins. (Un)conjugated bilirubin, coproporphyrin I (CPI) and III and (un)conjugated bile acids are for instance transported by OATP1B1/1B3 into the hepatocyte. Clinical studies[42,43] showed that direct bilirubin, CPI and also the bile acids like glycochenodeoxycholic acid-sulphate (gCDCA-S) are elevated upon dosing the OATP1B1/1B3 inhibitor rifampicin. Utilization of endogenous biomarkers is particularly valuable in *in vivo* studies to enhance drug safety, serving as an early indicator for potential transporter-mediated drug-drug interactions. Besides the expression of OATPs, other important proteins expressed on the basolateral membrane are the organic cation transporters (OCTs) and the natrium taurocholate transporting peptide (NTCP) which are uptake transporters and MRP3 and MRP4 which are efflux transporters[44,45]. Next to basolateral transporters, CYP450 enzymes are abundantly present in the liver, to a higher extent than in the intestine[26]. This higher abundance in the liver plays a crucial role in the hepatic extraction (E_H), which represents the fraction of a drug that is extracted by the liver (converted to metabolites of excreted

into the bile) during one passage through the liver[46]. The clearance of a drug by the liver is affected by transporter mediated uptake, CYP450 metabolism and hepatic blood flow[46]. The hepatic clearance refers to the amount of drug removed from the blood flow per unit of time and a greater (portal) blood flow will therefore lead to faster absorption into the hepatocytes. Liver diseases as non-alcoholic-fatty liver disease (NAFLD), currently known as metabolic associated fatty liver disease (MAFLD), alcoholic liver disease (ALD) and primary sclerosing cholangitis (PSC) can affect the hepatocellular function and lead to fibrosis which can progress to liver cirrhosis[47]. Subsequently, cirrhosis can affect the hepatic blood flow and lead to an increased resistance in the hepatic vascular bed resulting in a decreased portal hepatic flow[48,49]. Rane et al.[50] reported that the clearance of hepatically cleared drugs with a high extraction ratio are related to blood flow and thus a major decrease in portal flow as in cirrhosis can dramatically affect the first passage across the liver[50,51]. Therefore, it is of major importance and of interest to include (portal)flow into hepatic preclinical models to better predict clinical outcome.

After CYP450 metabolism (phase I metabolism), drugs and endogenous compounds can undergo phase II metabolism (conjugation) which involves glucuronidation or sulfation by uridine diphosphate-glucuronosy-ltransferases (UGTs) or sulfotransferase (SULT) enzymes respectively[52,53]. Thereafter, carrier mediated processes are required to transport phase II conjugated across the canalicular or basolateral membrane or transport the parent compound in unchanged form[53,54]. Drugs and endogenous compounds can also be excreted into the bile which is a carrier-mediated and energy-dependent process. BCRP, MRP2, P-gp, multidrug and toxin extrusion proteins 1 (MATE-1) and bile salt export pump (BSEP) are located on the canalicular membrane of the hepatocyte and are involved in the excretion of drugs, metabolites and endogenous compound as unconjugated and conjugated bile acids[55]. BSEP, and MRP2 to a lesser extent, are considered important transporters involved in the efflux of conjugated bilirubin and bile acids into the bile making them noteworthy endogenous biomarkers[56]. After a meal, the gallbladder contracts and the bile is excreted into the duodenum enhancing digestion of lipids and aid in the absorption of fat-soluble nutrients[57]. In the intestine the biliary excreted drug metabolite can undergo hydrolysis back to parent compound by the microbiome, whereafter the parent compound can be re-absorbed by the intestine ending up in the portal venous blood again. This is known as the enterohepatic circulation (EHC). These dynamic liver processes which include

portal and arterial flow, interplay between drug transporters and phase I, phase II metabolism and biliary excretion is challenging to mimick in preclinical models[12]. In *in vitro* studies, microsomal fractions and hepatocyte cultures are often applied to determine the hepatic clearance of drugs. By measuring the rate of unbound drug elimination followed by scaling the incubation cell content to average liver cell content, an estimate of the hepatic clearance is generated[58,59]. More advanced models which are used are precision cut liver slices, the isolated perfused liver, sandwich cultured hepatocytes or the liver-duct-on a chip which have the ability to study phase I and II metabolism and/or biliary excretion[12,60,61]. Although the isolated perfused liver model closely mimics the *in vivo* conditions by incorporating flow and allowing the measurement of biliary excretion, its primary application in studying drug pharmacokinetics is currently restricted to rodents[62].

