Characterisation of Hepatic Uptake Transporters in the Minipig.

2023

'A thesis submitted to the University of Strathclyde, Strathclyde Institute of Pharmacy and Biomedical Sciences for the degree of Master of Philosophy'

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Declaration

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ABBREVIATIONS

ABC	ATP binding cassette
ADME	Absorption, Distribution, Metabolism and Excretion
apaB	apolipoprotein B
APCI	Atmpospheric chemical ionisation
ATP	Adenosine Triphosphate
BCRP	Breast Cancer Resistance Protein
BSEP	Bile salt export pump
CA	Cholic acid
CCK	Cholecystokinin
CDCA	Chenodeoxycholic acid
CEM	Continuous electron multiplier
CHMP	Committee for Human Medicinal Products
Clint	Intrinsic Clearance
CNS	Central nervous system
CPS	Counts per second
CsA	Cyclosporin A
Cyp	Cytochrome
DCA	Deoxycholic acid
DDI	Drug-drug Interaction
DILI	Drug-Induced Liver Injury
DMPK	Drug Metabolism & Pharmacokinetic
DMSO	Dimethyl sulfoxide
DNP	Deoxycholic acid modified nanoparticels
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extarcellular Matrix
EG	Estradiol-17β-D-Glucuronide
EMA	European Medicines Agency
ES	Estrone-3-Sulfate
ESI	Electronspray ionisation
FA	Fatty acids
FDA	Food & Drug Administration agency

FTIH	First Time In Human
GC	Gas Chromotography
GST	Glutathione s transferases
HDL	High-density lipoprotein
HGNC	Gene Nomeclature Committee
HMGRI	HMG-CoA reductase inhibitor
HTS	High throughput screening
HUGO	Human Geneome Organisations
ICH	International Council for Harmonisation
IC_{50}	Half maximum inhibitory concentration
IDL	Intermediate-density lipoprotein
IND	Investigational New Drug application
IS	Internal standard
ITC	International Transporter Consortium
IVIVC	In vitro In vivo correlation
JPMDA	Japanese Pharmaceuticals Medical Devices Agency
Km	Michaelis-Menten constant
LC	Liquid choromotography
LCA	Lithocholic acid
LDL	Low-density lipoprotein
LC-MS	Lquid chromotography-Mass Spectrometry
MATE	Multidrug and toxin extrusion
MDR1	Multi Drug Resistance 1
MRP	Multi Drug Resistance Protein
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NAT	N-acetyl transferase
NCE	new chemical entity
NDA	New Drug Application
NTCP	Sodium Taurocholate Co-transporting Polypeptide
OAT	Organic Anion Transporter
OATP	Organic Anion Transporting Polypeptide

OCT	Organic Cation Transporter
PBPK	Physiologically Based Pharmacokinetic
PD	pharmacodynamic
Pdiff	Passive diffusion clearance
P-gp	P-Glycoprotein
PK	pharmacokinetic
PXR	Pregnane C Receptor
RSV	Rosuvastatin
SSA	Specific Support Action
SLC	Solute carrier
TCDC	Taurochenodeoxycholic acid
TG	Triglycerides
VLDL	very-low-density lipoprotein
Vmax	Michaelis-Menten maximum uptake rate

ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge the support and guidance I have received from my industrial supervisor Gareth Lewis and my academic supervisors Marie Boyd, Simon MacKay and Mary Grant. Thank you all for your time, input, review and objective discussion which has been extremely valuable in enabling the completion of this thesis. I would like to say a special thank you to Gareth for his continuous encouragement, patience and understanding throughout the project.

I would like to acknowledge and thank Kunal Taskar for his encouragemnt and support in my decision to embark on this MPhil.

Finally, I would like to acknowledge and thank my family for their continuous support, understanding, encouragment and belief in me. Helping me to believe in myself and realise I can take ownership to carve my own future.

ABSTRACT

The Göttingen minipig is a popular strain (Heining and Ruysschaert, 2016, Lignet *et al.*, 2016) used in toxicological studies. It is used based on its anatomical and physiological similarities to humans with respect to a variety of organs and functions (Forster *et al.*, 2010a).

Further evaluation needs to be carried out on the predictability of the minipig to human specifically on absorption, distribution, metabolism and excretion (ADME) processes. This project will aid in this predictability of the minipig to human correlation to facilitate the development and validation of mathematical models.

The liver plays a major role in the ADME process of xenobiotics, and hepatic transporters can be the rate limiting step for the clearance of xenobiotics from the body, playing a critical role in their elimination (Funk, 2008, Treiber *et al.*, 2007).

The aim of this project was to characterise minipig hepatic uptake transporters by investigating the uptake of three known organic anion transporter protein (OATP) substrates, estrone sulfate (ES), estradiol glucuronide (EG), and a clinically relevant probe, rosuvastatin. The uptake of the probe substrates was assessed across three species, human, rat and minipig. This was to give an understanding on the variance in the uptake of the probe substrates across the three different species and if minipig hepatic uptake transporters can be used to predict human pharmacokinetics and be used for *in vitro in vivo* correlation (IVIVC).

The cryopreserved hepatocytes were plated onto collagen coated plates to give a final well concentration of 0.35 x 10^6 cells per well. Plates were placed into a 37°C humidified incubator with 5% CO2 for approximately 4 hours to allow cells to attach. After approximately four hours the hepatocytes are incubated with the substrate at 37^0C. Experiments were conducted to investigate time dependent carrier-mediated uptake and concentration dependent uptake to determine kinetics (Km and Vmax). To determine the amount of substrate taken up, cells were lysed with 1% triton or water and the contents measured via LC-MS/MS or liquid scintillation counting dependent if the substrate was radioactive. To assess the amount of passive diffusion, the cells

were incubated using the same conditions as above, however the pre-incubation and working solutions contained the cocktail of inhibitors, rifamycin and imipramine.

As drug transporters are highly expressed in rodents, rat hepatocytes had the highest intrinsic clearance for all three substrates (Table 9). Uptake of the probe substrates was also assessed in different batches of minipig and human hepatocytes, therefore minipig hepatocyte batches GBV and IKL had lower Km values for ES compared to EG. The Intrinsic clearance (ml/min/Kg) determined for minipig and human hepatocytes was similar across both species for EG (4.78 to 10.54 ml/min/Kg). Minipig hepatocytes have a similar substrate affinity for EG and ES as human cells. Kinetics was determined in two out of the three minipig batches (RZX and IKL) for rosuvastatin and a fold change of >2 was only observed in the time linearity experiment for batches GBV and IKL which gave a fold change of just over 2, the third batch RZX gave a fold change of 1.5. There is not a huge difference in the fold change for all three minipig hepatocyte batches, which were around the acceptance threshold of a fold change of ≥2 between inhibited and uninhibited cells. This could be due to the poor affinity of rosuvastatin for the uptake transporters present in minipig hepatocytes. To assess if this was a phenomenon with just rosuvastatin, the uptake of pitavastatin was assessed and no kinetics could be determined in minipig hepatocytes.

This project has highlighted further work is required to fully characterise minipig hepatocytes and the experimental design in looking at pre-incubation times, choice and concentration of inhibitors.

The experimental data from this project demonstrated minipig uptake transporters, in particular OATPs are not fully representative of human hepatocytes.

1 INTRODUCTION

This project is concerned with the characterisation of minipig hepatic uptake transporters and the interspecies differences, by assessing the uptake of three known OATP substrates into cryopreserved minipig, rat and human hepatocytes. The minipig is becoming the preferred secondary species in toxicology studies with the Göttingen minipig being a popular strain (Heining and Ruysschaert, 2016, Lignet *et al.*, 2016). Hepatic transporters can be the rate limiting step for the clearance of xenobiotics from the body, and therefore play a critical role in their elimination (Funk, 2008, Treiber *et al.*, 2007). This characterisation will therefore improve the prediction of human pharmacokinetics from minipig *in vitro* and *in vivo* experiments.

1.1. Regulatory Perspective

The ultimate goal for pharmaceutical companies is to deliver new medicines to patients in order to improve patient quality of life through prevention or to cease progression or regression of a particular disease state. The role of regulatory agencies is to ensure that pharmaceutical companies develop medicines safely for patients by adhering to strict guidance. The main world regulatory agencies are the Food & Drug Administration agency (FDA), European Medicines Agency (EMA) and Japanese Pharmaceuticals Medical Devices Agency (JPMDA), that cover the US, Europe and Japan/Asia Pacific, respectively.

There are numerous regulatory guidance documents to drug development with a key focus on obtaining human clinical information for a new medicinal product with respect to safety, tolerability and pharmacokinetics (Phase I, First Time in Human trial) and then subsequent pharmacokinetic and pharmacodynamics in the patient/disease indication (Phase II proof of concept and phase III commit to medicine development). Regulatory agencies stipulate that the new medicinal product must be adequately characterised through a series of *in vitro* and non-clinical *in* vivo drug metabolism and safety studies prior to administration to humans. This guidance is regularly reviewed and updated as technology, techniques and acceptance criteria change.

This is highlighted by the FDA and EMA through two key strategic documents from the

International Council for Harmonisation (ICH) "*M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals*" (U.S. Dept of Health and Human Services, Feb 2013) and the Committee for Human Medicinal Products (CHMP) on "*Strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products*". (European Medicines Agency, 2017)

Several documents of guidance relate specifically to the safety assessment of the drug product, with respect to carcinogenicity, genotoxicity and systemic toxicity exposure assessment (ICH S1, S2 and S3A). These are further supported by a core battery of safety pharmacology studies outlined in the ICH S7A document which provides the general principles and recommendations for assessment of drug impact on the central nervous system (CNS), cardiovascular system, and respiratory system (ICH S7A Safety pharmacology studies for human pharmaceuticals CPMP/ICH/539/00 (European Medicines Agency, 2001).

It may be necessary for drug metabolites to be assessed through some or all the above guidance, depending on their pharmacological activity, relative exposure or safety liability (e.g., reactive metabolites). Directly related to this project is the guidance for *in vitro* metabolism, transporters and drug-drug interactions. It is important to understand and characterise how a new chemical entity (NCE) is metabolised, for example by cytochrome P450 enzymes such as CYP3A4, and also how the NCE is distributed and eliminated from the body, by active tissue processes for drug uptake and efflux through transporters such as OATP and/or P-glycoprotein (P-gp). Knowing the adsorption distribution metabolism and excretion (ADME) effects of an NCE whether it is a substrate or inhibitor of certain proteins will give a better understanding about the clinical implications it may pose on the intended patient population, as well as any risks associated with other co-medications and the intended drug therapeutic dose (both safety and efficacy).

Since as early as 2001, the Japanese authorities have highlighted methods to be used for Drug interaction studies (Methods of Drug Interaction Studies), (Japanese NIHS, 2001). More recently the FDA and EMEA issued draft guidance in 2012, providing recommendations about evaluating the inhibitory and substrate potential for NCEs.

(FDA: Federal Register :: Draft Guidance for Industry on Drug Interaction Studies-Study Design, Data Analysis, Implications for Dosing, and Labelling Recommendations; Availability (Draft Guidance for Industry on Drug Interaction Studies-Study Design, 2012) U.S. Dept of Health and Human Services, FDA, CDER. Feb 2012 and EMA: Guideline on the Investigation of Drug Interactions, EMA, CPMP/EWP/560/95/Rev. 1 (2012) (Guideline on the investigation of drug interactions, 2012).

The most recent FDA guidance related to "*In Vitro Metabolism- and Transporter-Mediated Drug-Drug Interaction Studies Guidance for Industry*" (In Vitro Drug Interaction Studies — Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions Guidance for Industry, 2020) (U.S. Dept of Health and Human Services, FDA, CDER. Oct 2017), outlines *in vitro* experimental approaches to evaluate the drug-drug interaction (DDI) potential, between investigational drugs that involve metabolizing enzymes and/or transporters. This guidance discusses how *in vitro* results can inform future clinical DDI studies and includes considerations when choosing *in vitro* experimental systems, conditions, and *in silico* model-based DDI prediction strategies. For example, HMG-CoA reductase inhibitors (HMGRIs) belong to a major drug class called statins, and they are both substrates and inhibitors of OATPs. Clinically significant drug-drug interactions have been linked to the modulation of these transporter proteins (Kellick, 2017).

A most recent update is the EMA 2017 "concept paper on a revision of the Guideline on the investigation of drug interactions" (EMEA/CHMP/694687/2016 Mar (2017)) which includes further recommendations and clarifications, primarily around inhibition and induction assays. This is supported by the latest FDA guidance for "*Clinical Drug Interaction Studies — Study Design, Data Analysis, and Clinical Implications Guidance for Industry*" (U.S. Dept of Health and Human Services, FDA, CDER. Oct (2017)),(Clinical Drug Interaction Studies —Study Design, 2017) evaluation of clinical drug-drug interactions (DDIs) during drug development and communication of the results and recommendations from DDI studies.

Thus, the guidelines provided by regulatory agencies are to ensure that all pharmaceutical companies follow the same stringent guidelines regarding the development of a drug ensuring the paramount safety of the patient. To be able to do

this, pharmaceutical companies generally follow a drug development process which is reviewed at certain stages by the regulatory agencies.

1.2. Drug Development Process

From when a molecule is identified through to clinical testing can take up to 15 years, and this section gives a brief overview of the drug development process from drug discovery (stage 1) to stage 4 (FDA review) (Figure 1 and Table 1). The Drug Metabolism and Pharmacokinetics (DMPK) of a molecule and its affects are also assessed as part of the drug development process, specifically studying the ADME of a molecule (section 1.3).

During drug discovery and target validation a gene or a protein is chosen to be the target of a drug. Multiple drug molecules are tested, to ensure they are targeting the disease in question, however, only a small number are selected to progress to pre-clinical testing (Robuck and Wurzelmann, 2005). Following target validation, we have the hit identification which falls in the lead discovery phase, where compound screening assays are developed. High throughput screening (HTS) is carried out on entire compound libraries directly against drug targets or cell-based assays, the activities of which are dependent upon the target (Hughes *et al.*, 2011).

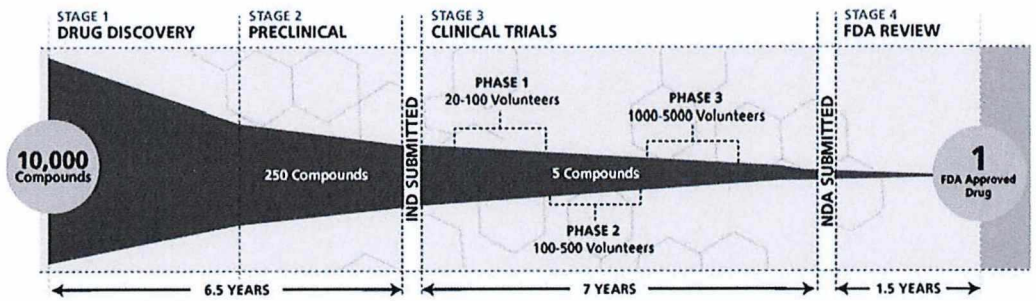

Figure 1. Drug development timeline (Robuck and Wurzelmann, 2005) Copyright (2022), with permission from Lippincott Williams & Wilkins.

Pre-clinical testing follows the drug discovery stage of a molecule: it involves *in vitro* and *in vivo* assessment of molecules. *In vivo* assessments are routinely carried out on

selected pre-clinical species, generally rodents and non-rodent species, where safety to humans is the focus. Pharmacological and toxicological effects of the molecule are assessed alongside the ADME properties (Robuck and Wurzelmann, 2005) including the efficacy of the molecule. The FDA and the EMA will not allow molecules to progress to human studies until extensive safety data are available. Prior to starting any clinical trials an Investigational New Drug applications (IND) is applied for to the FDA or EMA. The pre-clinical safety data for both *in vitro* and *in vivo* studies, including chemical structure, the manufacturing process, target biology of the drug, clinical research protocols of studies to be conducted and any previous clinical research data if available, are all scrutinised (Deore *et al.*, 2019). Once the IND has been approved the clinical trials of the drug development process begins, starting with the First Time In Human (FTIH) study followed by phase 1 to 3 of the clinical trials (stage 3) and then the FDA review (stage 4). An overview of each stage is described in (Table 1).

Table 1: Key Characteristics of Stage 3, NDA application and Stage 4 of the drug development process.

Phase	Characteristics of Stage 3, NDA application and Stage 4 of the drug development process.
First Time in Human (FTIH) (following IND approval prior to phase 1)	• Small group of healthy volunteers approximately 15 to 20 patients. • Focuses on the safety and tolerability of the drug • Escalating a single dose to a maximum, based on the toxicity, safety margin • ADME • Desired effect (pharmacodynamics) of the drug
Phase 1: Safety and dosage	• Drug candidate is administered to 20 to 80 healthy volunteers with the disease or condition. • Dose regime adjusted based on animal studies to establish tolerability of the drug and side effects. • Patients monitored closely and information collected on the pharmacodynamics in the human body. • An optimal dose is established with an increased focus to assess if the drug is having an effect on the disease and improving the patients condition or not.
Phase 2: Efficacy and side effects	• The drug candidate is administered to patients affected by the diseases. • Pool of patients increased to 100 or more. • A well-defined eligibility criteria is put into place and controlled comparisons with placebo or active control are incorporated into the study design. • Safety is still the paramount focus with side effects being closely monitored. • If the data indicate a positive effect, then the efficacy of the molecule is assessed with a focus on safety. • Dose-ranging studies are carried out, of short to medium duration (weeks to months). • The potential effectiveness is established of the drug for the specific population and disease.

Table 1 cont: Key Characteristics of Stage 3, NDA application and Stage 4 of the drug development process.

Phase 3: Efficacy and adverse drug reactions monitoring	• Large studies to investigate safety and efficacy are carried out involving hundreds to thousands of patients over longer duration of treatment (months to years). • The adequate population size needs to achieve efficacy power greater than 80%. • These studies are approved by the regulatory agencies with guidelines, where the endpoint of the study is clearly defined to determine the success or failure of the drug. • The comparator standard of care and the superiority of the drug needs to be demonstrated.

New Drug Application (NDA)	• When a NDA is filed with the agencies it shows the full story of the drug molecule and it describes all the data from all studies including the clinical trials.
	• The purpose of the NDA is to verify that a drug is safe and effective.
	• The following also has to be supplied to the agencies:
	  ○ Proposed labelling
	  ○ Safety updates
	  ○ Drug abuse information
	  ○ Patent information
	  ○ Institutional review board compliance information
	  ○ Directions for use
	• The agencies can deny the application or request further information.
	• Once the NDA is approved the drug becomes available for commercial production.

Phase 4: Post-Market Drug Safety Monitoring	• Post-marketing studies that provide additional safety and efficacy data • Must be conducted if the FDA approves the product on a ''fast track'' (i.e., before all premarketing data are compiled) • Evaluates adverse events, pharmaco-economic, and epidemiologic data

1.3. DMPK / ADME

DMPK is a scientific discipline associated with safety evaluation of xenobiotics in discovery, development through to post marketing, with particular emphasis on the ADME characteristics of drugs (Fan and de Lannoy, 2014). To assess the fate of a drug, the four disposition processes of ADME are required alongside the pharmacokinetics of the molecule. These processes are important in providing information from animal studies to the human situation (Caldwell *et al.*, 1995). Absorption is the process by which the xenobiotic enters the blood from the site of administration. The xenobiotic must be able to cross from the site of administration across the cell membranes to enter the circulatory system. Small lipophilic compounds can do this by passive diffusion whereas large polar or highly charged molecules may require an active carrier mediated process to cross the membrane barrier (Yang and Hinner, 2015). Distribution of a molecule is determined by blood flow, passive diffusion of the molecule across lipid membranes and the presence of carrier mediated processes. Metabolism itself looks at the metabolic breakdown of drugs following administration in order to make the drug amenable for excretion. The two main routes of excretion are via the kidney or the liver but there are other routes such as saliva, tears, and sweat (Barreto *et al.*, 2021).

Pharmacokinetics is the study of how an organism affects a substance once administered to a living organism. The substance can be xenobiotic drugs, food additives or even cosmetics. Pharmacokinetics analyses the metabolism and fate of the substance from administration to excretion. There are three phases of drug metabolism carried out by certain enzymes, which modify the chemical structure of xenobiotics in order to facilitate elimination from the organism (excretion) (Almazroo *et al.*, 2017). These reactions can act to detoxify compounds or in some instances the intermediates that are produced can have deleterious toxic effects or pharmacological effects from prodrugs or active metabolites (Stachulski *et al.*, 2013). In Phase I reactions, enzymes such as cytochrome P450 oxidases introduce reactive or polar groups into xenobiotics while in Phase II reactions the groups are conjugated to polar compounds, in Phase III

the conjugated xenobiotics may be further processed and pumped out by efflux transporters (Almazroo *et al.*, 2017).

Drug metabolism is predicted to produce metabolites with lower lipophilicity compared to the parent drug, yet pharmacologically, metabolism influences many things such as the biological activity, toxicity and safety of the drug. Therefore, drug metabolism plays a key role in the pharmacokinetics (PK), pharmacodynamics (PD), and safety properties of a molecule (He and Wan, 2018). Critically undesirable drug metabolism and pharmacokinetic effects are major reasons that drugs are discontinued (Sevior *et al.*, 2012). Some of the factors regarding attrition like poor absorption, PK, and bioavailability have been addressed by performing DMPK assays earlier in the discovery phase (Figure 2). Although this may have not been completely addressed, the high attrition rates which have been linked to adverse drug reactions are believed to be associated with reactive metabolites (Stachulski *et al.*, 2013). Having knowledge of the disposition of a molecule at all developmental stages will therefore aid in the clinical safety of the molecule and allow a more streamlined development process with a reduction in attrition (He and Wan, 2018).