Kidney

After gut-wall and hepatic metabolism, known as the first-pass effect, the drug reaches the systemic circulation. Systemic bioavailability after oral absorption is defined as (F) = fraction absorbed (F_a) * fraction escaping gut metabolism, (F_G) * fraction escaping hepatic metabolism (F_H)[63], showing the influence of the intestine (F_a and F_G) and the liver (F_H). After reaching the systemic circulation, the drug reaches various organs including the kidneys. The kidneys are responsible for the elimination of mainly hydrophilic drugs. The renal clearance is the result of glomerular filtration, tubular secretion and reabsorption[64]. The basolateral and apical membrane of the proximal tubule cell contain many different transporters which play a pivotal role in the elimination of drugs and metabolites which function in a secretive or a reabsorptive manner[64]. Organic anion transporter 1 (OAT1) and OAT3, and OCT2 and OCT 3 are important uptake transporters on the basolateral membrane. On the apical membrane, MATE1 and MATE2 and BCRP have a significant role in the elimination. The FDA and the international transporter consortium recommended evaluation of OAT1, OAT3, OCT2 and MATE transporter involvement when the active secretion of the drugs is ≥25% of the systemic clearance[39,65]. This is primarily since their significant role in drug clearance and inhibition of these transporters can result in potential DDI and renal toxicity[65]. Endogenous markers have also been established for several kidney transporters as early indicators in plasma and urine to investigate potential transporter mediated DDI. Taurine, the bile acid gCDCA-S and creatinine are known to be transporter into the renal proximal cells by OAT1, OAT3 and OCT2, respectively. Subsequently, creatinine

and thiamine are known to be excreted by MATE into the urine[66]. In general it is considered that the kidney has less metabolizing capacity compared to liver and intestine given the net weight of the organ and the microsomal content[67]. Preclinical renal clearance is often assessed using primary human cells or immortalized cells to study transporter affinity and transporter involvement in the renal uptake and efflux of compounds[68,69]. Nowadays, renal clearance is assessed in more complex preclinical models like the isolated kidney perfusion model, proximal tubule on a chip, or animal studies[70-74].

Currently, PBPK modeling is broadly applied in drug discovery to estimate the PK profile of a compound based on the preclinical absorption, distribution, metabolism and elimination (ADME) data[75]. Prediction could aid in the determination of the first dose for a clinical trial in the absence of *in vivo* data, assess dose regimen or to study potential population variability[76]. The more accurate the input data into these models, the more reliable the outcome which could facilitate early identification of drug with a high potential for DDI in the drug discovery process[77]. To exemplify, the use of isolated intestine, liver and kidney perfusion in the past have demonstrated to be value models gaining mechanistic insights into the physiology and the role of transporters and enzymes including their interplay in the organs[78]. Using *ex vivo* organ perfusion with rat organs, Pang et al., demonstrated important DMPK concepts as hepatic zonation, hepatic and renal metabolism and blood flow dependent hepatic elimination[79-83]. For the isolated organ perfusion experiments mainly rodent organs are used, however translation of these findings to clinical practice remains challenging due to, among others, species differences in transporter expression[11,84]. Currently, advancements in the development and application of *ex vivo* organ perfusion are occurring in the field of organ transplantation. The use of pressure driven machine perfusion facilitates organ preservation under hypothermic conditions and also provides the opportunity to assess organ viability and functionality under normothermic conditions[85-88]. The use of these novel pressure driven perfusion machines opens new opportunities for the field of pharmacology since it allows to study the function of human or porcine whole organ(s) under conditions that are as close as possible to the *in vivo* situation[89]. The inclusion flow, intact cellular morphology and preservation of excretion pathways make the model attractive to study pharmacological processes such as the hepatic first pass effect, renal clearance, biliary and urinary elimination or DDI. Compared to the isolated perfused-organ systems using rat organs, the human/porcine machine perfusion models have

relatively high circulating perfusion volume and urinary and biliary output which allows easy sample collection to assess drug PK[89]. Furthermore, and of utmost significance, the use of human organs enables direct translation of the findings to the human in vivo condition.

The objective of this thesis

The aim of this thesis was to explore the applicability of pressure driven normothermic organ perfusion to study pharmacological processes in the liver, intestine and kidney.

Outline of this thesis

Preclinical models are a crucial part of the drug development process, however to study complex ADME processes more advanced preclinical models are needed. The first part of this thesis, chapter 2, provides an overview of the currently used *ex vivo* models in drug development research. The review describes the novel developments using *ex vivo* tissues to improve the predictions of human ADME profiles and DDIs in health and disease.

In part II of this thesis, the focus is on *ex vivo* liver perfusion to study drug pharmacokinetic processes and endogenous substrate handling. Porcine organs are often used for method validation and device development and it has been shown that normothermic machine perfusion (NMP) of the porcine liver is an excellent platform to study hepatic processes[90,91]. Additionally, the pig liver model is considered a proper translational model because of anatomical, physiological and biochemical similarity to humans and nowadays this model is increasingly used in biomedical research[92,93]. In chapter 3, we evaluate the use of normothermic machine perfusion of porcine livers as a novel model to predict pharmacokinetic processes. Using three statins as OATP1B1/1B3 substrate model drugs (rosuvastatin, atorvastatin and pitavastatin) we studied the transporter mediated hepatic extraction and biliary excretion. Furthermore, we examined the effect of rifampicin on the disposition of these three statins. In clinical data, a rank-order relationship has been observed in the DDI with rifampicin and we aimed to study if the *ex vivo* liver perfusion model could mimick the rank-order relationship.

Established *in vitro* and animal models are often used to study the pathology and pharmacological characteristics of drugs of varying diseases. However,

translation of these findings to clinical practice remains challenging due to, among others, species differences in transporter expression and the difficulty to mimic dynamic liver processes[61,84]. In chapter 4, we developed a novel hepatic model using diseased explanted human livers. Four model drugs (rosuvastatin, digoxin, furosemide and metformin) with and without perpetrator drugs were used to study hepatic extraction, clearance, biliary excretion and DDI. These model drugs are known substrates for different important hepatic uptake and efflux transporters and enabled comparison of the model to *in vivo* reported data.

In the field of *ex vivo* liver perfusion, limited research is performed regarding characterization of bile acid composition, cholesterol homeostasis and transporter gene expression during *ex vivo* liver perfusion. Moreover, in the *ex vivo* perfused liver model the enterohepatic circulation is absent. Nevertheless, the bile acid recirculation plays a crucial role, particularly in supporting the function of specific drug transporters and homeostasis of cholesterol levels[94-96]. In chapter 5, we aimed to characterize the de novo bile acid production, cholesterol levels and transporter gene expression during *ex vivo* liver perfusion in pig and human livers. Additionally, we aimed to decreased the metabolic burden of the *de novo* bile acid synthesis by incorporating a pool of (un)conjugated bile acids during *ex vivo* liver perfusion and study subsequently its effects.

Part III is aimed to unravel drug pharmacokinetics through multi-organ perfusion. While the majority of organ perfusion studies focus on perfusion with a single organ, a few studies have investigated the possibility of a multi-organ perfusion model to study physiological processes[97-100]. The possibly to perfuse multiple organs allows in-depth analysis of ADME processes like gut wall metabolism, portal vein concentrations, hepatic uptake and biliary excretion and thus generating novel pharmacological insights. In chapter 6, we aimed to explore the applicability of a porcine *ex vivo* perfusion model to study gut-hepatobiliary metabolism by characterization of oral absorption, gut wall metabolism, pre-systemic hepatic metabolism and biliary excretion using midazolam as a CYP3A4 model compound.

In part IV – chapter 7, the results and conclusions of this thesis are summarized, discussed and future perspectives are presented. As the application of *ex vivo* organ perfusion for pharmacokinetic research is quite

novel, major potential lies ahead for future pharmacokinetic questions regarding DDI, studying the first-pass effect and the enterohepatic circulation in the multi-organ model and the use of explanted human diseased livers. Furthermore, the first steps towards translation of *ex vivo* data to *in vivo* PK profiles will be shown and discussed.

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