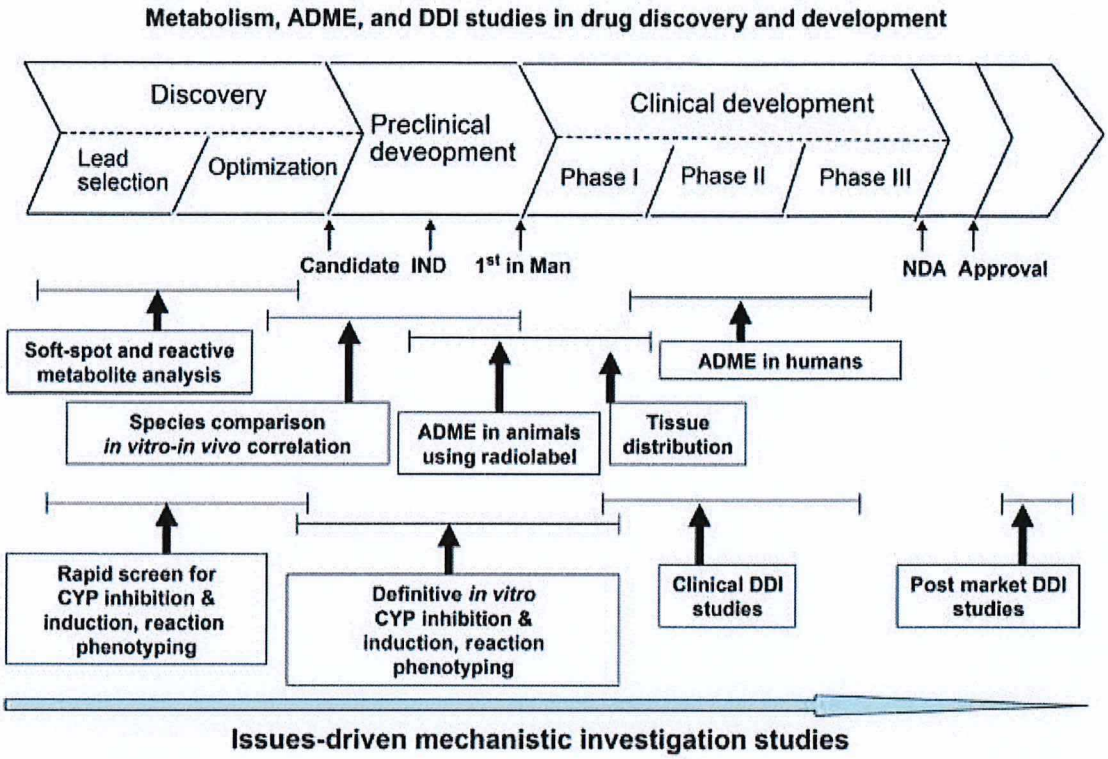

Figure 2: Schematic diagram of the DMPK process in discovery and development which follows the drug development process. Diagram shows the time frame of selected studies to determine the metabolism, ADME and DDI of a molecule (Zhang *et al.*, 2012). Copyright (2022), with permission from Elsevier.

1.4. Liver

1.4.1. Physiology & Structure

The liver is a major organ which carries out many functions which include ADME of drugs, xenobiotics, exogenous and endogenous compounds (Shitara *et al.*, 2006). Other functions of the liver include vitamin storage, immunity and production of bile which is essential for the breakdown of fats. The liver weighs 1.4 to 1.7kg and comprises around 2% of an adults body weight (Sibulesky, 2013).

The liver is connected by two large blood vessels: the hepatic artery, which carries blood from the aorta to the liver and the portal vein, which carries blood to the liver containing digested nutrients from the gastrointestinal tract, spleen and pancreas (Sibulesky, 2013). The liver is divided into four lobes left, right, caudate and quadrate

lobes. In 1957 a definitive descriptive classification was provided by Claude Couinaud known as the Couinaud classification (Sutherland and Harris, 2002). The system further subdivides the lobes into eight independent functional units known as segments which are numbered in a clockwise manner (Figure 3). Each of the segments has its own vascular inflow, outflow, biliary and lymphatic drainage. In the centre of each segment there is a branch of the portal vein, hepatic artery and bile duct; in the periphery of each segment there is vascular outflow through the hepatic veins. The division of the liver into self-contained units means that each segment can be resected without damaging those remaining. For the liver to remain viable, resections must proceed along the vessels that define the peripheries of these segments. The classification system uses the vascular supply in the liver to separate the functional segments (numbered I to VIII). The caudate lobe segment I is situated posteriorly, and it may receive its supply from both the right and the left branches of portal vein. It contains one or more hepatic veins which drain directly into the inferior vena cava (Sibulesky, 2013).

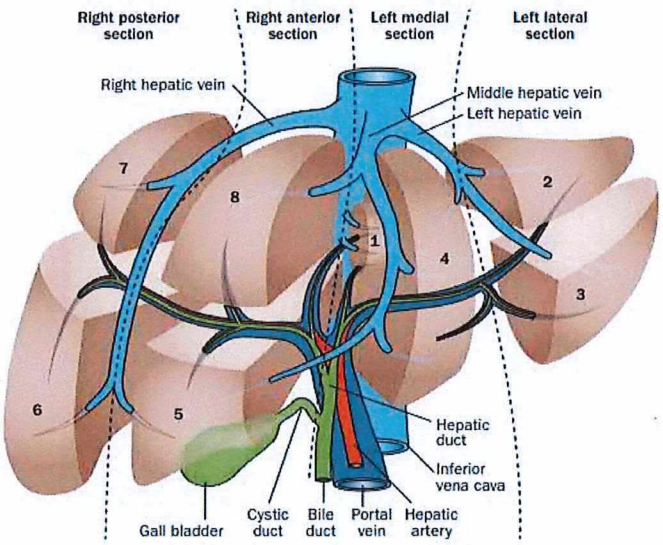

Figure 3: Segments of the human liver (Siriwardena et al., 2014). Copyright (2022), with permission from Springer Nature Customer Service Centre GmbH.

The functional unit of the liver is the lobule which is made up of hepatic lobules. The lobules are hexagonal in shape with the centre being the central vein. Hepatocytes are arranged like plates and in between these plates there is a vascular space with a thin fenestrated endothelium and a discontinuous membrane called the sinusoid. The

central vein joins to the hepatic vein to carry blood out from the liver (Trefts *et al.*, 2017). A distinctive component of a lobule is the portal triad, which can be found running along each of the lobule's corners. The portal triad consists of five structures: a branch of the hepatic artery, a branch of the hepatic portal vein, and a bile duct, as well as lymphatic vessels and a branch of the vagus nerve (Figure 4). Between the hepatocyte plates (Sevior *et al.*, 2012) are liver sinusoids, which are enlarged capillaries through which blood from the hepatic portal vein and hepatic artery enters via the portal triads, then drains to the central vein (Sibulesky, 2013).

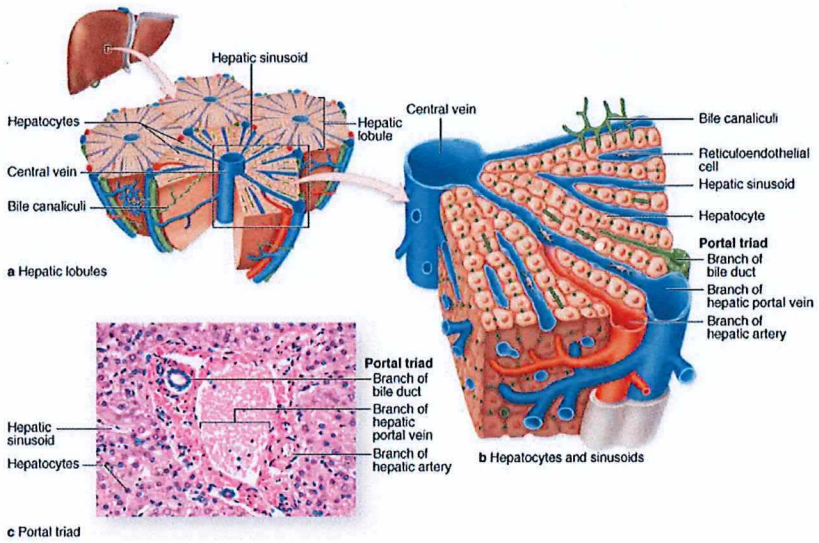

Figure 4: Hepatic lobule. (a) Depiction of the hepatic lobules. (b) Arrangement of hepatocytes and hepatic sinusoids. (c) Histology of a portal tract. (Mescher, 2009) Copyright (2022), with permission from McGraw Hill LLC.

1.4.2. Functions of the Liver

It is thought the liver carries out about 500 functions. Some of the primary functions include:

- Bile production and excretion.

Bile is an alkaline solution and the liver produces about 600ml per day. Bile contains water, electrolytes, bile acids, cholesterol, phospholipids, bilirubin, exogenous substances xenobiotics and environmental toxins. Bile acids are metabolites of cholesterol and are synthesised in the liver either by the classical pathway or acidic

pathway. The classical pathway occurs in the liver and accounts for approximately 90% of bile synthesis. The classical pathway starts with the rate-limiting 7-α hydroxylation of cholesterol catalysed by CYP P450 enzyme CYP7A1 (Monte *et al.*, 2009) and the12-α hydroxylation of the intermediates by CYP8B1 (Eggertsen *et al.*, 1996), followed by side chain oxidation by CYP27A1. The acidic pathway which produces 10% of bile acids is initiated by the hydroxylation of the cholesterol side chain by sterol 27 hydroxylase (CYP27A1), followed by 7-α hydroxylation of the oxysterol intermediates by oxysterol 7-α hydroxylase (CYP7B1) (Monte *et al.*, 2009) (Figure 5a). The two major bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are conjugated with the amino acids (glycine and taurine), sulphate or glucuronic acid to increase ionisation at acidic pH, prevent Ca^{2+} precipitation, minimise passive absorption and cleavage by pancreatic enzymes in the intestine (Chiang, 2014). Some bile acids can be deconjugated in the intestine by bacteria to produce secondary bile acids deoxycholic acid (DCA), a derivative of CA, and lithocholic acid (LCA), a derivative of CDCA. Both DCA and LCA are highly insoluble and toxic and are excreted into the faeces (Chiang, 2014).

Conjugated bile acids (bile salts) are amphipathic which aids in effectively emulsifying lipids and cholesterol to form spherical structures known as micelles (Hundt *et al.*, 2021). Micelles play an important role in the digestion of fats and transport their contents to the intestinal epithelium where they can be absorbed. The formation of bile is vital in the elimination of drugs, xenobiotics and endotoxins from the liver. Bile secretion is also a major route in the excretion of endogenous compounds such as cholesterol and bilirubin (Chiang, 2014) . Bile is secreted by hepatocytes into the bile canaliculi and travels through the bile ducts where it is eventually concentrated and stored in the gallbladder (Cai and Chen, 2014). During and after a meal, bile is released from the gall bladder by contraction, stimulated by the hormone cholecystokinin (CCK) and it passes into the duodenum through the common bile duct (Chiang, 2014). Approximately 5% of these bile acids are excreted but the majority are reabsorbed in the terminal ileum and returned to the liver via the hepatic portal vein a process known as enterohepatic recirculation (Figure 5b) (Hundt *et al.*, 2021). Enterohepatic recirculation regulates bile acid synthesis by a feedback

mechanism. This feedback pathway also plays a role in absorption and transport of nutrients from the intestine to the liver for metabolism and distribution to other organs. Bile acids are recycled 4 to 12 times a day and this circulation of bile acids involves bile acids and drug transporters.

Hepatocytes make up the majority of cells present in the liver. The transport of drugs and xenobiotics into the hepatocyte can be modulated by transport proteins. The sinusoidal membrane accounts for 70% of the hepatocytes surface in comparison to the canicular membrane which accounts for about 10-15% (Ayrton and Morgan, 2001). Hepatic uptake transporters are located on the sinusoidal membrane of hepatocytes and belong to the Solute Carrier (SLC) superfamily of transporter proteins (Faber *et al.*, 2003). The main function of the Na+/taurocholate co-transporting polypeptides (NTCP) is to facilitate the uptake of conjugated bile salts which is an important step in the enterohepatic uptake circulation of bile salts (Kullak-ublick *et al.*, 2004). The uptake of bile acids across the canicular membrane is an active process which requires energy. Efflux transporters which are part of the ABC transporter family, hydrolyse ATP and the energy is used to export substrates against steep concentration gradients 100-1000 fold into the canaliculus (Faber *et al.*, 2003). Many efflux transporters are involved in this process but the bile salt export pump (BSEP), is primarily responsible for the export of bile salts (Liu and Sahi, 2016).

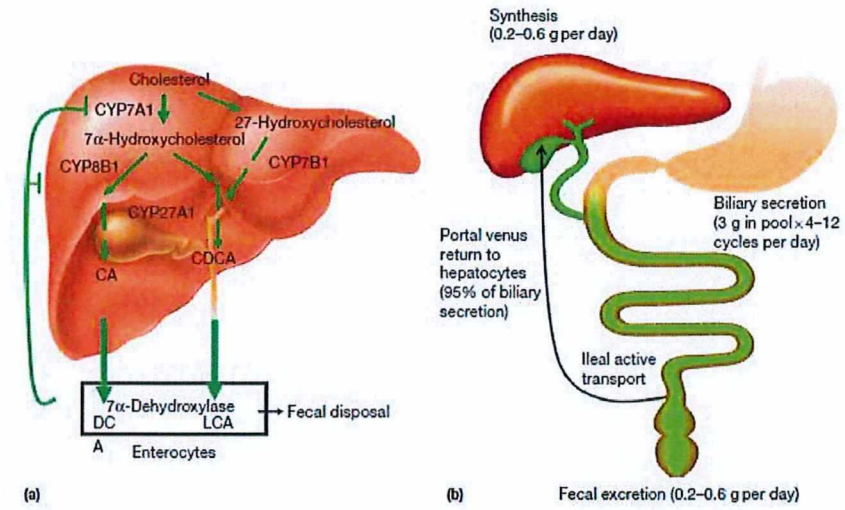

Figure 5a and 5b. Metabolism and enterohepatic circulation of bile acids. (Chiang, 2014) Copyright (2022), with permission from Elsevier.

- **Excretion of bilirubin, cholesterol, hormones, and drugs (xenobiotics).**

Macrophages break down haemoglobin in tissues to biliverdin, and the enzyme biliverdin reductase facilitates this conversion of biliverdin to bilirubin (Naito *et al.*, 2004). Bound to albumin the unconjugated bilirubin is transported to the liver from the circulation. In hepatocytes the bilirubin is conjugated with glucuronic acid rendering it soluble and then the conjugated bilirubin is secreted into the bile canaliculi as part of bile which ends up in the small intestine (Li *et al.*, 2017). Bacteria in the intestine metabolise bilirubin to urobilinogen, which is eliminated in faeces as stercobilin, a brown pigment giving faeces its typical colour. Some of the urobilinogen is oxidised to urobilin which is the main component of the straw-yellow coloured urine which is reabsorbed and excreted by the kidney (Hamoud *et al.*, 2018). If excessive quantities of either free or conjugated bilirubin accumulate in extracellular fluid, a yellow discoloration of the skin, sclera and mucous membranes is observed - this condition is called icterus or jaundice (Fargo *et al.*, 2017).

Cholesterol is an important component of biological membranes and functions in intracellular transport, cell signalling and nerve conduction (Amir and Fessler, 2013). It is a precursor molecule for many pathways such as the synthesis of vitamin D and hormones including the adrenal gland hormones cortisol and aldosterone, sex

hormones progesterone, oestrogens, testosterone and their derivatives. Cholesterol is hydrophilic so to be transported effectively, it is packaged within lipoproteins. There are several types of lipoproteins in the blood. In order of increasing density, they are chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Häussinger, 1996) .

The liver plays a critical role in maintaining a homoeostatic cholesterol balance in the body. Humans absorb approximately 300 – 500 mg cholesterol from diets and synthesise approximately 600-900mg cholesterol per day (Chiang, 2014). Daily the liver synthesises approximately 500-600mg of bile acids from cholesterol, which facilitates approximately 600mg cholesterol biliary secretion (Chiang, 2014). Cholesterol is either synthesised in hepatocytes *de novo* from acetyl -CoA, or is derived from intestinal absorption and reaches the liver through chylomicron endocytosis. Dietary cholesterol or its metabolic derivatives can inhibit de novo cholesterol synthesis by inhibiting the transcription of HMG-CoA reductase which is the rate controlling step in cholesterol synthesis (Häussinger, 1996). Microsomal HMG-CoA reductase can also be induced when there are low levels of dietary cholesterol, therefore additional cholesterol can be supplied to cells by *de novo* synthesis and the receptor-mediated uptake of LDL from the blood (Lennernäs and Fager, 1997). HMG-CoA reductase can also be inhibited by statins which are a group of compounds used to lower levels of (LDL) cholesterol in the blood (Lennernäs and Fager, 1997, Jamei *et al.*, 2014). There are four enzymes that are involved in hepatic cholesterol metabolism HMG-CoA reductase, 7α-hydroxylase, acetyltransferase and ester hydroxylase and their regulation is important in the maintenance of cholesterol synthesis (Häussinger, 1996).

A critical function of the liver is metabolism and/or detoxification of drugs or xenobiotics (Fernández-Murga *et al.*, 2018). This process occurs within the hepatocytes making the compounds more water soluble enabling them to be more easily excreted from the body. There are three phases of drug metabolism. Phase I and phase II involve the metabolism of the molecule whilst phase III is concerned with the transport of the molecule via drug transporters. (Xu *et al.*, 2005). In phase I CYP P450s

are responsible for the majority of the enzymatic reactions such as hydrolysis, oxidation or reduction of the xenobiotic. Transferase enzymes such as glutathione S-transferase (GST) are mainly responsible for phase II metabolism. Phase II metabolism occurs if during phase I metabolism the molecule is not completely polarised, or if reactive metabolites are formed (Badolo *et al.*, 2011). Large polar groups are added (conjugation) to increase the hydrophilicity of the molecule and making it more soluble to be able to be excreted by the kidneys (Soars *et al.*, 2007b). In phase III the conjugated molecule may be further processed before elimination by efflux transporting proteins (Mittal *et al.*, 2015).

- **Metabolism of fats, proteins, and carbohydrates (and drugs)**

The body utilises two sources of fats for energy either from dietary food or they are synthesized by the liver by a process called lipogenesis. Fats enter the liver by being absorbed as fatty acids (FA) and glycerol, they are esterified and transported to the circulation via the lymphatic system as chylomicrons which are packages of triglycerides (TG), phospholipids and proteins (Kawano and Cohen, 2013). Fatty acids are utilised for energy and ketone body production via mitochondrial β-oxidation, esterification into TGs and storage in lipid droplets, or TG synthesis and VLDL formation with apolipoprotein B (apoB) for secretion into the circulation (Kawano and Cohen, 2013). Recent studies indicate that the transport of FAs, particularly long-chain FAs, is regulated by translocases and transporters (Hajri and Abumrad, 2002). The rate of FA uptake from plasma into hepatocytes depends on the plasma FA level as well as on the hepatocellular capacity for FA uptake. In the fasting state, a reduction in insulin levels stimulates TG hydrolysis in adipose tissue. FAs are thereby released and transported to the liver. The process of FA uptake depends on the number and activity of transporter proteins expressed on the sinusoidal plasma membrane of the hepatocytes (Enjoji *et al.*, 2016). Fatty acid catabolism occurs in the mitochondria where long chain fatty acids are converted to fatty acyl-CoA in order to pass across the mitochondria membrane. Fatty acid catabolism begins in the cytoplasm of cells as acyl-CoA synthetase uses the energy from cleavage of an ATP molecule to catalyse the addition of coenzyme A to the fatty acid. The resulting acyl-CoA crosses the mitochondria membrane and enters the process of beta oxidation. The main products

of the beta oxidation pathway are acetyl-CoA (which is used in the citric acid cycle to produce energy), NADH and FADH (Enjoji *et al.*, 2016, Kawano and Cohen, 2013)

Dietary proteins are hydrolysed to amino acids and dipeptides by various enzymes. These amino acids are further broken down to α-keto acids which can be recycled in the body for generation of energy, and production of glucose or fat or other amino acids (Häussinger, 1996). Protein accretion in the form of muscle, occurs in response to exercise, anabolic steroids or β-agonists (Campbell, 2006). Proteins are continuously broken down and synthesised to amino acids in the body where they undergo the following reactions in the liver (Charlton, 1996). Oxidative deamination is where amino acids are broken down so that they can be converted to sugars. The amino group is removed from the amino acid (glutamate) a reaction catalysed by glutamate dehydrogenase resulting in the formation of ammonium. The ammonia generated is converted to urea by hepatocytes through the urea cycle (Mitra and Metcalf, 2012). The remainder of the amino acid is oxidized, resulting in an alpha-keto acid which proceeds into the TCA cycle, in order to produce energy. The acid can also enter glycolysis, where it is converted into pyruvate. The pyruvate in turn is converted into acetyl-CoA so that it can enter the TCA cycle and convert the original pyruvate molecules into ATP, or usable energy (Chiang, 2014). Transamination essentially leads to the same end result as deamination where the amino group of one amino acid is transferred to alpha-ketoglutarate, so that it can be converted to glutamate. Glutamate transfers the amino group to oxaloacetate where it is converted to aspartate or other amino acids. This eventually will proceed into oxidative deamination (Charlton, 1996). Hepatocytes are responsible for synthesis of most plasma proteins. Albumin, the major plasma protein, is synthesized almost exclusively by the liver and the liver also synthesizes many of the clotting factors necessary for blood coagulation.

Carbohydrates are central to many essential metabolic pathways. The liver plays a critical role in the breakdown of carbohydrates from the diet to form predominately glucose but also fructose and galactose. Glucose is the primary structure that is distributed to cells in tissues, where it is broken down for energy, or stored as glycogen in the liver and muscle (Campbell, 2006). In hepatocytes, glycolysis only consumes

about 20-30% of glucose taken up by the liver for energy metabolism, the remaining glucose is synthesised into glycogen, fatty acids and ketone bodies which are stored inside hepatocytes and transported to other tissues for energy metabolism (Chiang, 2014). Glucose transporter 2 (GLUT2) located on the hepatocyte membrane is a key transporter for the transfer of glucose between the liver and the blood following a meal when blood glucose levels have increased to above 10mmol/L (Campbell, 2006). There are three main metabolic functions the liver carries out regarding carbohydrate metabolism. Firstly, glycogenesis where excess glucose following a meal is converted to glycogen, a process which is stimulated by insulin released from the pancreas during the fed state (Mitra and Metcalf, 2012). Glycogenesis consists of the release of glucose 1-phosphate from glycogen, and the modifying of the glycogen substrate to allow further degradation, and the conversion of glucose 1-phosphate into glucose 6-phosphate for further metabolism. The glucose 6-phosphate derived from the breakdown of glycogen is the initial substrate for glycolysis. Glucose 6-phosphate can be processed by the pentose phosphate pathway to yield NADPH and ribose derivatives, and it can also be converted into free glucose for release into the bloodstream (Chiang, 2014).

Glycogenolysis is the biochemical breakdown of glycogen to glucose whereas glycogenesis is the opposite, the formation of glycogen from glucose. Secondly, glycogenolysis takes place in the cells of muscle and liver in response to hormonal and neural signals. It plays a significant role in an adrenaline-induced response and the regulation of glucose levels in the blood. In muscle, glycogenolysis serves to provide an immediate source of glucose-6-phosphate for glycolysis to provide energy solely for muscle contraction but not for other body tissues. Muscle cells lack the enzyme glucose-6-phosphatase and thus cannot convert glucose-6-phosphate to glucose (Chiang, 2014). Adrenaline, which is released in response to stress response, and glucagon, released by pancreatic alpha cells in response to low blood glucose levels, stimulate glycogenolysis by binding to their respective receptors.

Thirdly, gluconeogenesis is the opposite of glycolysis where non-carbohydrate molecules like pyruvate, lactate, glycerol, alanine, and glutamine are converted into glucose. Gluconeogenesis is one of the two main mechanisms humans and many other

animals use to keep blood glucose levels from dropping too low (hypoglycaemia) (Mitra and Metcalf, 2012). This pathway is regulated by multiple different molecules and is activated in the fasted state by glucagon and is inhibited in the fed sate by insulin (Campbell, 2006). Gluconeogenesis takes place mainly in the liver and, to a lesser extent, in the cortex of kidneys. The process occurs during periods of fasting, starvation, low-carbohydrate diets, or intense exercise and is often associated with ketosis (Campbell, 2006)

1.5. In vitro assays systems in DMPK

In vitro assays can provide potential information on the ADME of a molecule. Many DMPK *in vitro* assays are carried out in the discovery phase of a molecule, due to the quick turnaround time of these assays, decisions can be made on the progression of a molecule through the development phase relatively quickly. At an early stage of molecule development large quantities of the compound are not available, therefore *in vitro* assays are important to provide the maximal information using small quantities of compound. Many studies are carried out to determine the ADME properties of a molecule and Table 2 gives a brief representation of the currently utilised ADME studies and the assay systems used.

Table 2: Model systems for studies

Study Type	Assay Description
ADME Study	Assay Test Systems utilised in DMPK studies
Metabolic stability	Liver preps, enzymes
Metabolite ID	Liver preps, bioreactors, in vivo
Reaction phenotyping	Microsomes, hepatocytes, enzymes
CYP inhibition	Microsomes, hepatocytes, enzymes
CYP induction	Microsomes, hepatocytes, ex vivo
Transporters	Hepatocytes, Caco-2 and other cell lines
Plasma protein binding	Plasma
Mass balance	Animals and human subjects
Metabolite profiling	Animals and human subjects
Disposition	Healthy subjects or patients
Species comparison	Animals and humans
Tissue distribution	Rats
(Zhang *et al.*, 2012). Copyright (2022), with permission from .Elsevier.	

All the assays described in Table 2 provide a PK and PD picture of the molecule: for example, the metabolic stability microsome assay is carried out early in the discovery phase to give an understanding of the clearance of the molecule. Each assay serves a purpose and is carried out in a certain order to answer a specific question. The next few sections will cover some of the enzyme and cellular systems that are used in the above assays.

1.6. Subcellular fractions

Subcellular fractions described below are used for metabolic stability assays, metabolite identification assays, reaction phenotype assays, CYP induction and CYP inhibition assays (Figure 6). The liver and gut are used for the preparation of enzyme systems, and subcellular fractions such as S9 fractions, cytosolic fractions and microsomal fractions (Figure 6). Certain co-factors, such as NADPH, necessary for

the biological activity for the enzyme to work are lost during the isolating process of subcellular fractions. Therefore, these co-factors may need to be added to the enzyme preparation to initiate the various enzymatic reactions. The use of expressed enzyme systems aids in the identification of the CYP isoforms involved in the clearance path of a molecule and their contribution to overall clearance. They are also used to identify the qualitative involvement of CYPs in the metabolic pathway of a molecule, assisting, not only in the identification of which CYP isoform is involved in the metabolism of the molecule, but also providing information on whether there are multiple isoforms or polyphormic enzymes involved.

The cytosol is isolated from the S9 fraction (Figure 6) and contains soluble drug metabolising enzymes, which are responsible for specific routes of drug metabolism e.g. N-acetyl transferase (NAT) and certain glutathione transferases (GST).

The S9 fraction consists of both the cytosol and microsomes (Figure 6) where nearly of all the drug metabolizing enzymes are represented. The advantages of using S9 fractions over microsomes is that metabolism mediated by non-CYP enzymes, such as sulfation and acetylation can be captured. The other advantages are S9 fractions are that they are cheaper and easier to handle than hepatocytes which also provide a complete collection of enzymes.

The microsomal fraction (Figure 6) is the most widely used of the sub-cellular fractions in drug metabolism. It contains membrane-bound CYPs and primary conjugation enzymes such as UGTs. Microsomes are relatively inexpensive and easy to handle, they are used for DDI studies and to determine intrinsic clearance of a compound to establish an *in vitro* correlation between animals and humans. CYP enzymes in microsomes are concentrated and therefore they are a useful tool for kinetic assessment of a substrate. Although microsomes are useful, hepatocytes are increasingly being used for metabolism studies. The application of hepatocytes to higher throughput assays has been improved by sophisticated automation instruments and miniaturization methods using cryo-preserved cells. Isolated hepatocytes are used to study drug metabolism and transporter interactions. Handled correctly they can express a broad complement of metabolizing enzymes and transport proteins.

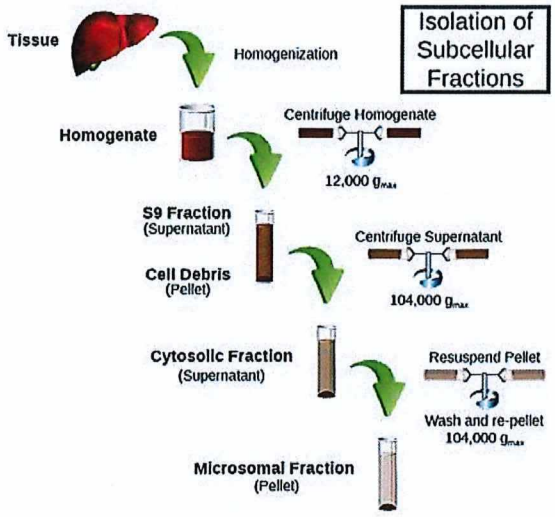

Figure 6: Isolation of Subcellular Fractions for use in ADME studies (Bohl, 2019). Image used with the permission of SEKISUI XenoTech www.xenotech.com

1.7. Hepatocytes

The liver performs metabolic and endocrine functions and is made up of approximately 80% hepatocytes (Häussinger, 1996, Rui, 2014). Hepatocytes contain a cell nucleus, other organelles such as rough and smooth endoplasmic reticulum (RER & SER), Golgi apparatus, mitochondria, cytoplasm, membrane, ribosomes, centrioles, vesicles, lysosomes and peroxisomes (Sevior *et al.*, 2012). Hepatocytes are polygonal in shape and their sides are in contact either with sinusoids or other hepatocytes to form bile canaliculi. Microvilli are present abundantly on the sinusoidal face and project into bile canaliculi. Hepatocytes contain round single nuclei but binucleated or multinucleated cells are not uncommon.

Hepatocytes are involved in many liver functions such as protein synthesis but also synthesis of cholesterol, bile salts and phospholipids, detoxification, modification and excretion of exogenous and endogenous substances, bile formation and secretion (Sevior *et al.*, 2012). The cell membranes of hepatocytes contain various uptake or efflux transporters. Drugs as well as endogenous compounds can passively diffuse through the cell membrane or be taken up by SLC transporters, such as OATPs. Once a drug enters the hepatocyte, ABC transporters, such as P-gp, BCRP and MRP, can

efflux the drug out, to reduce the intracellular drug concentration (Akram *et al.*, 2010). Freshly isolated hepatocytes still express relevant enzymes and co-factors and can therefore mimic the *in vivo* environment closely under the correct physiological conditions, providing holistic and reliable models to investigate both enzyme- and transporter-mediated intrinsic clearances and their interplay (Soars *et al.*, 2007b). However, intrinsic variability between hepatocyte donors can cause inconsistency in experiments (Tsamandouras *et al.*, 2017, De Bruyn *et al.*, 2011).

Freshly isolated hepatocytes have their limitations: once isolated, hepatocytes need to be used within a finite time limit, experiments cannot be repeated and especially regarding human hepatocytes- they are not readily available (Zhang *et al.*, 2012). With the availability of cryopreserved hepatocytes, these limitations have been largely overcome and the use of cryopreserved hepatocytes has provided huge advantages in drug discovery. Pooled cryopreserved hepatocytes can be obtained from suppliers who can provide characterisation data for metabolising enzymes in human and pre-clinical species whereas characterisation data for drug transporters is currently only available for single human donors. This characterisation information allows scientists to choose specific lots of hepatocytes that would provide the best platform for their assays.

A sandwich culture is a system where hepatocytes are grown between two layers of an extracellular matrix (ECM), for example the hepatocytes may be plated on a collagen coated plate and a layer of ECM such as Matrigel is added on top (De Bruyn *et al.*, 2013). Compared to the conventional monolayer culture, a sandwich culture is more representative of the *in vivo* situation. The sandwich culture environment allows hepatocytes to maintain their polarity, morphology, and liver-specific activities (Dunn *et al.*, 1989). The advantages of sandwich cultures are the formation of bile canaliculi which allows the determination of clearance via biliary excretion. Due to the time frame required in setting up a viable sandwich culture system, a 2D hepatocyte plated system provides a platform within approximately 4 hours to assess the uptake of drugs. Therefore, the plated hepatocyte system was used in this project.

Hepatocytes can be used as suspension cultures to assess metabolic stability or in the gold standard oil spin method to determine the uptake of drugs (Petzinger and Fückel,

1992, Paine *et al.*, 2008). As a suspension, hepatocytes have limited viability, typically 4 hrs, but cultured on collagen coated plastic plates hepatocytes can be viable for up to 7-10 days (De Bruyn *et al.*, 2013). There is a decline in CYP activity over this culture period, but an insight into the effects of increased drug exposure and to the formation of secondary metabolites can be observed. Plated hepatocytes are used for uptake studies, but due to decline in transporter expression the assay is best performed approximately 4 hours after plating. Cultured hepatocytes can also be used for CYP induction studies as enzyme induction may involve gene transcription and translation (Zhang *et al.*, 2012).

1.8. Drug Transporters

The cellular membrane transport system allows the movement of all endogenous substances, essential nutrients and ions into cells (Nigam, 2014) e.g., hormones and fatty acids, as well as transfer or elimination of metabolic products or waste e.g., urea, bilirubin and CO_2. This transport is controlled by large trans-membrane proteins embedded within the plasma membrane called transporters or channels which are pore-forming proteins. Channels open a gate in a ligand or electric potential-dependent manner. Transporters are proteins which change their confirmation thereby transport a ligand from one side of a membrane to other. There are three major types of carrier transport proteins: primary active, secondary active and facilitative transporters (Figure 7). Primary active pumps utilize the energy released by ATP hydrolysis to move substrates against their electrochemical gradient. Uniporters, also known as facilitative transporters, transport the substrate down its concentration gradient. Secondary active transporters (symporters and antiporters) catalyse the movement of a substrate against its concentration gradient, driven by the movement of one or more ions down an electrochemical gradient (Schweizer *et al.*, 2014).

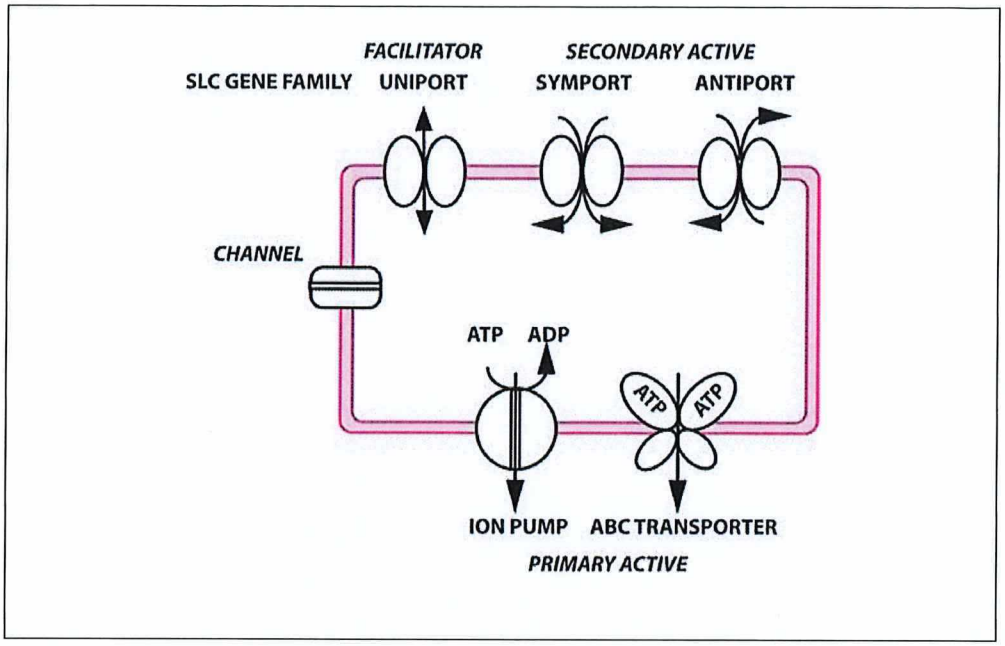

Fig 7: Summary of how transmembrane transporter proteins transport ligands from one side of a membrane to the other. Taken from (Schweizer *et al.*, 2014). Copyright (2022), with permission from S. Karger AG.

There are two super families of transporters, the ATP binding cassette (ABC) of transporters and the solute carrier family of transporters (SLC) (Liu, 2019, Keogh, 2012). Between both super families there are more than 400 members (Giacomini *et al.*, 2010). Transporter nomenclature is complex, however the Human Genome Organisations (HUGO) has a Gene Nomenclature Committee (HGNC) which is responsible for approving unique gene symbols and protein names https://www.genenames.org/. Symbols for human and rodent proteins are given in all capitals e.g., OATP1B1, whilst the corresponding gene symbols are in italics and all capitals for human e.g., *SLCO1B1* and lowercase for rodent except the initial letter being in capital e.g., Slco1b1.

Exogenous substances like drugs and environmental toxins can be substrates or inhibitors for transporters. Understanding if a drug is a substrate or an inhibitor can provide essential information on its PK/PD profile along with any clinical significances, such as increased toxicity or altered efficacy. *In vitro* guidelines are

provided by regulatory agencies (FDA, EMA and PMDA) (Liu and Sahi, 2016), recommending drug transporters that have shown clinical involvement in drug interactions (Table 3) Guidance for Industry (fda.gov). Other transporters can also be considered depending on factors such a drug being co-administered with a known substrate of another transporter. For the purpose of this thesis, both ABC and SLC hepatic transporters will be discussed but the focus will be on the SLC transporters.

Table 3: Summary of drug transporters to be studied as recommended by the FDA, EMA and PMDA (Japanese agency) (Liu and Sahi, 2016). [a] Drug transporters not located in the liver incorporated in table for completeness. Copyright (2022), with permission from SAGE SCIENCE PRESS (US)

Transporter	FDA	EMA	PMDA
P-gp	✓	✓	✓
BCRP	✓	✓	✓
OATP1B1	✓	✓	✓
OATP1B3	✓	✓	✓
OAT1[a]	✓	✓	✓
OAT3	✓	✓	✓
OCT1		Consider	Consider
OCT2[a]	✓	✓	✓
BSEP	Consider	Consider	Consider
MRP's	Consider	Consider	Consider
MATE	Consider	Consider	✓

1.9. The ABC Superfamily.

The ABC superfamily is one of the largest protein families encoded within the human genome: more than 48 genes encoding human ABC transporters have been identified and sequenced (Toyoda *et al.*, 2008). The ABC transporters are made up of four characteristic domains, two transmembrane domains (TMDs), and two nucleotide-binding domains (NBDs) (Figure 8). These four domains may be present within one

polypeptide chain (Locher, 2016) spanning the membrane multiple times ("full transporters"), or within two separate proteins ("half transporters") (Figure 9).

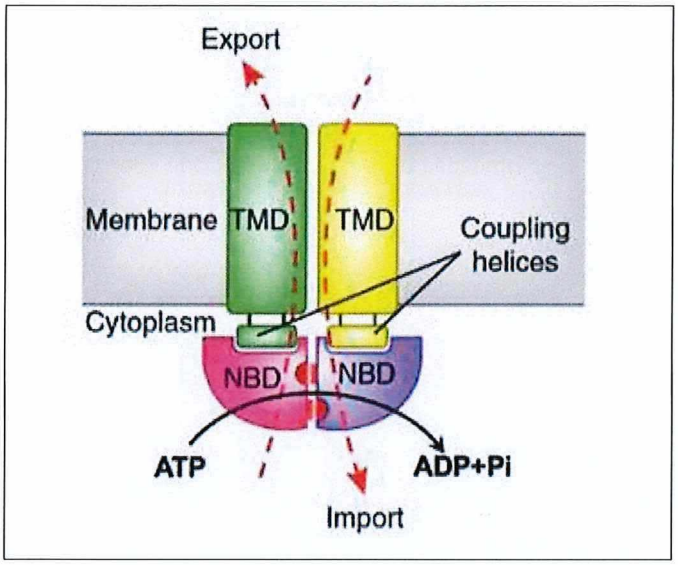

Figure 8: Conserved NBD architectures Taken from (Locher, 2016) Copyright (2022), with permission from Springer Nature

ABC transporters are referred to as efflux transporters and transport is driven by ATP hydrolysis, and they often act as protective/preventative barriers in organ functionality/toxicity. They transport xenobiotics and a wide range of endogenous substrates including conjugated bile salts, steroid hormones, cholesterol and unconjugated bilirubin often against a steep concentration gradient (Keogh, 2012).

ABC transporters discussed here are located on the apical or basolateral membrane and share a highly conserved ABC sequence motif (Lai, 2013) (Figure 9). Based on the sequence and organization of their ATP-binding domains, ABC transporters are phylogenetically classified into seven subfamilies of 49 transporter genes designated ABCA to ABCG (Table 4) (Dean and Allikmets, 2001). These transporters share similar homology over 12 transmembrane and two ATP-binding domains except for BCRP/ABCG2. Their main function is to eliminate endobiotics and xenobiotics out of a cell (Figure 9) (Schinkel and Jonker, 2012). Based on predicted structure and amino acid seoquence homology four classes can be seen in figure 9. P-glycoprotein consisting of two transmembrane domains, each containing 6 transmembrane segments, and two nucleotide binding domains (NBDs). It is N-glycosylated

(branches) at the first extracellular loop; MRP1, 2 and 3 have an additional amino terminal extension containing 5 transmembrane segments and they are N-glycosylated near the N-terminus and at the sixth extracellular loop. The amino terminal extension in MRP1-3 is absent in MRP4 and 5 and they are N-glycosylated at the fourth extracellular loop. BCRP is a 'half transporter' consisting of one NBD and 6 transmembrane segments, and it is most likely N-glycosylated at the third extracellular loop. In contrast to the other transporters, the NBD of BCRP is at the amino terminal end of the polypeptide and BCRP almost certainly functions as a homodimer.

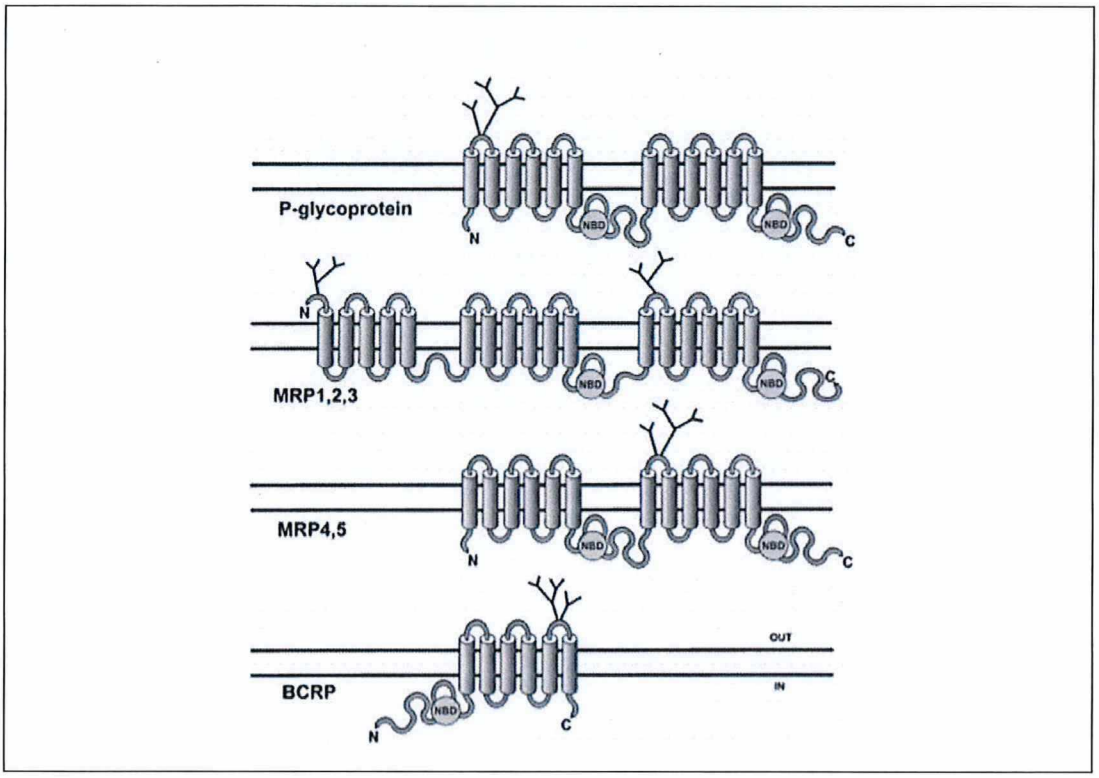

Figure. 9: Predicted secondary structures of drug efflux transporters of the ATP-binding cassette family. Four classes are distinguished here, based on predicted structure and amino acid sequence homology. (1) P-glycoprotein consists of two transmembrane domains, each containing 6 transmembrane segments, and two nucleotide binding domains (NBDs). (2) MRP1, 2 and 3 have an additional amino terminal extension containing 5 transmembrane segments (3) MRP4 and 5 lack the amino terminal extension of MRP1–3, (4) BCRP is a 'half transporter' consisting of one NBD and 6 transmembrane segments. N and C denote amino- and carboxy-terminal ends of the proteins, respectively. Cytoplasmic (IN) and extracellular (OUT) orientation indicated for BCRP applies to all transporters drawn here. Taken from (Schinkel and Jonker, 2012). Copyright (2022), with permission from Elsevier

Table 4: Human ABC transporters expressed in hepatocytes.

Transporter	Gene Symbol	Substrates	Inhibitors
MDR1/P-gp	*ABCB1*	Digoxin, fexofenadine, dabigatran, vincristine, loperamide, doxorubicin, paclitaxel	Cyclosporine A, quinidine, verapamil, amiodarone, clarithromycin, itraconazole, lapatinib, ritonavir Elacridar (GF120918)
BCRP	*ABCG2*	Estrone-3-sulfate, methotrexate, topotecan, imatinib, rosuvastatin, sulfasalazine, doxorubicin	Elacridar (GF120918), cyclosporine A, K0143, sulfasalazine.
BSEP	*ABCB11*	Taurocholic acid, pravastatin, bile acid	Cyclosporin A, rifampicin, glibenclamide
MRP2	*ABCC2*	Glutathione and glucuronide conjugates, methotrexate, doxorubicin, etoposide, valsartan, olmesartan, indinavir, cisplatin, paclitaxel, Topotecan	Cyclosporine A, delavirdine, efavirenz, MK-571, tamoxifen,
MRP3	*ABCC3*	Methotrexate Estradiol-17β-glucuronide, leukotriene C4, monovalent bile salts, morphine-3-glucuronide, fexofenadine, etoposide	tenofovir, indomethacin, furosemide, and probenecid, delavirdine, efavirenz, and lamivudine, MK-571
MRP4	*ABCC4*	Estradiol-17β-glucuronide, Methotrexate, 6-mercaptopurine, Topotecan	Indomethacin, verapamil, probenecid, MK-571, diclofenac
Adapted from (Sodani *et al.*, 2011, Faber *et al.*, 2003, Zhou, 2008) Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers | FDA			

P-gp (ABCB1) P-glycoprotein commonly abbreviated to P-gp belongs to the ATP-binding cassette transporter superfamily (Keogh, 2012). In humans P-gp is encoded by the Multi Drug Resistance 1 (MDR1) gene or ABCB1 and its expression is regulated by the nuclear hormone receptor, pregnane X receptor (PXR). P-gp is expressed in many tissues such as on the luminal membrane of the small intestine, the renal proximal tubule, the placenta, the blood–brain barrier and on the bile canalicular membrane of hepatocytes (Schinkel and Jonker, 2012). Multiple binding sites for

substrates and inhibitors have been identified. One of the key functions of P-gp is to protect cells against harmful compounds and hence it limits the entry of endo- or exo-toxins including some drugs, but it can also prevent pharmacotherapeutic agents from entering the systemic circulation or the site of action like the brain (Giacomini *et al.*, 2010). Another important role is in intestinal absorption or eliminating xenobiotics entering excretory cells from the blood side of the organ by transporting them into the bile, urine, or gastrointestinal tract. P-gp is responsible for the efflux of a broad range of substrates some of which are clinically important drugs e.g. digoxin, loperamide, fexofenadine, dexamethasone and endogenous substances such as steroids and bilirubin (van de Steeg *et al.*, 2012).

The breast cancer resistance protein BCRP (ABCG2). was initially cloned from a multidrug resistant breast cancer cell line. BCRP, like P-gp, is expressed in many tissue barriers throughout the body, including the intestine, the blood–brain barrier (BBB), the blood–placenta barrier, the blood–testis barrier and the bile canalicular membrane of hepatocytes. It is unidirectional and is localised on the apical membrane of polarised cells where it transports its substrates such as estrone 3-sulfate to the luminal side of the organ. BCRP is co-expressed with P-gp and shares many of its substrates, inhibitors and inducers. BCRP is known for its involvement in cytostatic drug resistance but it also plays a wider role in the disposition of drugs (Mao, 2005) e.g. rosuvastatin is a known substrate of BCRP (Zhang, 2018).

The bile salt exporter pump BSEP (ABCC11) is a unidirectional transporter and is expressed in the liver specifically the apical (canalicular) membrane of hepatocytes with much lower levels reported in the kidney. BSEP shows a high affinity for conjugated bile acids and mediates the hepatic excretion of bile acids from the hepatocyte into the bile canaliculi for export into the gastrointestinal tract (Cheng *et al.*, 2016). Inhibition of BSEP can result in the build-up of bile salts in the liver which in turn can lead to cholestasis and drug-induced liver injury (DILI) (Yucha *et al.*, 2017). As there are only few identified drug substrates and inhibitors of BSEP, knowledge of its involvement in drug-drug interactions (DDI) is limited.

The multi resistance protein2 MRP2 (ABCC2) is a unidirectional efflux transporter that transports organic anions, drug conjugates and conjugated bilirubin into bile and into the lumen of excretory organs. MRP2 is regulated by nuclear receptors PXR and Constitutive and Androstane Receptor (CAR) which can be induced by numerous drugs. MRP2 is exclusively located on the apical plasma membrane of polarized cells such as hepatocytes, pneumocytes, kidney proximal tubules, and specialized cells in the intestine and brain. MRP2 facilitates the elimination of bilirubin glucuronides, positively charged drugs and conjugates into the bile from hepatocytes, and into urine from the renal proximal tubule. While in other organs such as the placenta and the gastrointestinal tract it limits the distribution of its substrates. Therefore, the primary role of MRP2 is to limit cellular exposure to its substrates. Dubin-Johnson syndrome (DJS) is a condition where there is an inherited mutation of the ABCC2 gene, therefore giving rise to a non-functional MRP2, causing chronic hyperbilirubinemia (Gilibili *et al.*, 2017).

MRP3 (ABCC3) is also an efflux transporter located in the basolateral membranes of polarized cells such as cholangiocytes and hepatocytes. MRP3 is predominantly expressed in small intestine, pancreas, placenta, colon and adrenal cortex, whereas lower levels are found in liver, kidney and prostate. MRP3 mediates the transport of organic anions such as bile acids and drug-glucuronide conjugates e.g. estradiol-17β-glucuronide (Zeng *et al.*, 2000). It enables the oral absorption of conjugated forms of some dietary oestrogens and antioxidants. In conditions such as DJS where MRP2 is impaired, MRP3 can be up-regulated (expression increased) to mediate the efflux of organic anions from liver into blood when secretion into bile via MRP2 is blocked (König *et al.*, 1999). These adaptive responses serve as a compensatory mechanism to minimize the hepatocellular accumulation of toxic biliary constituents.

MRP4 (ABCC4) is an efflux transporter expressed in the kidney, BBB and liver, it is either located on the basolateral or apical membrane dependent on the tissue type. Similar to MRP3, MRP4 has a wide substrate specificity, including nucleoside analogues and antiviral drugs. Alongside MRP3 it plays an important role in

alleviating the impact of cholestasis on hepatocytes by efflux of bile acids into the blood and can be up-regulated in the liver in this instance (Zhou, 2008).

1.10. The Solute Carrier (SLC) family.

The SLC family consist of >400 transporters in approximately 65 sub families organised based on similarities and differences in transport mechanisms (Bai *et al.*, 2017). Progress has been made in understanding SLC transporter-mediated drug-drug interactions (DDIs). The International Transporter Consortium (ITC) in 2010 reviewed SLC transporters which are clinically relevant to DDI Guidance for Industry (fda.gov) (Table 5) and recommended candidate drugs should be assessed for substrate and inhibition potential of these transporters. SLC transporters not only serve as "gate keepers" but can be of great use to the pharmaceutical industry in the facilitation of a drug binding to a target for example the use of nanoparticles, deoxycholic acid-modified nanoparticles (DNPs) can be used for the delivery of insulin (Su *et al.*, 2019).

SLC proteins are expressed on cellular plasma membranes but can also be expressed on membranes of intracellular organelles such as the mitochondria, they consist of 7-14 putative membrane-spanning domains and their molecular mass is approximately 50 to 100 kDa (Russel, 2010, Roth *et al.*, 2012). The nomenclature for SLC carriers is complex and the HUGO Nomenclature Committee Database provides a list of transporters families of the SLC gene series (Table 5). SLC transporters in Table 5 are hepatic uptake transporters who are known to have an affinity for estrone 3 sulfate (ES), estradiol glucuronide (EG), rosuvastatin and pitavastatin. The table provides additional information on other substrates and inhibitors drugs and gives some information on additional hepatic transporters that are listed in the regulatory guidance.

Table 5: Solute Carrier (SLC) proteins listed in the FDA guidance, showing specific hepatic SLC transporter and corresponding gene with examples of specific substrates and inhibitors.

Transporter	Gene Symbol	Substrates	Inhibitors
NTCP	*SLC10A1*	Bile Salts, thyroid hormones	Antihyperlipidemics
OATP1B1 OATP1B3	*SLCO1B1* *SLCO1B3*	Repaglinide, valsartan, olmesartan, bilirubin, bile acids, glyburide, methotrexate, asunaprevir, atorvastatin, bosentan, danoprevir, docetaxel, fexofenadine, nateglinide, paclitaxel, pitavastatin, pravastatin, rosuvastatin, simvastatin acid	Saquinavir, ritonavir, cyclosporine A, clarithromycin, erythromycin, gemfibrozil, Rifampicin, Rifamycin SV, Estradiol-17β-glucuronide
OATP2B1	*SLCO2B1*	Taurocholate, statins, fexofenadine, glyburide	Rifampicin, cyclosporine A
OCT1	*SLC22A1*	Acyclovir, agmatine, berberine, daunorubicin, dopamine, furamidine, ganciclovir, imatinib irinotecan, lamivudine, metformin, morphine, MPP^+, norepinephrine, oxaliplatin, paclitaxel,	Quinine, quinidine, Ciprofloxacin, fleroxacin, amprenavir and ritonavir, imatinib and verapamil
(Boxberger *et al.*, 2014, Patel *et al.*, 2016), (Shen *et al.*, 2017), Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers | FDA			

Many of the SLC family members facilitate the cellular uptake or influx of substrates, either by facilitated diffusion down the electrochemical gradient acting as a channel or uniporter; or by secondary active transport against a diffusion gradient coupled to the symport or antiport of ions to provide the driving force (Schweizer *et al.*, 2014, Koepsell and Endou, 2004). Certain SLC transporters exhibit efflux properties or are bidirectional, depending on the concentration gradients of substrate and coupled ion across the membrane. Understanding the interplay between transporters located both on the apical and basolateral membrane is essential in determining the extent and direction of drug movement in organs such as the intestine, liver, and kidney. The uneven distribution of influx and efflux transporters in the epithelia can either impede or facilitate the transport of molecules, therefore ultimately contributing to the pharmacokinetic profile of a drug substrate in the body.

Organic cation transporter1 (OCT1) (SLC22A1) is expressed on the basolateral membrane of hepatocytes, renal proximal cells, with much lower expression in the heart, brain, skeletal muscle and lung (Roth *et al.*, 2012). It mediates the uptake of small hydrophilic cationic molecules and Type II cations e.g. Fe^{3+}. OCT1-mediated organic cation transport is mediated by electronegative membrane potential (Patel *et al.*, 2016) and metformin, an antidiabetic, is transported by OCT1. OCT1 polymorphisms have shown to cause a reduced hepatic uptake of metformin and its pharmacodynamic effect by reducing oral glucose tolerance.

Currently, the FDA and EMA guidelines do not state OCT1 liabilities should be investigated but OCT2 is advised, this is to evaluate interactions of drugs which are likely to be co-administered with OCT and multidrug and toxin extrusion (MATE) substrates. OCTs were discovered in 1995 whereas MATEs were discovered much later around 2005, therefore, there have not been as many DDIs associated with MATEs. Given the strong association of substrates and inhibitors of OCTs and MATEs e.g., metformin and cimetidine, some DDIs associated with OCT1 are under re-evaluation.

The specific transport mechanism of the organic anion transport protein (OATP) has not been established (Ali *et al.*, 2020), but it has been proposed to occur through a central, positively charged pore in a rocker-switch type of mechanism (Figure. 10). In this mechanism, the transport protein is assumed to have two major alternating conformations: inward-facing and outward-facing (Schweizer *et al.*, 2014). At any moment, a single- binding site in a pore is accessible from only one side of the membrane. Interconversion between the two conformations is only possible via a substrate-bound form of the transport protein (Liu, 2019).

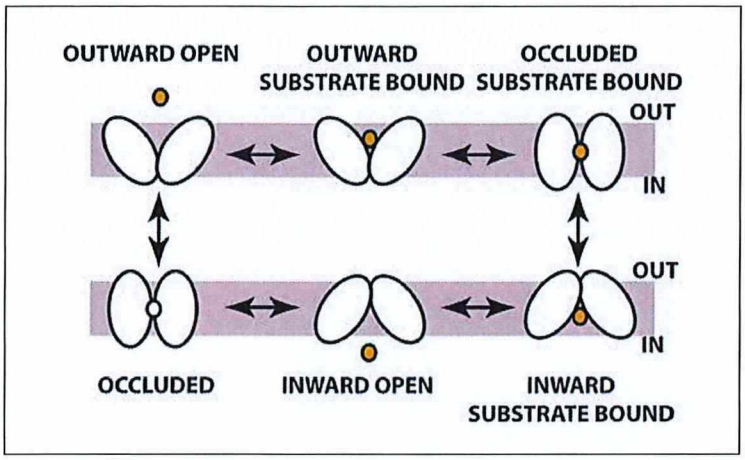

Figure 10: Rocker switch model of transmembrane transport. Transporters pass through a sequence of conformations, which allow solutes alternate access to a central binding site from the two faces of the membrane. Shown is the example of a uniporter, but secondary active transport is easily incorporated in this model. taken from (Schweizer et al., 2014) Copyright (2022), with permission from S.Karger AG.

OATPs are encoded by genes in the SLCO/Slco superfamily but originally were named SLC21A (Hagenbuch and Meier, 2004). Eleven human OATPs have been identified and are classified into six families based on their amino acid identity. The different proteins are named OATP (Oatp for the rodent proteins) followed by the family number, a subfamily letter and then a consecutive number identifying the individual members within the family (e.g., Oatp1a1, OATP1A2 and Oatp1a3). The corresponding gene symbols are SLCO followed by the same number–letter– number combination. A significant amount of gene duplication and divergence has occurred in this family, especially in rodents, complicating direct comparisons between human (OATP) and rodent (Oatp) studies. OATP1A2 has five rodent orthologues: Oatp1a1, Oatp1a3 (in rats only), Oatp1a4, Oatp1a5 and Oatp1a6. OATP1B1 and OATP1B3 have a single rodent orthologue (Roth *et al.*, 2012).

OATP1B1 (SLCO1B1) is a liver specific uptake transporter exclusively expressed on the basolateral membrane of hepatocytes. It is responsible for the hepatic uptake of a broad spectrum of substrates which include anionic, zwitterionic, neutral lipophilic drugs, endogenous compounds including bile acids, bilirubin, glucuronide conjugates, and therapeutic drugs such as HMG-CoA inhibitors (Table 5) (Shitara, 2011). A wide

range of food and herbal products such as grapefruit juice, black tea and St John's Wort have been found to cause adverse reactions when co-administered with certain medications (Ali *et al.*, 2020)

Nuclear hormone receptors FXR, HNF1α, HNF3β and HNF4α regulate the expression of OATP1B1and other important OATPs such as OATP1B3 and OATP2B1. OATP1B1 shares 80% of its amino acid sequence with OATP1B3 (Faber *et al.*, 2003).

Inhibition of OATP1B1 can result in a high systemic exposure (circulating levels) in patients to drugs such as statins which can result in myopathy and rhabdomyolysis. For example, in rotor syndrome where there is complete and simultaneous deficiency of OATP1B1 and OATP1B3, the hepatic reuptake of conjugated bilirubin is disrupted and clinically this is presented as mild hyperbilirubinemia (van de Steeg *et al.*, 2012). The functional loss of OATP1B1 due to polymorphisms can result in altered PK of the substrate drug. Cerivastatin was withdrawn from the market due to lethal interaction between cerivastatin and gemfibrozil, where gemfibrozil glucuronide inhibited OATP1B1 and CYP2C8 (Graham *et al.*, 2004) (Shitara *et al.*, 2004).

OATP1B3 (SLCO1B3) is expressed only in the liver on the basolateral membrane of hepatocytes.; however, expression was much stronger in the pericentral region compared with the periportal region. OATP1B3 is involved in the clearance of drugs and endogenous substrates from blood. Many of these molecules such as HMG-CoA inhibitors (i.e. statins), rifampin/rifamycin, cyclosporine, HIV protease inhibitors and endogenous substances e.g. bilirubin, bile acids and conjugated steroids are both substrates and inhibitors of OATP1B1 (Table 3) (Kullak-Ublick *et al.*, 2001). Therefore, OATP1B1 and OATP1B3 are often assessed together regarding any DDI liabilities. Other specific OATP1B3 substrates include docetaxel, digoxin, paclitaxel (Kalliokoski and Niemi, 2009), and the mushroom toxin amanitin (Letschert *et al.*, 2006).

OATP1B3 is also over-expressed in a multitude of carcinoma types and it has been identified that the OATP1B3 protein in cancer tissues is missing the first 28 amino acids, therefore the transporter function of the cancer-type OATP1B3 is greatly reduced. Many anticancer drugs are substrates for OATP1B3 and so it has been

suggested that OATP1B3 can be used as a target in cancer therapy (Obaidat *et al.*, 2012, Schulte and Ho, 2019) .

OATP1B1 and OATP1B3 play key roles in the hepatic uptake and disposition of drugs, including many drug interactions. Assessment of any potential drug interactions involving OATP1B1, other uptake/efflux transporters, and drug-metabolizing enzymes (DMEs) are vital. Populations that may have genetic polymorphisms should also be considered (Keogh, 2012). For drugs that are eliminated by the liver the regulatory authorities such as the FDA and EMA recommend evaluation of the drug interaction liability on OATP1B1 and OATP1B3 for all drug candidates as inhibitors, and as substrates. These assessments are used to predict DDI potential and aid in the development of a clinical drug interaction strategy. They can also be used for label recommendations, dose adjustments, and product withdrawals.

1.11. Species differences

Interspecies differences can cause difficulty in translating PK data from preclinical species into humans and therefore creating challenges in DDI prediction. Over time it has become evident that there are significant differences between the rodent, dog, monkey, pig and human specifically in their substrate specificity, tissue distribution, and relative abundance of metabolising enzymes and drug transporters (Chu *et al.*, 2013). The variability in the expression levels of drug metabolising enzymes and transporters across species can cause the same drug to be cleared rapidly in one species compared to another. Therefore characterising these differences in vitro can help improve predictions and therefore assist in drug development (Chu *et al.*, 2013) and provide quantitative knowledge of species differences in enzymes and transporters, especially at the protein and functional level.

When looking at species selection the closest species to humans in terms of genetic homology are non-human primates. Dogs as pre-clinical models have an advantage as they have comprehensive background data for toxicological safety assessment and they are easy to handle. Pigs have been used less than dogs and monkeys as a model in safety assessment of drug candidates (Dalgaard, 2015).

Protein expression quantified by tandem mass spectrometry by Wang *et al.,* (2015) has shown OATPs are the most abundant transporters in dogs, humans, rats and monkeys. However, there is interspecies variability, with the relative abundance of OATPs being greater in dog (69%), compared to only 29% in humans. There are also differences in absolute expression, with transporters being expressed more highly in rats (Figure 11) (Wang *et al.*, 2015c).

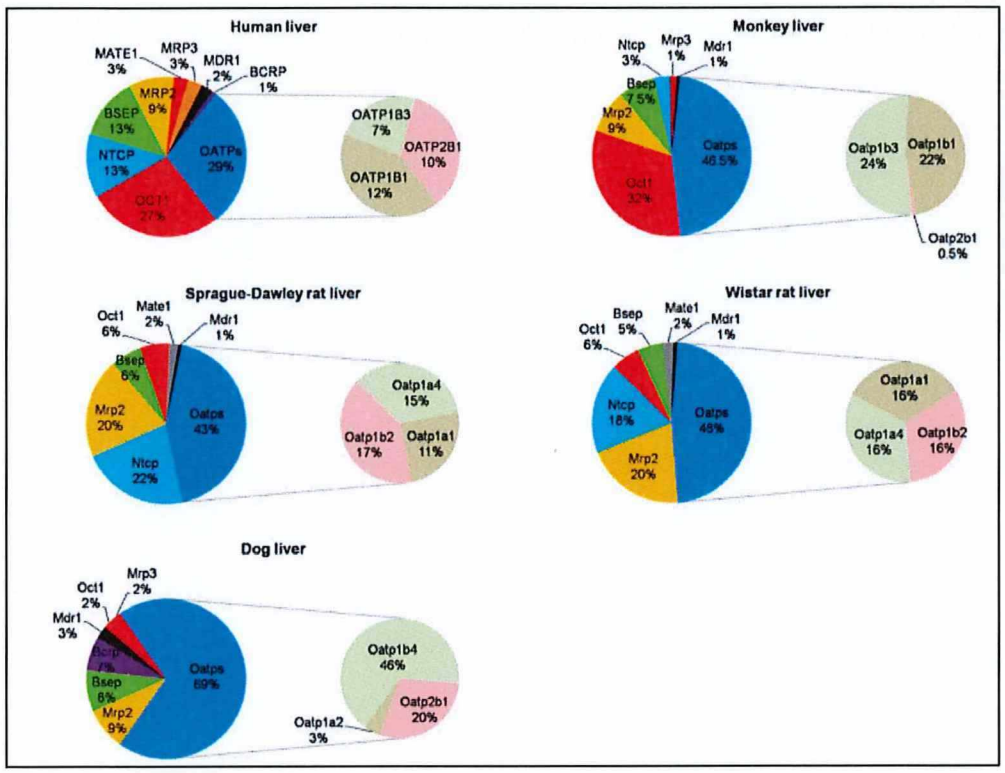

Figure. 11: Relative abundance of quantifiable transporters in liver tissue of humans, beagle dogs, cynomolgus monkeys, Sprague-Dawley rats, and Wistar rats. Data are expressed as percent of total transporter protein expression in the liver tissue of the respective species. Expression data of human BCRP, MDR1, MRP2, and OATPs were obtained from our previous publications (Deo *et al.*, 2012; Prasad *et al.*, 2013, 2014). (Wang et al., 2015c) The expression of MRP4/Mrp4 was below LLOQ (0.6 fmol/mg membrane protein) in all the species studied. Copyright (2022), with permission from Am Soc for Pharmacology & Experimental Therapeutics.

Additionally, OATP1B1 and OATP1B3 are poorly conserved, with no direct orthologues being found in either dogs or rats. Instead, Oatp1b2, Oatp1a1 and Oatp1a4 are expressed in rat hepatocytes, where, collectively, they have a similar role to the human OATP1B1/OATP1B3 (Chu *et al.*, 2013). In dogs, the predominant OATP is

Oatp1b4, which is thought to be a single orthologue of OATP1B1 and OATP1B3. However, its substrate specificity is more similar to that of OATP1B3, which could result in interspecies differences for some substrates (Wilby *et al.*, 2011). These differences result in variation in uptake, with active hepatic uptake being generally slower in humans than in rats (Soars *et al.*, 2007a, Ménochet *et al.*, 2012b), characterising these differences *in vitro* can help improve predictions and therefore assist in drug development (Chu *et al.*, 2013).

1.12. Pigs as a model for assessing elimination

There is a requirement from regulatory authorities regarding evaluation of new chemical entities, that they are tested in rodent and non-rodent species in order to ensure safety in humans (Tang and Mayersohn, 2018). Due to the knowledge available generally, dogs and non-human primates are used as the non-rodent species. Non-human primates are used in scientific experiments when it has been demonstrated that none of the other non-rodent species used in safety testing are appropriate for the study. The use of dogs can be restrictive due to their physiological difference to humans and also, they are considered as a pet in society (Webster *et al.*, 2010). The pig was introduced in drug development approximately 20 years ago, giving the option to select another non-rodent species, based on its anatomical and physiological similarities to humans with respect to a variety of organs and functions (Forster *et al.*, 2010a).

In the 1960s the University of Gottingen in Germany developed the Gottingen minipig from three breeds, the Minnesota minipig, Vietnamese Potbelly Pig and German Landrace. The minipig was rarely used in industry, but in the 1980s the Canadian health Authority and the FDA were cited in their use of the pig (Alder and Zbinden, 1988). In 1992 an exclusive licence contract was made between Ellegaard Göttingen minipigs ApS in Denmark and the University of Göttingen (Simianer and Köhn, 2010). The Gottingen minipig strain is similar to the human in terms of its anatomy, physiology and biochemistry, and its smaller size makes it favourable in terms of its use in research.

The RETHINK project was funded by the European community 6th Framework Programme as a Specific Support Action (SSA), Its objective was to evaluate the

impact of toxicity testing in the minipig (Forster *et al.*, 2010b). Expert working groups reviewed and reported on different areas related to the use of minipigs in regulatory safety toxicology studies (Forster *et al.*, 2010a) These areas investigated ethical issues, welfare and animal care, development of new medicines and chemicals safety testing issues and emerging technologies in safety testing (Forster *et al.*, 2010b). A survey by the International Consortium for Innovation and Quality in Pharmaceutical Development on factors that are considered in the selection of the minipig as a nonrodent species for safety assessment, confirmed the outcome of a survey carried out in 2010 by the RETHINK group (Heining and Ruysschaert, 2016). The outcome of this survey was the amount of drug substance required and the associated costs relating to a toxicology study was a major driver in deselecting the minipig as a non-rodent species for projects.

A review carried out by Lars Dalgaard in 2015 on drug metabolism and distribution, summarised similarities and differences in metabolic enzyme and transporter activity of the minipig compared to human (Dalgaard, 2015). This study suggested that the minipig should be the preferred animal model when assessing compounds that are metabolized by aldehyde oxidase, N-acetyl-transferases, or CYP2C enzymes. Studies that focused specifically on cytochrome P450 mediated metabolism in minipig showed CYP1A, CYP2A and CYP3A catalysed reactions are very close to those in humans (Dalgaard, 2015).

Furthermore, the presence of CYP3A and P-gp in Gottingen minipigs was demonstrated by (Van Peer *et al.*, 2014) and their ontogeny was similar to humans. Table 6 shows a comparison of human transporter homology with monkey, pig and dog. Drug transporters in pigs are comparable to humans with amino acid sequences greater than 72% identical (Table 6), except that SLCO1B1 expressed in human hepatocytes does not have an orthologue in pigs or dogs (Dalgaard, 2015).

Table 6: Comparing human transporter homology to monkey, pig and dog. Adapted from (Dalgaard, 2015)

Selected Human Transporter	% homology of amino acids.		
	Monkey	Pigs	Dogs
P-gp	96	89	90
BCRP	97	84	83
OATP1B1	92	No Homology	
OATP1B3	93	72	72
OAT1	95	90	90
OAT3	96	82	78
OCT1	91	75	74
OCT2	94	86	84

Non-human primates are probably the closest to man in terms of transporter homology compared to dog and minipig (Dalgaard, 2015), but the selection of the right species for specific studies needs to be considered on an individual basis dependent upon study requirements. Further evaluation needs to be carried out on the predictability of the minipig for human specifically on ADME processes. There is a reluctance in the use of minipigs for these studies, due to the amount of test compound required and there are still knowledge gaps that exist. Filling in these knowledge gaps will enable the predictability of the minipig to human correlation to facilitate the development and validation of mathematical models.

1.13. Non-Clinical Translation

Modelling at the early discovery preclinical stage is often used to aid selection of the candidate drug for progression into the clinic, aid dose selection and assess safety aspects (Wishart, 2007, Wang *et al.*, 2015d). Prediction of the human dose-response relationship is essential for such decisions and requires techniques for accurate scaling of animal data. Data from compounds of the same class can be used as comparators, with the relative difference between the comparator's efficacy and potency in animals

compared to man and applied to the new drug of interest. Confidence can be gained from predictive models scaling between other non-clinical species (Riviere *et al.*, 1997). For example, PK parameters are usually scaled allometrically or physiologically. However, for PD in the absence of comparator data, this type of empirical scaling would not be feasible and a mechanistic approach would be required (Huang and Riviere, 2014).

General methods for predicting human PK include extrapolation of intrinsic clearance (Clint) from *in vitro* experiments. The experiments can be carried out in primary isolated or cryopreserved hepatocytes, hepatic microsomes and recombinant expressed enzyme systems (De Buck and Mackie, 2007). *In Vitro In Vivo* Extrapolation (IVIVE) is the extrapolation of *in vitro* data to predict whole organ (hepatic) clearance and whole body clearance of a molecule by using scaling factors which are species specific. Methods such as allometric scaling is one way where ADME parameters such as clearance (Cl), volume of distribution (Vd), and elimination half-life ($t_{1/2}$) are used to predict PK parameters across species, another way is to use whole body physiologically-based pharmacokinetic (PBPK) modelling. These models can predict safety and efficacy data across animal models using *in vitro* data and data from across species (Huang and Riviere, 2014).

The method used may differ depending on the time point when the human PK prediction is required and the corresponding amount of available data. With every method there is uncertainty, but the goal is to get "in the ballpark" (e.g., within 3-fold of observed exposure) (Ring BJ, *et al* 2011) for the perceived PK/PD exposure-response relationship in the clinic. This aids the likelihood of the success in combination with the 4-pillars of drug discovery, i) does the drug have good pharmacokinetic properties?, ii) can the drug reach the target with sufficient engagement?, iii) does the drug elicit a pharmacological response? and iv) is the PD response translatable in man and does it alter the desired disease state?

1.14. Intrinsic Clearance and IVIVE

Intrinsic clearance is an important parameter in understanding a drug's kinetics. It is a measure of the cellular or subcellular capacity rate to remove the drug. This is

primarily associated with the main clearance organs such as the liver, kidney and intestinal wall.

Intrinsic clearance (Clint), which is experimentally determined *in vitro* is the theoretical unrestricted maximum clearance of unbound drug by an eliminating organ using whole cells or microsomal subfractions (e.g., mL/min/mg microsomal protein or mL/min/million hepatocytes) that can be extrapolated to the whole organ *in vivo* CLint (mL/min or L/h). Most commonly, this is hepatic clearance, which can then be used (e.g., by substitution into the 'well-stirred' model or the parallel tube model) to determine the contribution of organ clearance to total blood clearance. e.g.:

mL/min/mg microsomal protein X mg microsomal protein/g liver = mL/min/g liver

mL/min/g liver X liver wt (g) = mL/min

There is a standardised and scientifically accepted set of liver scaling factors, namely microsomal protein per gram liver, hepatocyte million cells per gram liver and liver weights (gram / kg bodyweight) as shown in the Table 7 below (SimCyp Certara). Previously, many pharmaceutical companies and academic institutions would use generic scaling factors or internally derived values for protein per gram of liver and cellularity, which is still observed for rabbit and guineapig, and even liver weights. Investigative work identified that values are different between species which can lead to different propagation of the factors and differing interpretations.

Table 7: Harmonised New Scaling Factors to be used Worldwide

Species	Microsomal (protein per gram liver (MMPGL))	Hepatocytes (million cells per gram liver)	Liver weights (gram / kg bodyweight)
Human (70 kg)	39.7	117.5	24.5
Rat (0.25 kg)	46.0	108	36
Mouse (0.025 kg)	48.0	135	51
Dog (10 kg)	40.41	170	32.5
Cyno Monkey (5 kg)	31	122	30
Minipig (10-20 kg)	34.4	124	16.7
Rabbit (2.5 kg)	52.5	120	30.8
Guinea pig (0.5 kg)	52.5	120	43.1

The limitations in determining intrinsic clearance are drug solubility and stability in matrix, the turnover rate, k can be low sensitivity for slowly metabolised drugs and require longer experimental incubation such as in the new HUREL system. Microsomes lack phase II metabolism, therefore cofactors need to be added. The potential correction factors that need to be used are the fraction unbound in plasma, the fraction unbound *in vitro* (microsomal or hepatocyte cells), blood to plasma ratio (RBP) and blood cell partitioning.

1.15. Bioanalysis

1.15.1. Background to Bioanalysis

Bioanalytical approaches have been employed within this project to enable the generation of data from different compounds and biological study designs. Bioanalytical assay methods utilised within this project were qualified based upon existing validated assays that have been adapted from regulated studies or from literature, such that bioanalytical peak-area-ratios or concentration data was produced to support study interpretation and integration.

In the context of analytical chemistry, this is the intentional separation of a substance into one or more constituents (e.g., elements, compounds or ingredients), usually by chemical means, to ascertain the kind, composition or quantity of component parts

whether obtained in separate intact form or not. Bioanalysis is a sub-discipline of analytical chemistry covering the identification or measurement of xenobiotic substances (such as drugs and/or their metabolites) or biotics (such as macromolecules, proteins, DNA, large molecule drugs, metabolites) in a biological system (such as blood plasma, urine, or tissue).

Bioanalysis is an important facet to the measurement of physicochemical and metabolic properties that are crucial to the discovery and development of new drug candidates. If these properties fit the target therapeutic profile required of a commercial drug, they are considered to have "drug-like properties" which may be exhibited by good absorption after oral dosing, bioavailability, pharmacokinetics and stability. Biological profiling of drug candidates may be achieved through qualitative or quantitative assessment and by a combination of analytical separation techniques using chromatographic resolution (e.g. TLC, GC or HPLC) and detection techniques (e.g. UV, fluorescence, radioactivity, MS) (Ding *et al.*, 2013).

Quantitative bioanalysis involves determining the concentration of drugs and/or metabolites in a biological matrix such as plasma or blood. Methodologies must generate reproducible and reliable data to allow valid interpretation, especially when attempting to develop appropriate dosing regimens, therapeutic strategies and safety exposure in patients. Therefore, regulatory guidance has been an equally important component which has accompanied bioanalytical methodologies to ensure high quality and integrity (Shah and Bansal, 2011). Calibration lines are prepared over an analytical range in order to quantify the anticipated concentration levels of unknown samples. To monitor the performance of an analytical method during an analytical run quality control samples (QCs) are also prepared and incorporated as part of the assay. Acceptance criteria are set for the calibration standards and QCs in a local standard operating procedure, if the assay fails to meet these acceptance criteria then a repeat analysis would be required.

1.15.2. Bioanalytical Regulatory Landscape

Prior to 1990, there were only regulations requiring bioanalytical methods to be sensitive, specific, accurate and precise (US 21 Code of Federal Regulations, 2002)

where bioanalysts provided their own interpretations of procedures and specifications to meet the regulatory requirements. As a result, there was a lack of uniformity to the approaches to validation of bioanalytical methods, submission of data to the regulatory agencies and evaluation of the data submitted.

At a workshop, subsequently known as "Crystal City Bioanalytical Workshops", dedicated to bioanalytical method validation (BMV) the first concept 'white paper' of a harmonised approach was made (Shah *et al.*, 1992) The conference focussed on defining the essential parameters (accuracy, precision, selectivity etc) and addressed a 'how to evaluate' approach as well as defining the standard curve, recovery and replicate analysis for BMV However, it was not until 10 years later that a draft regulatory guidance was issued (FDA, 1999). A second workshop reported on the progress made within the decade and formed the basis of the first formal FDA Guidance on BMV (FDA, 2001). At the time the EMA produced a brief directive in 2003 and then comprehensive guidance in 2009 with minor differences highlighted as result of field progression (Smith, 2010). Numerous workshops since the inaugural Crystal City meeting have led to white papers which recognise the continual advancement in methods and techniques (Savoie *et al.*, 2009, Savoie *et al.*, 2010, Fluhler *et al.*, 2014). The need for a 'global' harmonisation from constituent bioanalytical bodies, such as European Bioanalytical Forum (EBF) and Japanese Bioanalysis Forum (JBF), was recognised in the formation of the Global Bioanalytical Consortium (GBC). The GBC is currently working towards a harmonised document between various global regulatory agencies and bioanalytical groups (van Amsterdam *et al.*, 2010, Imazato, 2013). To ensure consistency in the analysis of the samples in this project all the samples analysed using bioanalytical methods will conform to regulatory requirements.

With regulatory guidance documents in place, a considered best practice approach as part of the bioanalytical toolbox can be applied to appropriate studies within early discovery (Timmerman *et al.*, 2015). Quantitative drug bioanalysis measures drug concentrations in biological specimens for different purposes. It can be divided into non-regulated (non-GLP) and regulated (GLP) bioanalysis. In the pharmaceutical industry, regulated studies refer to bioavailability/ bioequivalence, GLP, toxicokinetic

and human PK studies that are used for clinical drug toxicology and therapeutic monitoring. Non-regulated studies refer to early discovery and development pharmacokinetics, investigative and preliminary safety toxicology studies that are not critical in a regulatory submission (Huang *et al.*, 2012). The in-house guidance document will be used to ensure samples are analysed correctly and all relevant control criteria are met.

Within discovery sample bioanalysis there is the need to balance data quality with speed for 'fast turnaround' decision making. This is not simplified regulated bioanalysis with relaxed acceptance criteria, but a more pragmatic approach to balance the level of assessment of BMV reporting and generic method suitability to deliver sample analysis (Ho, 2014).

The most robust and abundant bioanalytical tool used in the pharmaceutical industry currently is reversed-phase ultra-high performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS) (Ramanathan *et al.*, 2011). Referred to as LC-MS, it meets all the prerequisite requirements for analysing drug concentrations in biological fluid samples in-line with current standard approaches (Ding *et al.*, 2013, Zhang *et al.*, 2013).

1.15.3. Ultra-High-Performance Liquid Chromatography (UHPLC)

Chromatographic methods in the pharmaceutical industry fall into three categories; high throughput, high productivity, and high resolution. These are defined by the objective of the separation and application area, which include run time, efficiency and resolution (Chesnut and Salisbury, 2007). HPLC is a common versatile technique that has a wide range of applications and compatible with numerous detection techniques for determining the assay or impurity/ metabolic profiles of drugs. There are two variants of HPLC dependent on the relative polarity of the solvent and the stationary phase (silica column packing material). Normal phase chromatography is similar to thin layer chromatography or column chromatography, where polar compounds in the mixture pass through a column and 'stick' longer to the polar silica phase than non-polar compounds. The non-polar compounds therefore pass more quickly through the column. Reverse phase (RP) is the opposite, where a polar solvent

is used and polar molecules elute more rapidly due to the non-polar components forming interactions with the hydrocarbon column residues, and this is the most common technique deployed in the pharmaceutical industry (Dejaegher *et al.*, 2010, Pieters *et al.*, 2010).

RP-UHPLC utilised in quantitative or qualitative bioanalysis falls into the category of high productivity where reduced run time and efficiency is the most important factor. Traditional isocratic or gradient methods can be developed and applied with specific column phase chemistries and mobile phases to further aid efficiencies (Molnar, 2002, Krisko *et al.*, 2006). These traditional methods can simply be applied to UPLC for improved productivity by systems now utilising columns that are smaller in length, packed with smaller diameter particle sizes (<2 μm), resulting in higher backpressure, and termed "ultra" HPLC (Chesnut and Salisbury, 2007). As a result, UHPLC chromatography greatly enhances MS sensitivity through reduced dispersion at lower flow rates and increased source ionisation as shown by a diverse application in quantitative studies (Gosetti *et al.*, 2013, Wang *et al.*, 2015a).

1.15.4. Mass Spectrometry (MS)

Mass spectrometry (MS) is an analytical chemistry technique that helps identify the amount and type of chemicals (analytes) present in a sample by measuring the mass-to-charge (m/z) ratio and abundance of gas-phase ions under vacuum. In the early days MS detected precursor-product ion pairs to allow the identification of different structures by fragmenting molecules into smaller building blocks. MS technology has evolved considerably identifiying analytes based on acurate masses which can be obtained by using high resolution mass spectrometry. These new developments have lead to more sensitive, robust and repeatable analyses not only for identification of analytes but also their quantification. MS is now a fundamental application to pharmaceutical analysis (Loos *et al.*, 2016).

There are three basic components to a mass spectrometer; firstly under atmospheric conditions there is the in-let, which receives the sample either directly or from a separation system (such as gas or liquid chromatography (GC or LC)), and ionisation source, where the analytes become negatively or positively charged as a result of

source method used, such as electrospray ionisation (ESI) or atmospheric chemical ionisation (APCI). The second is the mass analyser quadrupole, which separates and focuses gas ions based on *m/z* using 4 electro-magnetic rods under vacuum (for example instruments such as TOF, Ion Trap and Orbitrap). The final component is the detector and recorder, which can be in the form of counts per second (CPS), continuous electron multiplier (CEM) with or without deflection. A mass spectrum plot is generated of the ion signals detected as a function of the *m/z* ratio, which for example is achieved when analysing full scan spectra for potential metabolites.

Quantitative MS analysis is highly selective as it can be optimised to 'filter' the molecule of interest based on its *m/z* ratio and input parameters for the mass spectrometer components. Further specificity is achieved by fragmenting the parent molecule in the MS to its 'daughter ions' that give a characteristic product ion profile which can be further focussed for multiple reaction monitoring (MRM) in MS/MS mode (tandem). Sensitivity for each individual analyte of interest can be achieved by optimising a range of custom parameters within the MS, such as temperature, curtain gas, collision energy, declustering potential, and cell exit potential (Higton, 2001). An internal standard (IS) is used as a reference point to calculate peak area ratios of drug-related material of interest and determine its relative ratio or concentration (based on a calibration line). An IS is also used to correct any inter-sample variability in signal response, extraction and identify issues. In combination with sample preparation (extraction solvents) and chromatographic resolution (column chemistry, gradient and solvents) a highly selective, sensitive and reliable bioanalytical method is achieved (Kerns and Di, 2006, Plumb, 2008, King *et al.*, 2014).

Therefore UPLC-MS was employed for the analysis of biological samples (plasma, blood and heart tissue) from studies conducted within this thesis to deliver fast, sensitive, reproducible qualitative and quantitative data in line with current practices for bioanalytical methods in the pharmaceutical industry.

1.16. Radiodetection

Radiodetection has aided the study of drug metabolism, allowing DMPK scientists the ability to follow a molecule through tissues and organs of the body. The use of

radiolabel tracers has alleviated the difficulty in being able to detect small quantities of molecules that are of interest amongst an array of other compounds, also allowing the detection of molecules following metabolism. Radioactivity is routinely detected and quantified in laboratories using liquid or solid scintillation counting. The basic principle of scintillation counting is when nuclear decaying energy is converted to photons of light by a scintillator and it is the photons that are detected and measured. Solid and liquid scintillation counters can be used to analyse radioactive samples. With liquid scintillation counting the sample is dissolved in a cocktail containing a solvent and the liquid scinitillantor. Energy from the nuclear decay is transferred to the solvent first then to the scintillator. On receiving the energy from the solvent the scintillator becomes excited and moves to a higher energy state. Returning to the ground state the energy is released as photons of light by fluorescence. The photons are detected by photomultiplier tubes, the light emitted can be directly related to the energy of the nuclear decay. Solid scintillation is similar to liquid scintillation except the nuclear decay is absorbed and trapped directly by the solid scintillator, which then emits photons (Lappin, 2006).

1.17. SLC substrates and inhibitors

EG, ES , rosuvastatin and pitavastatin are known to be OATP substrates and used as control substrates in rat and human hepatic uptake assays. The statins are also used as clinical probes due to the DDI implications around statins and co-medication with other drugs, food or endogenous compounds. As there is plenty of in-house and literature data available around rat and human uptake, these molecules were selected to determine their uptake in minipig hepatocytes as currently no information is available.

Rifamycin SV, imipramine and cyclosporin A were selected as known OATP inhibitors.

1.17.1. Rosuvastatin (RSV)

RSV belongs to a class of medications called statins: the structure of RSV is shown in Figure 12. It is sold under the trade name Crestor and is used to reduce plasma cholesterol levels in the prevention of cardiovascular disease.

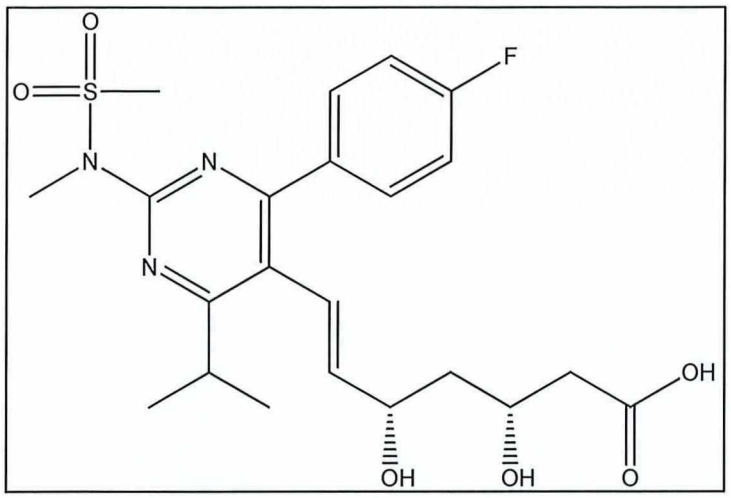

Figure 12: Rosuvastatin Structure.

Rosuvastatin is a competitive inhibitor of HMG-CoA reductase, which catalyses the conversion of HMG-CoA to mevalonate, a rate-limiting step in cholesterol biosynthesis. Rosuvastatin can also inhibit the synthesis of very low-density lipoprotein (VLDL). A decrease in hepatic cholesterol concentrations stimulates the upregulation of hepatic low-density lipoprotein (LDL) receptors which increase hepatic uptake of LDL. Rosuvastatin is not extensively metabolized: approximately 10% is excreted as a metabolite (Quirk *et al.*, 2003). CYP 2C9 is primarily responsible for the formation of rosuvastatin's major metabolite, *N*-desmethylrosuvastatin, which has approximately 50% of the pharmacological activity of its parent compound *in vitro*. Inhibitors of CYP2C9 increase the Area Under the Curve (AUC) by less than 2-fold. This interaction does not appear to be clinically significant

1.17.2. Estradiol glucuronide, or estradiol 17β-D-glucuronide (EG)

EG is a natural human metabolite of 17-beta-estradiol generated in the liver by UDP glucuronosyltransferase: the structure of EG is shown in Figure 13. Glucuronidation is a process by which toxic substances, drugs or other substances that cannot be used as an energy source are excreted.

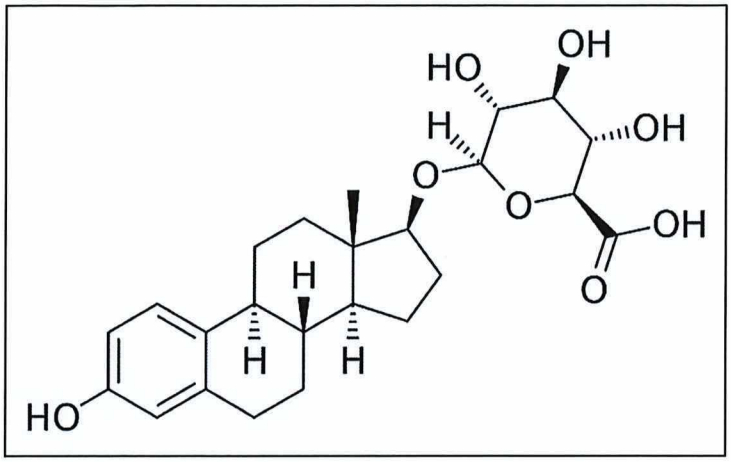

Figure 13: Estradiol glucuronide structure.

When estradiol is administered orally, it goes through approximately 95% first-pass metabolism in the intestines and liver (Oettel and Schillinger, 2012). It is a naturally occurring hormone that circulates endogenously within the human body and is the most potent form of mammalian estrogenic steroids and acts as the major female sex hormone. As such, estradiol plays an essential role in the regulation of the menstrual cycle, in the development of puberty and secondary female sex characteristics, as well as in ageing and several hormonally mediated disease states. Estradiol is commercially available as hormone therapy for managing conditions associated with reduced oestrogen production such as menopausal and peri-menopausal symptoms as well as hypoestrogenism. It is also used in transgender hormone therapy, and as the pill preventing pregnancy.

1.17.3. Estrone 3-sulfate (ES)

ES belongs to the class of organic compounds known as sulphated steroids: the structure of ES is shown in Figure 14. These are sterol lipids containing a sulfate group attached to the steroid skeleton. Estrone sulfate (as estropipate) is a form of oestrogen and has several uses such as: alleviating symptoms of menopause as hormone replacement therapy, treatment of some types of infertility, treatment of some conditions leading to underdevelopment of female sexual characteristics, treatment of vaginal atrophy, treatment of some types of breast cancer (particularly in men and postmenopausal women), treatment of prostate cancer and prevention of osteoporosis.

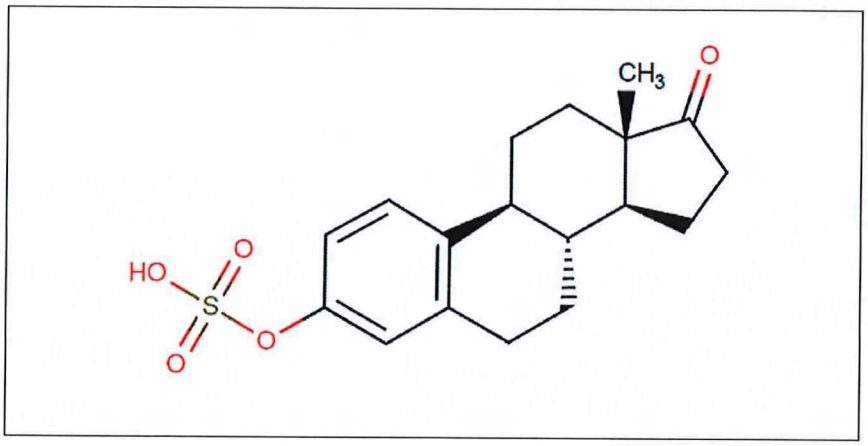

Figure 14: Estrone 3-sulfate structure.

Hydrophilic sulphated steroids are actively transported by SLC transporters like OATP1B1 or OATP1B3, or ABC transporters like MRP2 or BCRP (Mueller *et al.*, 2015).

1.17.4. Imipramine

Imipramine belongs to the class of organic compounds known as dibenzoazepines; these are compounds with two benzene rings connected by an azepine ring: the structure of imipramine is shown in Figure 15.

Imipramine is sold under the brand name of Tofranil, it is a tricyclic antidepressant (TCA), used for the treatment of depression. It works by enhancing and prolonging the activity of neurotransmitters such as serotonin and noradrenaline. Monamine neurotransmitters such as serotonin, norepinephrine, and dopamine can be blocked by a monoamine reuptake inhibitor (MRI). It does this by blocking the action of one or more of the respective monoamine transporters (MATs) resulting in an increase in the synaptic concentration of one or more of the neurotransmitters and thus leading to an increase in monoaminergic neurotransmission (Walter, 2005).

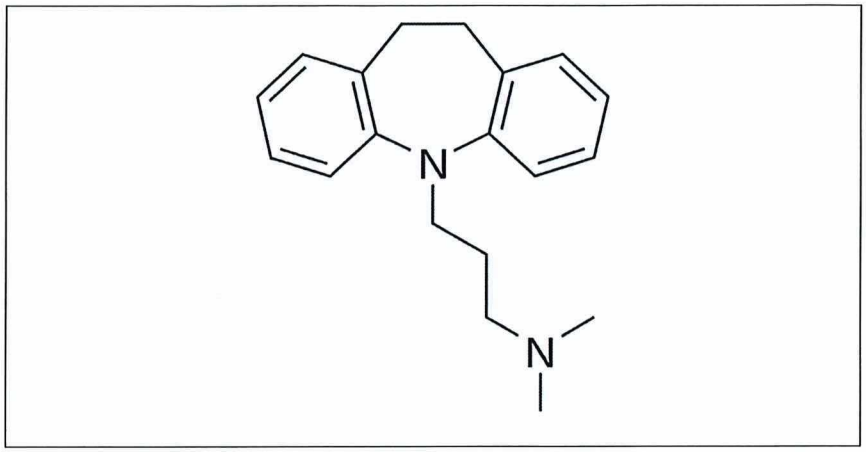

Figure 15: Imipramine structure.

Imipramine is mainly metabolized by the liver and it is converted to desipramine by CYP1A2, CYP3A4, CYP2C19 (Sallee and Pollock, 1990). Both imipramine and desipramine are hydroxylated by CYP2D6. Less than 5% of orally administered imipramine is excreted unchanged.

1.17.5. Rifamycin SV

Rifamycin is the product of fermentation from gram-positive bacterium known as *Streptomyces mediterranei*. The parent compound of rifamycin was rifamycin B which was originally obtained as a main product in the presence of diethylbarburitic acid. Rifamycin has several derivative products e.g. rifamycin SV, rifaximin, rifampin and rifamycin CV, all have slightly different physicochemical properties when compared to the parent structure. The structure of rifamycin SV is shown in Figure 16 below.

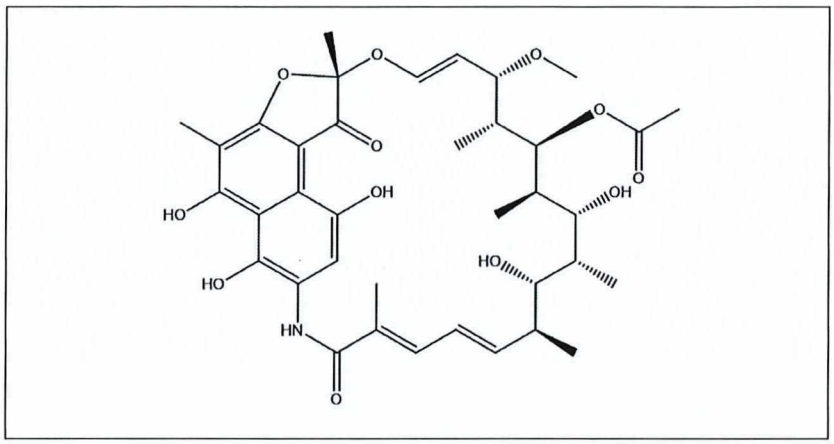

Figure 16: Rifamycin SV structure.

Rifamycin SV was the first antibiotic used intravenously for the treatment of tuberculosis (Sensi, 1983). Rifamycin, as well as all the other members of this group, present an antibacterial mechanism of action related to the inhibition of RNA synthesis.

1.17.6. Cyclosporin A

Cyclosporin A is a non-steroidal drug that has immunosuppressive and immunomodulatory properties, it is a lipophilic cyclic polypeptide formed by 11 amino acids. It is isolated from the fungus *Beauveria nivea* (Forsythe and Paterson, 2014), and was originally manufactured by Sandoz and approved for use by the FDA in 1983. The structure of cyclosporin A is shown in Figure 17.

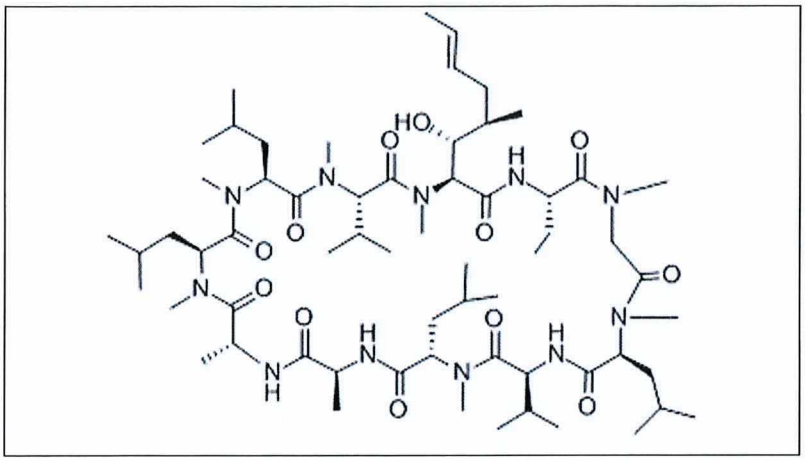

Figure 17: Cyclosporin A structure.

It is used in organ and bone marrow transplants and in the treatment of inflammatory conditions such as ulcerative colitis, rheumatoid arthritis, and atopic dermatitis.

1.17.7. Taurochenodeoxycholic acid

Taurochenodeoxycholic acid (TCDC) is a bile acid produced in the liver, by conjugation of chenodeoxycholic acid with taurine. It is secreted into bile and then into the intestine. As a bile acid it acts as detergent to emulsify lipids such as cholesterol in the bile, the structure of TCDC is shown in Figure 18.

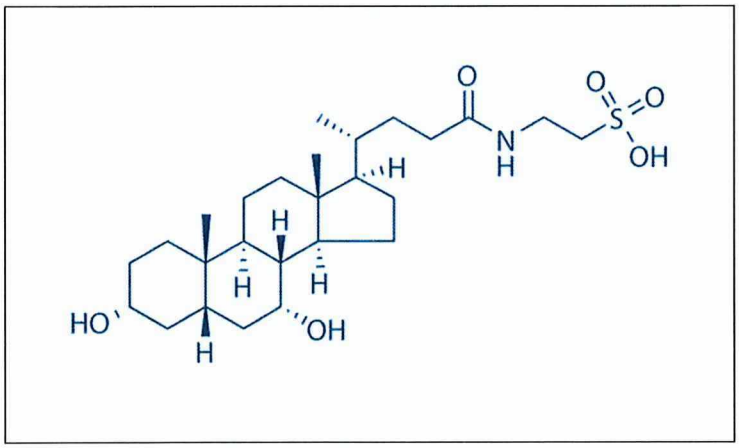

Figure 18: Taurochenodeoxycholic acid structure.

Taken as a medication, TCA reduces cholesterol formation in the liver, and is used as a choleretic to increase the volume of bile secretion from the liver and as a cholagogue to increase bile discharge into the duodenum.

2 PROJECT AIMS AND OBJECTIVES

The aims of this project are to assess and compare the uptake of three known organic anion transporter protein substrates into cryopreserved hepatocytes across different species (minipig, rat and human).

We hypothesise that minipig uptake transporters, in particular OATP, are representative of human hepatocytes. In order to test this hypothesis, the specific aims are to-

1. Confirm if the known hepatic uptake substrates EG, ES and rosuvastatin are substrates of minipig hepatic OATP transporters via a time dependent linearity assessment and comparing the data from rat and human cryopreserved hepatocytes. If the compounds appear to be substrates of minipig hepatic transporters, concentration dependent analysis will be carried out.

2. Determine the concentration dependent analysis of the substrates therefore enabling determination of kinetics to generate a Km and Vmax of all three compounds in all three species.

3. Assess the concentrations used in the concentration dependent analysis for cytotoxicity.

3 MATERIALS AND METHODS

3.1. Materials

3.1.1. Compounds

Probe substrates - Estradiol [6,7-3H(N)] 17β-D-glucuronide (3H-EG) (catalogue no ART 1320, calculated purity 98.12 to 98.50%) Estrone 3-sulphate [6,7-3H(N)] ammonium salt (3H-ES) (catalogue no ART 0821, calculated purity 98.31%) supplied by American Radiochemicals (ARC), β-Estradiol 17-(β-D-glucuronide) sodium salt (catalogue no E1127, assigned purity 99.6%), Estrone 3-sulfate potassium salt (catalogue no E9145, assigned purity 99%) supplied by Sigma Aldrich, Rosuvastatin Calcium CRS (lot no batch 2, catalogue no Y0001719, assigned purity 96.2%.) supplied by European Directorate for the quality of medicines & HealthCare (EDQM) (European Pharmacopeia).

Inhibitors - Rifamycin SV Sodium (catalogue no R508200, assigned purity 96%), supplied by Toronto Research Chemicals, Imipramine Hydrochloride (catalogue no 17379, assigned purity 100%) supplied by Sigma Aldrich.

3.1.2. Reagents

Dimethyl sulfoxide (DMSO) (catalogue no D/4125/PB08) supplied by Fisher Scientific, Dulbecco's Phosphate Buffered Saline (DPBS) with Ca2+, Mg2+ (catalogue no 14040-091) supplied by Gibco, Trypan Blue (catalogue no 93595) supplied by Sigma Aldrich, InVitroGRO CP rodent medium (catalogue no Z990028), InVitroGRO CP medium (catalogue no Z99029) supplied by BioIVT, Collagen I coated plates (catalogue no 354408) supplied by Corning, Scintlogic U (catalogue no SG-BXX-01, Flowlogic U (catalogue no SG-BXX-05) supplied by LabLogic, methanol (catalogue no M/4056/PB17) supplier Fisher Scientific, Cell maintenance cocktail A (catalogue no A13447), Acetonitrile (catalogue no 34851) supplied by Sigma Aldrich, FBS (catalogue no A13450) supplied by Gibco, CellTitre-Glo ® Luminescent Cell Viability Assay (catalogue no G7571).

3.1.3. Cells

All hepatocytes were cryoplatable, pooled and supplied by BioIVT. Gottingen Minipig male hepatocytes (3 donors, lot no's GBV, IKL, and RZX catalogue no M00615-P). LiverPool™ Human mixed gender hepatocytes (10 donors, lot no CYN and 10 donors lot no AMH, catalogue no X008001-P). Han Wistar Rat male hepatocytes (29 donors, lot no GEA, catalogue no M00065-P).

3.2. Methods

3.2.1. Radiochemical purity

The radiochemical purity of both 3H-EG and 3H-ES was determined prior to use via high pressure liquid chromatography (HPLC). Details shown in Appendix 1 and 2.

3.2.2. Preparation of Stocks Solutions for Time Dependent and Concentration dependent Uptake.

Stock solutions spanning 7 concentrations (0.1mM to 30mM) of rosuvastatin, estrone sulfate, and estradiol glucuronide were prepared in DMSO. Stocks of rifamycin SV sodium (50mM) and imipramine hydrochloride (50mM) were also prepared in DMSO. All stock solutions were used within one month of preparation and stored at -20°C.

3.2.3 Preparation of Working solutions for Time Dependent and Concentration dependent Uptake.

For time dependent uptake, rosuvastatin stock (1mM) was diluted to 1μM in DPBS whereas radiolabelled 3H-ES and 3H-EG (1mCi/mL) were diluted in DPBS to a concentration of 0.02μM.

For concentration dependent uptake, rosuvastatin stocks were diluted in DPBS to make working solutions of 0.3μM, 1μM, 2μM, 3μM, 5μM, 10μM, 30μM, 100μM. Working solutions of radiolabelled 3H-ES and 3H-EG were diluted in DPBS to prepare a concentration of 0.02μM, 0.1μM, 0.3μM, 1μM, 3μM, 5μM, 10μM, 30μM. Working solutions above 0.1μM of 3H-ES and 3H-EG required the addition of cold ES and EG mM stocks respectively.

All working solutions were prepared with and without inhibitor. The inhibitor stocks were added to make up a final concentration of 100μM rifamycin and 100μM imipramine. The solvent content was maintained at <1%. The specific concentration of the working solutions with and without inhibitor were determined via either liquid scintillation counting for 3H-ES and 3H-EG or LC/MS/MS for rosuvastatin.

Pre-incubation solutions for the time and concentration dependent experiments were prepared as above with and without inhibitor minus the substrate.
For the cell health assay, the pre-incubation solutions contained the respective substrate (rosuvastatin 0 μM and 100μM, 3H-ES and 3H-EG 0μM, 5μM,10μM and 30μM) minus the inhibitors. All working solutions and pre-incubation solutions were prepared on the day of the assay.

3.2.4 Rosuvastatin Analysis by LC-MS/MS

The concentration of rosuvastatin in cell lysates and working solutions was measured using Liquid Chromatography – Mass Spectrometry (LC-MS/MS). Two stock solutions of rosuvastatin (100μg/ml) in DMSO were prepared from independent weighing's. For the concentration dependent uptake exact concentrations of rosuvastatin were determined. Quality Control (QC) checks were prepared in DPBS and matrix on the day of assay, at 3ng/ml, 200ng/ml and 800ng/ml. On the day of analysis, a eight point calibration line (1-1000ng/ml) was prepared in DPBS or the appropriate matrix. The raw data was integrated using Applied Biosystems/MDS Sciex software Analyst v 1.6.1. which was used to calculate peak area ratios (weighted $1/x^2$ linear regression was applied to analyte/internal standard peak area ratios versus analyte concentration data) to construct the calibration curves from which the concentration of all the respective samples in the study were determined. Whereas, for the time dependent uptake, only the peak area ratios were calculated (Appendix 2 Table 1).

3.3. *In Vitro* Methods

3.3.1 Hepatocyte culture

Vials of the cryopreserved hepatocytes were thawed rapidly in a 37^{0}C water bath before being decanted into pre-warmed medium. InVitroGRO CP medium was used for human and minipig hepatocytes, whereas InVitroGRO CP Rodent medium was used for the rat hepatocytes. The hepatocytes were re-suspended by gently inverting the tube several times. For all species, a small aliquot (25µL) was removed to determine post thaw viability via the trypan blue exclusion assay. Using the respective medium, the hepatocytes were diluted to a cell density of $0.7x10^6$ viable cells/mL. The cell suspension (500 µL/well) was seeded in 24 well collagen coated plates. Plates were gently agitated to spread the cells evenly before being incubated at 37^{0}C in a humidified incubator with 5% CO_2 for approximately 4 hours to allow the hepatocytes to attach. Cells were checked using a light microscope prior to use.

3.3.2 Uptake experiments

Monolayers of hepatocytes were washed twice with 1mL of warm DPBS, before being incubated with 1mL of pre-incubation solution for 30 minutes at 37^{0}C. The pre-incubation solution was removed and 400µL of working solution containing the substrate was added. The hepatocytes were incubated with the substrate at 37^{0}C for a set amount of time and or concentration to determine time linearity or concentration dependence of the compound. Uptake was terminated via removal of the working solutions followed by the addition of ice-cold DPBS. The cells were then washed a further 2 times with ice-cold DPBS. To assess the amount of passive diffusion, the cells were incubated using the same conditions as above, however the pre-incubation and working solutions contained the cocktail of inhibitors, rifamycin and imipramine. All conditions were assessed in triplicates. To lyse the cells treated with rousuvastatin 200µL millipore water was added to each well and the plates were stored in the freezer (-20°C) until analysis. The uptake of rosuvastatin was determined by using LC/MS/MS. Plates treated with 3H-ES and 3H-EG 400µL of 1% triton X-100 was added to each well to lyse the cells. The plates are incubated with triton at 37^{0}C for 30

mins, 100µL of the lysate was added to a topcount microplate which was left overnight to dry prior to determining the amount of radioactivity via microplate scintillation counter (TopCount, PerkinElmer).

3.3.3 Time dependent uptake

To determine the time dependent uptake, experiments were performed as above, with a set concentration of substrate (1µM for rosuvastatin, 0.02µM for 3H-ES and 3H-EG) incubated with the hepatocytes for 0.5, 1, 2, 5 and 10 minutes. This was done for each substrate and species combination.

For the time dependent uptake of rosuvastatin into minipig hepatocytes, an additional experiment was run with plates at 37^{0}C and 4^{0}C. Both plates were run without inhibitors present, with the plate at 4^{0}C being used to assess passive diffusion.

3.3.4 Concentration dependent uptake

To determine concentration dependent uptake, hepatocytes were incubated, with a range of substrate concentrations at a time determined from the time dependent uptake experiments. The optimum timepoint for each substrate and species combination was determined from the linear portion of the time dependent experiments.

Concentrations of 0.3µM, 1µM, 2µM, 3µM, 5µM, 10µM, 30µM, 100µM were used for rosuvastatin. Concentrations of 0.02µM, 0.1µM, 0.3µM, 1µM, 3µM, 5µM, 10µM, 30µM were used for ES and EG.
Vmax and Km were determined via Michaelis-Menten simulation using GraphPad Prism version 8.1.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Cellular Intrinsic clearance was calculated using the equation:

Equation 1:

$$Intrinsic\ clearance\ (ul/min/million\ cells) = \frac{Vmax}{Km}$$

The cellular intrinsic clearances were scaled up, using standardised species-specific scaling factors (Appendix 2) and equations 2 and 3.

Equation 2:

$$Intrinsic\ clearance\ (ml/min/g\ tissue) = \frac{ul/min/\ million\ cells}{1000}\ x\ no.million\ cells/g.tissue$$

Equation 3:

$$Intrinsic\ clearance\ (ml/min/kg\ body\ weight) = ml/min/\ g\ tissue\ x\ g.of\ tissue/kg\ bodyweight$$

3.3.5 CellTiter-Glo® Luminescent Cell Viability Assay

To determine the integrity of the cells, Adenosine Triphosphate (ATP) was quantified using a Promega CellTitre-Glo Luminescent Cell Viability assay. Relative light units (RLU), indicative of metabolically active cells was recorded using a Fluostar Omega luminometer. To evaluate cell viability, hepatocytes were plated as per uptake experiment and treated with 0.4 mL of preincubated transport medium without the substrate (negative control) and also with the following target concentrations of Rosuvastatin (5, 10, 30 and 100μM), EG and ES (5, 10, and 30μM) for 15 minutes at 37°C and then 15 minutes at room temperature. Following the incubation period 300μL of the pre-incubation solution was removed from each well and 300μL of the reconstituted cellTiter-Glo® reagent added. The contents of the plates were mixed gently on a gyro rocker for approximately 10 minutes and 100μL of the contents transferred onto a 96 well luminescence plate. The Luminescence plate was left to stand for 10 minutes before being read on the luminometer (FluoStar, BMG LABTECH).

3.3.6 Rosuvastatin Analysis by LC-MS/MS

The concentration of rosuvastatin in cell lysates and working solutions was measured using Liquid Chromatography – Mass Spectrometry (LC-MS/MS). Two stock solutions of rosuvastatin (100µg/ml) in DMSO were prepared from independent weighings. For the concentration dependent uptake exact concentrations of rosuvastatin were determined. Quality Control (QC) checks were prepared in DPBS and matrix on the day of assay, at 3ng/ml, 200ng/ml and 800ng/ml. On the day of analysis, a calibration line, 1-1000ng/ml, was prepared in DPBS and appropriate matrix.

Samples were diluted using a solution of acetonitrile/methanol/formic (95/5/0.1v/v/v) containing stable isotopically labelled GSK123 compound (20 ng/mL GSK123) as the analytical internal standard (IS). Mass spectrometry parameters were optimised for both rosuvastatin and the internal standard before samples were run. For each sample, 50µl of lysate was diluted in acetonitrile containing the (IS), before being mixed thoroughly and centrifuged at approximately 4000g for 10 minutes.

Rosuvastatin samples were analysed by negative ion turbo ionspray via MS/MS with the Applied Biosystems/MDS Sciex API 5000 (Appendix 2 Table 1). The raw data was integrated using Applied Biosystems/MDS Sciex software Analyst v 1.6.1. which was used to calculate peak area ratios (weighted 1/x2 linear regression was applied to analyte/internal standard peak area ratios versus analyte concentration data) to construct the calibration curves from which the concentration of all the respective samples in the study were determined.

4 RESULTS

4.1. Radiochemical purity

The radiochemical purity was determined to be greater than 97% for both 3H-ES and 3H-EG. No single impurity was greater than 1% for 3H-EG. However, for 3H-ES there was a single impurity of 1.68% (data not shown) it was however deemed that the material was fit for purpose and therefore used in the experiments.

4.2. Cell health

There was no significant depletion observed in hepatocyte viability (>20%) for ES or EG treatments across all three species of hepatocytes. Rat and human hepatocytes treated with rosuvastatin demonstrated no significant depletion in cell viability. However, minipig hepatocytes showed a depletion of 23.3% at the highest concentration of rosuvastatin administered, therefore this concentration was excluded from any concentration dependent analysis. Appendix 3 Table 1.

4.3. Time dependent uptake

The time dependent uptake of all three substrates was observed across all three species to various degrees. The presence of the inhibitor cocktail (100μM Rifamycin and 100μM Imipramine) was to observe the passive uptake of the substrates and determine if uptake transporters play a role in the uptake of the three substrates and to what degree. Interspecies differences were observed for all probe substrates, with variability in the fold change between active and passive uptake being observed (Table 8). EG uptake into rat hepatocytes gave a fold difference of 31.4, whereas minipig and human showed a fold difference of 5.0 and 4.1 respectively. Human hepatocytes treated with ES showed a 12.9 fold difference between the inhibited and unhibited treatment followed by rat and minipig hepatocytes with fold changes of 5.8 and 2.8 respectively. Minipig and human hepatocytes showed similar fold changes for the rosuvastatin treatment 2.3 and 2.7 whereas rat hepatocytes had a fold change of 24.1 between the inhibited and uninhibited treatment.

The optimum time point for the concentration dependent experiments to determine the kinetics of the substrate is selected from the linear section of the time dependent graphs (figures 19, 20 and 21). For both ES and EG a time point of 2 minutes was selected across all species. However, for rosuvastatin the optimum time point was 1 minute for both rat and minipig, whereas 5 minutes was chosen for human.

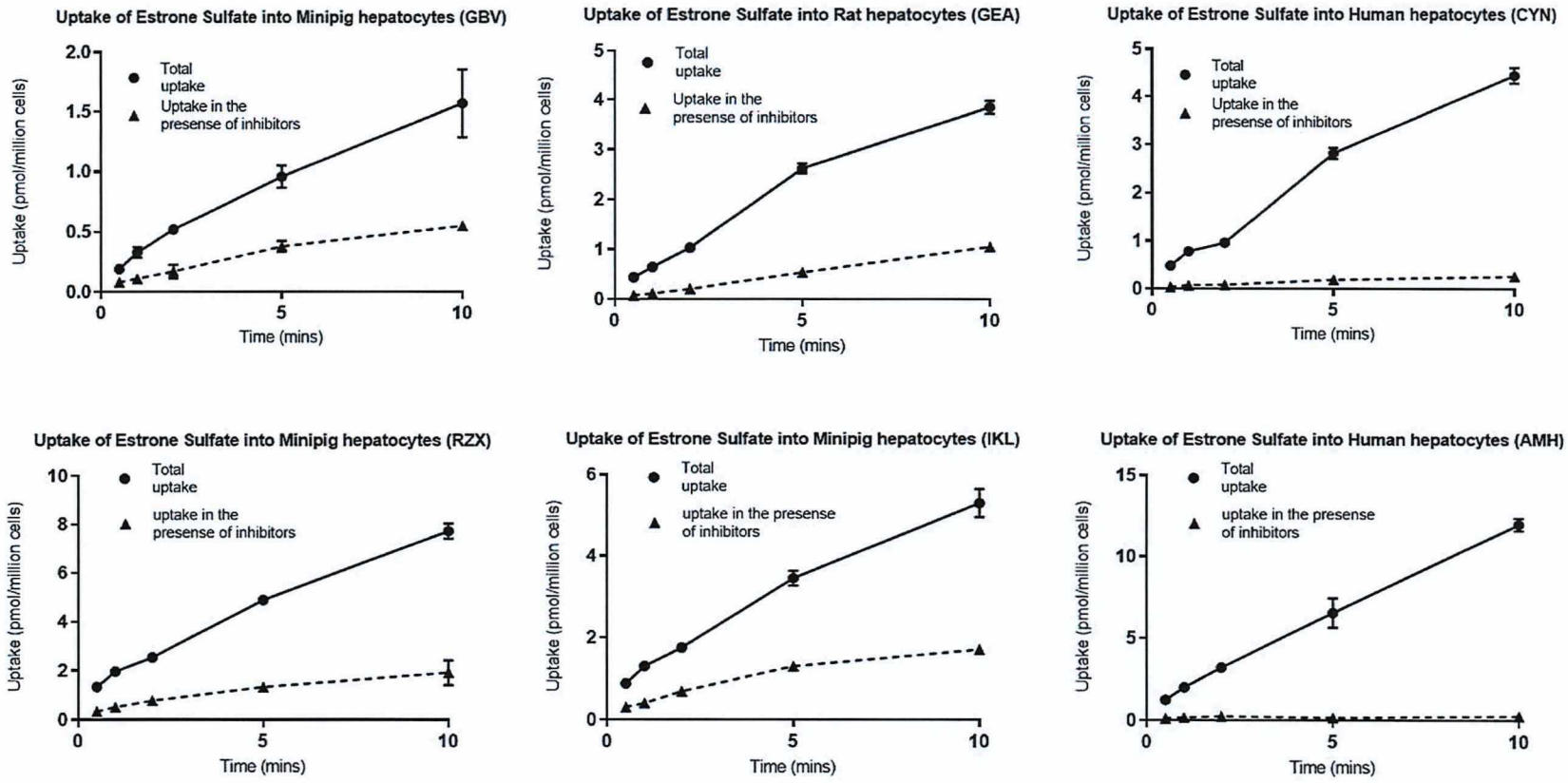

Figure 19: The time dependent uptake of estrone sulfate into cryopreserved minipig lot GBV, rat lot GEA and human lot CYN cryopreserved hepatocytes. Each value represents mean ± SD (n=3).

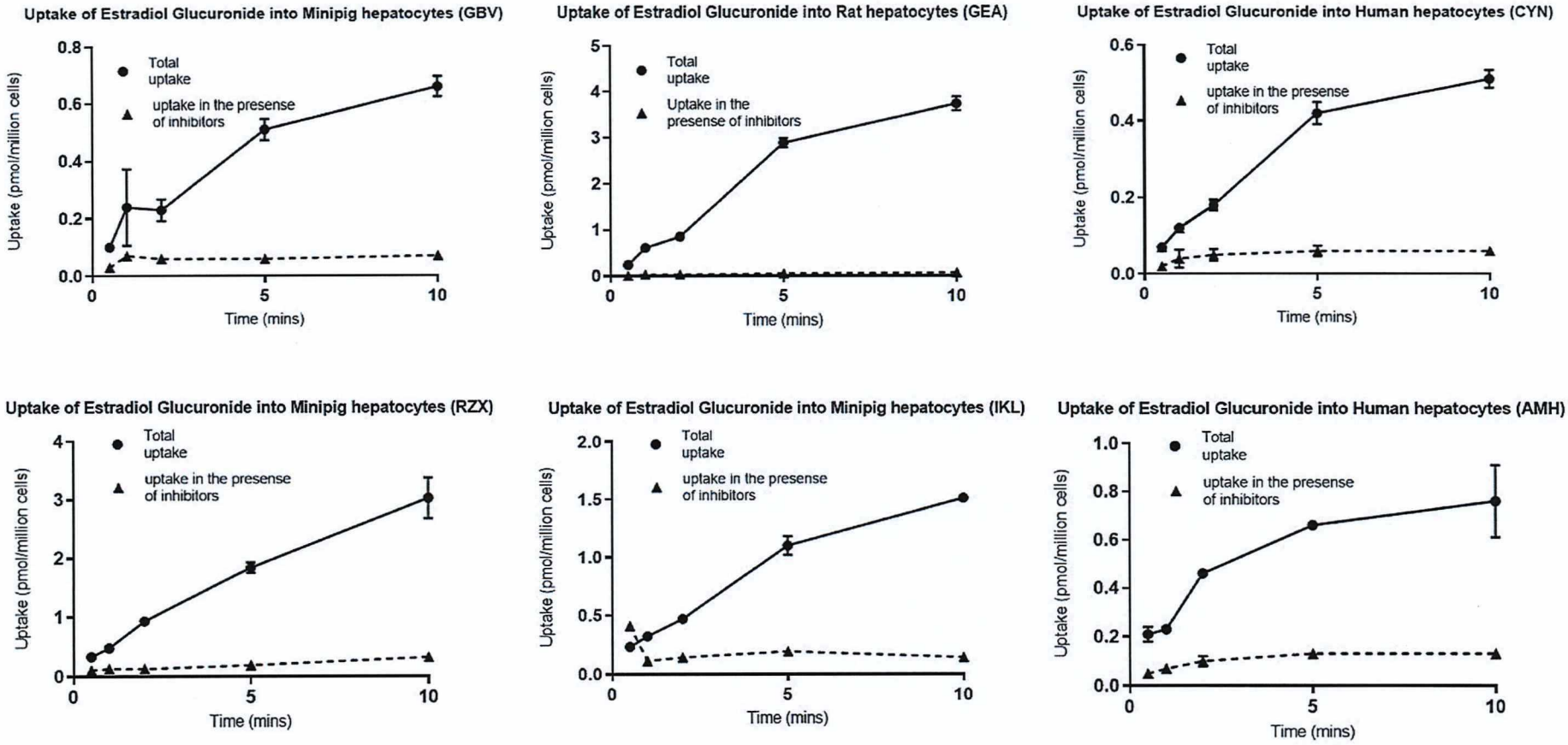

Figure 20: The time dependent uptake of estradiol glucuronide) into cryopreserved minipig lot GBV, RZX and IKL, rat lot GEA, human lot CYN and AMH cryopreserved hepatocytes. Each value represents mean ± SD (n=3).

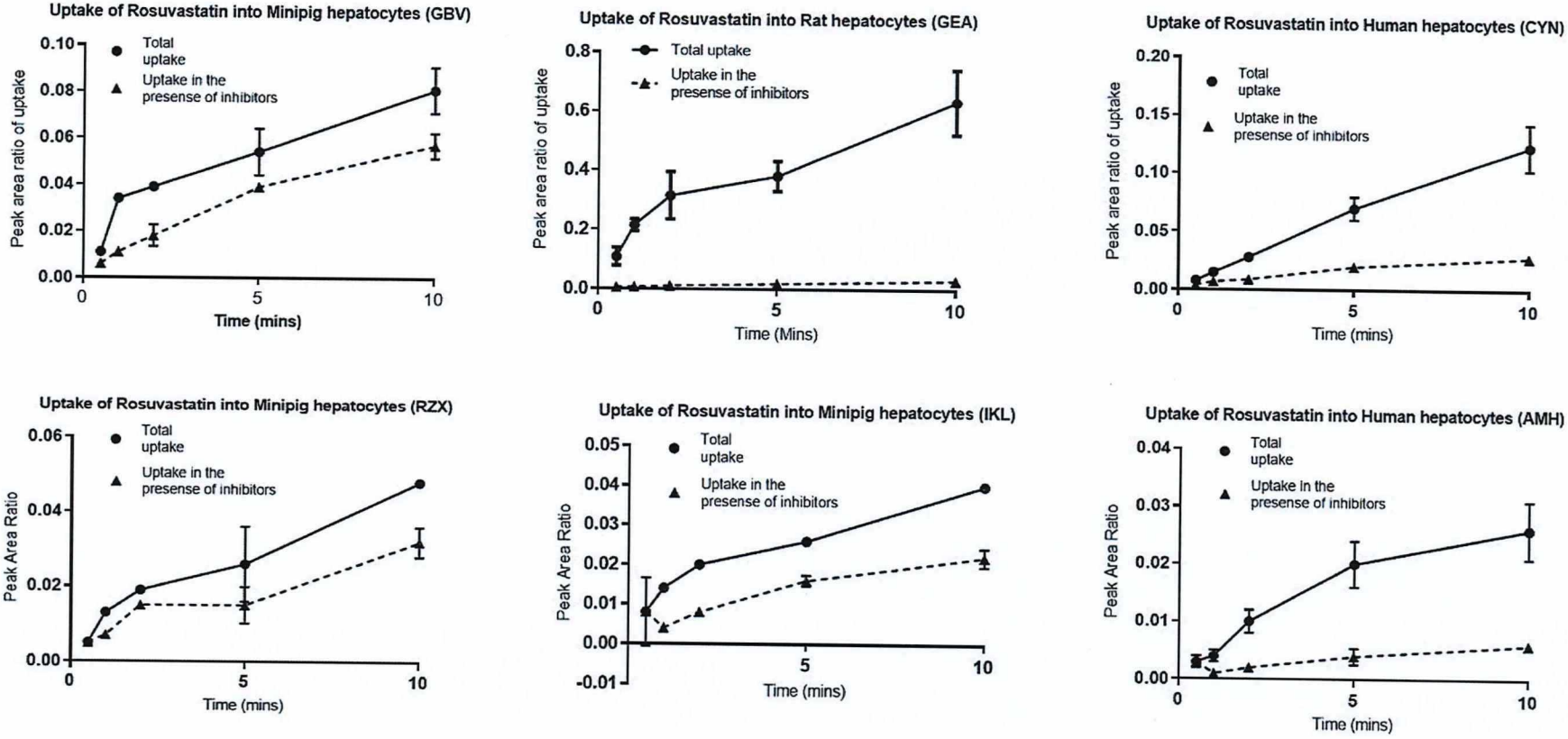

Figure 21: The time dependent uptake of rosuvastatin into cryopreserved minipig lot GBV, RZX and IKL, rat lot GEA, human lot CYN and AMH cryopreserved hepatocytes. Each value represents mean ± SD (n=3). Note for rosuvastatin peak area ratio of uptake is plotted.

Table 8: The fold difference between total and passive uptake of OATP probe substrates into Rat, Minipig and Human cryopreserved hepatocytes. Fold difference is the average difference between passive and total uptake for all time points in the linear portion of the time dependent uptake.

Substrate	Species	Fold difference
Estrone Sulfate	Rat	5.8
	Minipig (GBV)	2.8
	Minipig (RZX)	3.8
	Minipig (IKL)	2.9
	Human (CYN)	14
	Human (AMH)	25
Estradiol Glucuronide	Rat	31
	Minipig (GBV)	5.0
	Minipig (RZX)	6.6
	Minipig (IKL)	4.7
	Human (CYN)	4.1
	Human (AMH)	4.8
Rosuvastatin	Rat	24
	Minipig (GBV)	2.3
	Minipig (RZX)	1.5
	Minipig (IKL)	2.1
	Human (CYN)	3.0
	Human (AMH)	3.2

4.4. Temperature Dependent Uptake of Rosuvastatin

The time dependent plot of rosuvastatin uptake in minipig hepatocytes shows there is a passive element over the 10 minute incubation, potentially suggesting that another transporter is playing a role in the uptake of rosuvastatin in minipig hepatocytes. Rifamycin inhibits OATPs and imipramine inhibits OCT1, therefore to assess if another transporter was involved in rosuvastatin uptake in minipig hepatocytes, a time dependent experiment was carried out at 37°C and 4°C (ice). Lowering the

temperature will reduce the activity of all hepatic uptake transporters. The mean fold difference between 37°C and 4°C in the linear portion of the graph was 5.4-fold. The level of total uptake was comparable across both time dependent assays. However, the level of uptake at 4°C was lower than the level of uptake at 37°C in the presence of inhibitor cocktail (Figure 22).

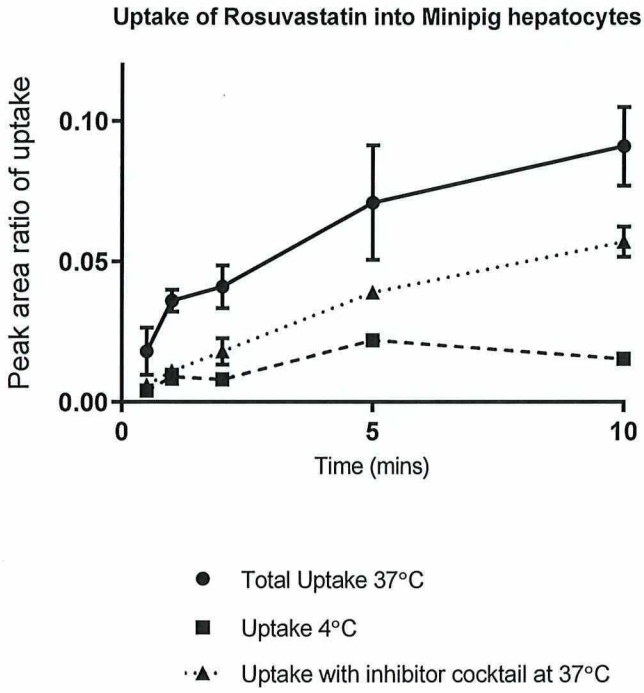

Figure 22: Temperature dependent uptake of rosuvastatin into minipig lot GBV cryopreserved hepatocytes. Uptake at 37°C was average across the two experiments with ±SD (n=6). Uptake at 4°C and with inhibitor are mean ± SD (n=3), from the respective experiments.

Although uptake transporters have been inhibited by the use of the cocktail of inhibitors, the level of inhibition at 4°C is far greater.

4.5. Concentration dependent uptake

The concentration dependent active uptake of ES, EG and rosuvastatin for all species-substrate combinations is shown in Figures 23, 24, 25 and 26. Active uptake was calculated for each concentration by subtracting the passive diffusion (inhibited uptake) from the total (non-inhibited) uptake. From these results the Vmax, Km and intrinsic clearance were determined in accordance to the methods, shown in Table 9. Kinetic parameters for rosuvastatin concentration dependence could not be determined for minipig as the data did not fit the Michaelis Menten equation. Rat hepatocytes

showed the highest uptake rate for all three substrates (Figures 23, 24 and 25). The ES uptake rate (Vmax) was the lower in all three minipig hepatocytes compared to rat, but only one minipig hepatocyte batch (RZX) was observed to be higher in its uptake rate compared to human. Minipig batches GBV and IKL were similar in their uptake rates to the human hepatocyte batches CYN and AMH (Figure 23). The Km is an indication of the affinity of the substrate to the transporter, therefore a low Km value is an indication that the substrate has a high affinity to the transporter/s. A low Km also means less substrate is required to reach Vmax. Minipig hepatocyte batch GBV and human hepatocyte batch AMH show the greatest affinity with Km values of 0.78 and 0.7. When both tissue cellularity and body weight are accounted for, the minipig clearance is very similar to human (Table 9). A similar pattern is demonstrated for the uptake of EG, where minipig hepatocytes batch GBV and human batch AMH have the lowest Km values. When both tissue cellularity and body weight are accounted for, the minipig clearance for ES and EG is very similar to human than rat (Table 9).

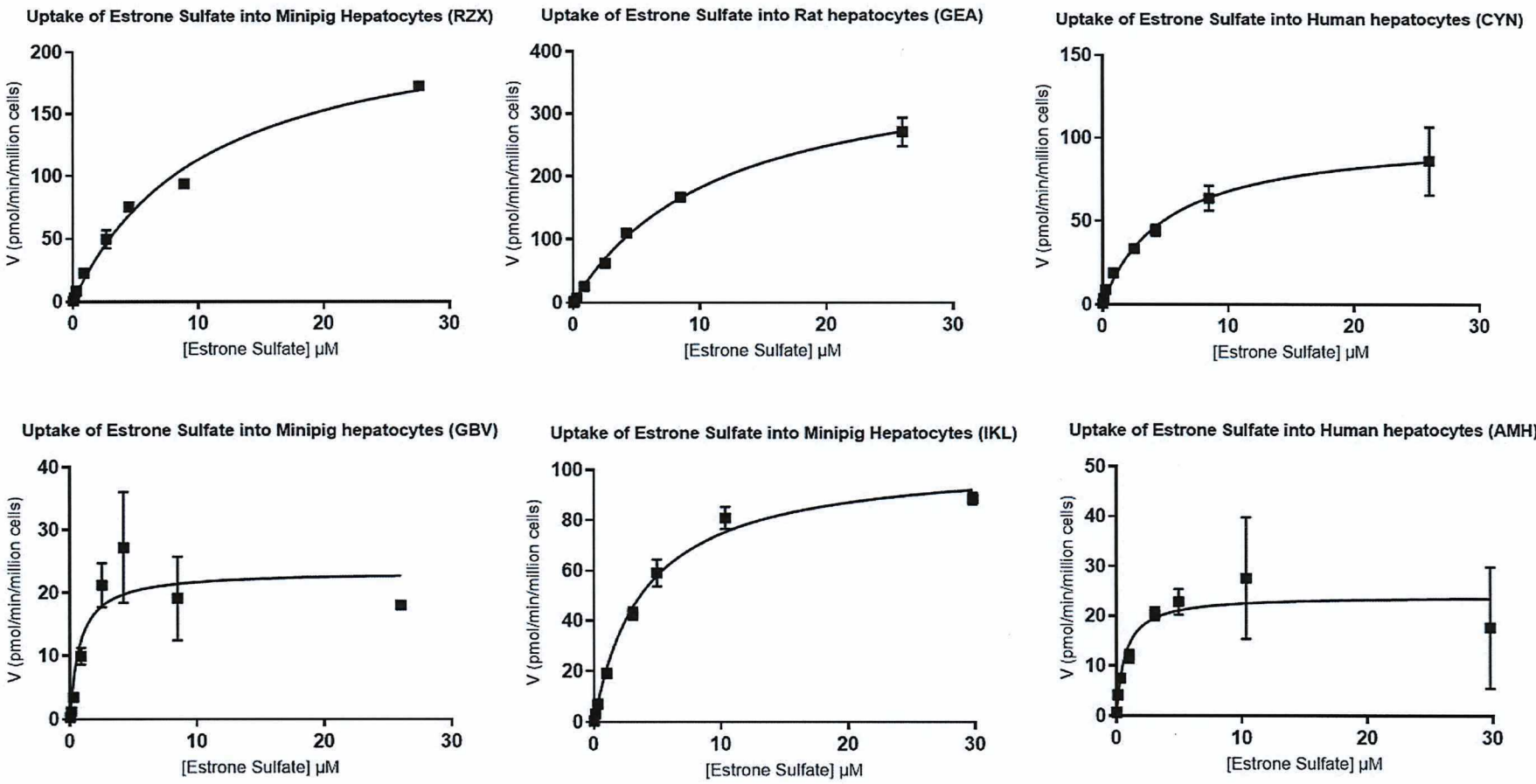

Figure 23: Concentration-dependent active uptake of estrone Sulfate into minipig lots GBV, RZX and IKL, rat lot GEA and human lots CYN and AMH cryopreserved hepatocytes. Active uptake was determined by subtraction the passive from the total uptake. Each value represents mean ± SD (n=3), excluding 30µM estrone sulfate in minipig where n=2, standard deviation was not calculated. The 100µM

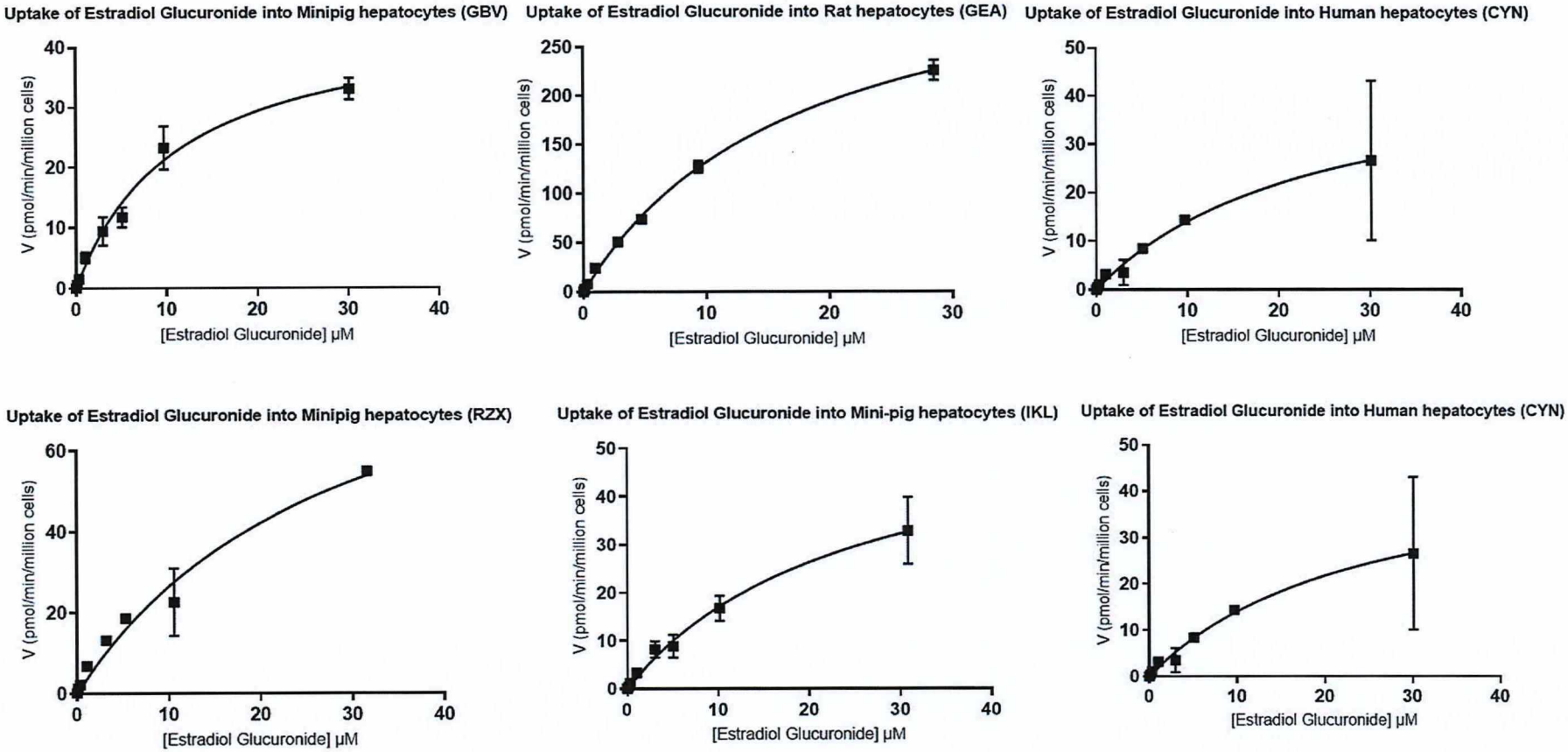

Figure 24: Concentration-dependent active uptake of estradiol glucuronide into minipig lots GBV, RZX and IKL, rat lot GEA and human lots CYN and AMH cryopreserved hepatocytes. Active uptake was determined by subtraction of the passive uptake from the total uptake.

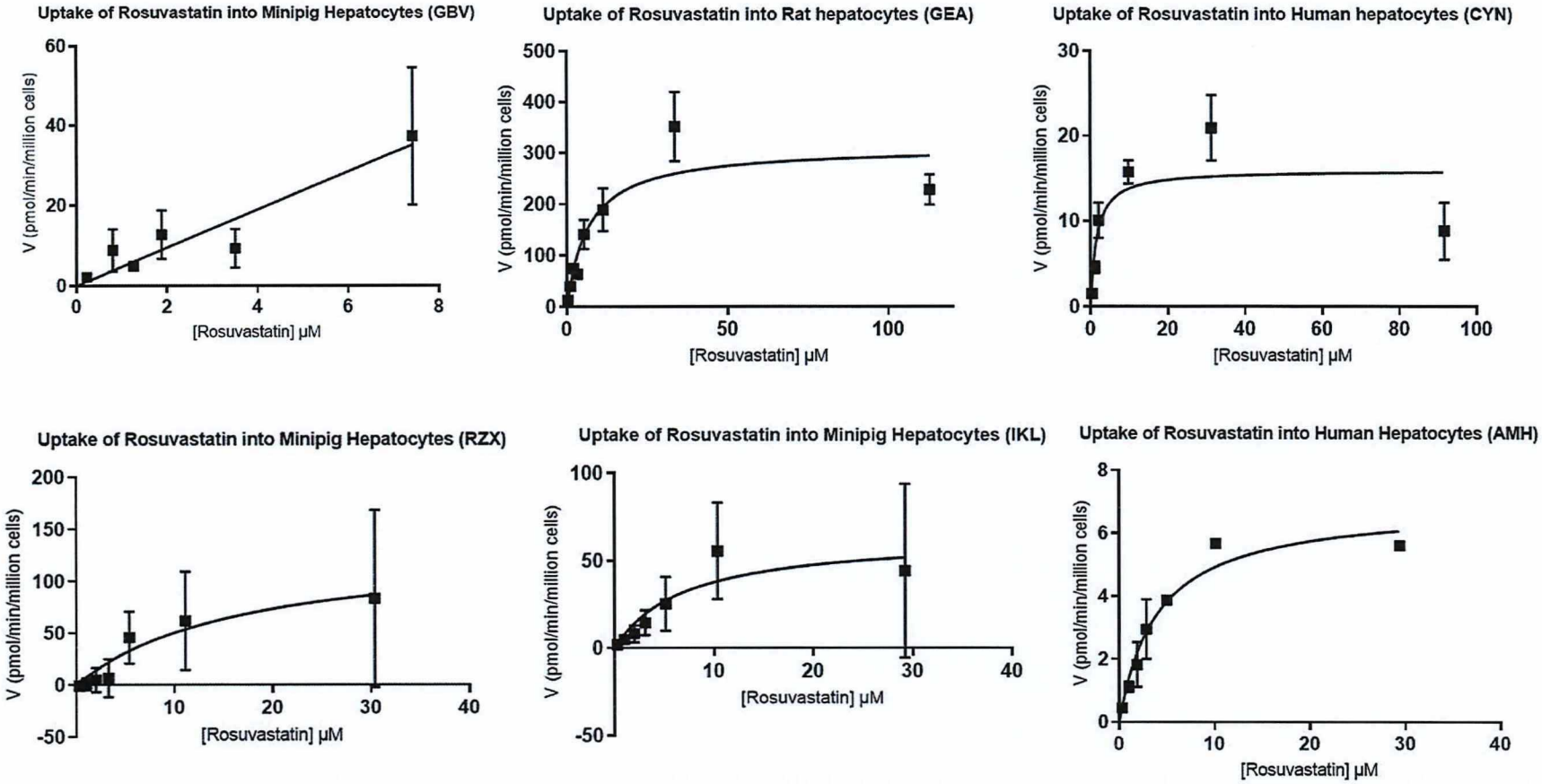

Figure 25: Concentration-dependent active uptake of rosuvastatin into minipig lots GBV, RZX and IKL, rat lot GEA and human lots CYN and AMH cryopreserved hepatocytes. Active uptake was determined by subtraction of the passive uptake from the total uptake Due to cell health minipig hepatocytes (GBV) were only tested up to 7µM, saturation was not observed therefore kinetic parameters could not be determined.

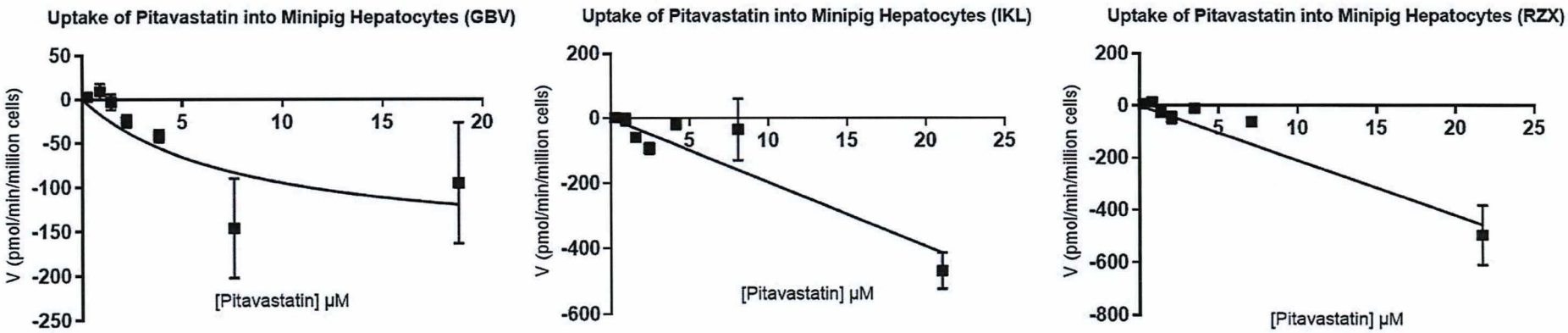

Figure 26: Concentration-dependent active uptake of Pitavastatin in three minipig hepatocytes batches lots GBV, IKL and RZX.

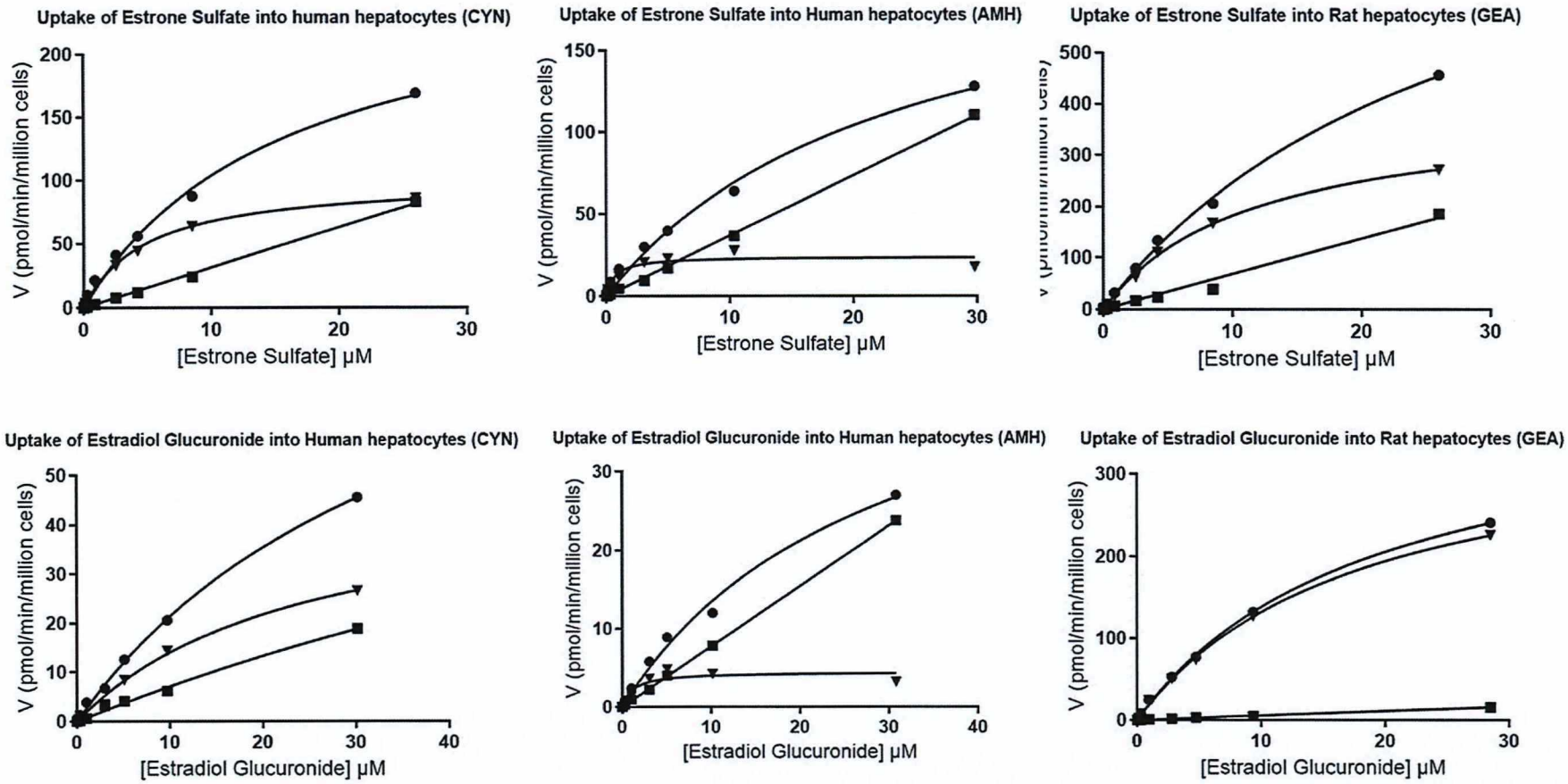

Figure 27: Concentration-dependent uptake of ES and EG in human and rat hepatocytes. Key ● Total Uptake, ■ Passive uptake, ▼ Active uptake.

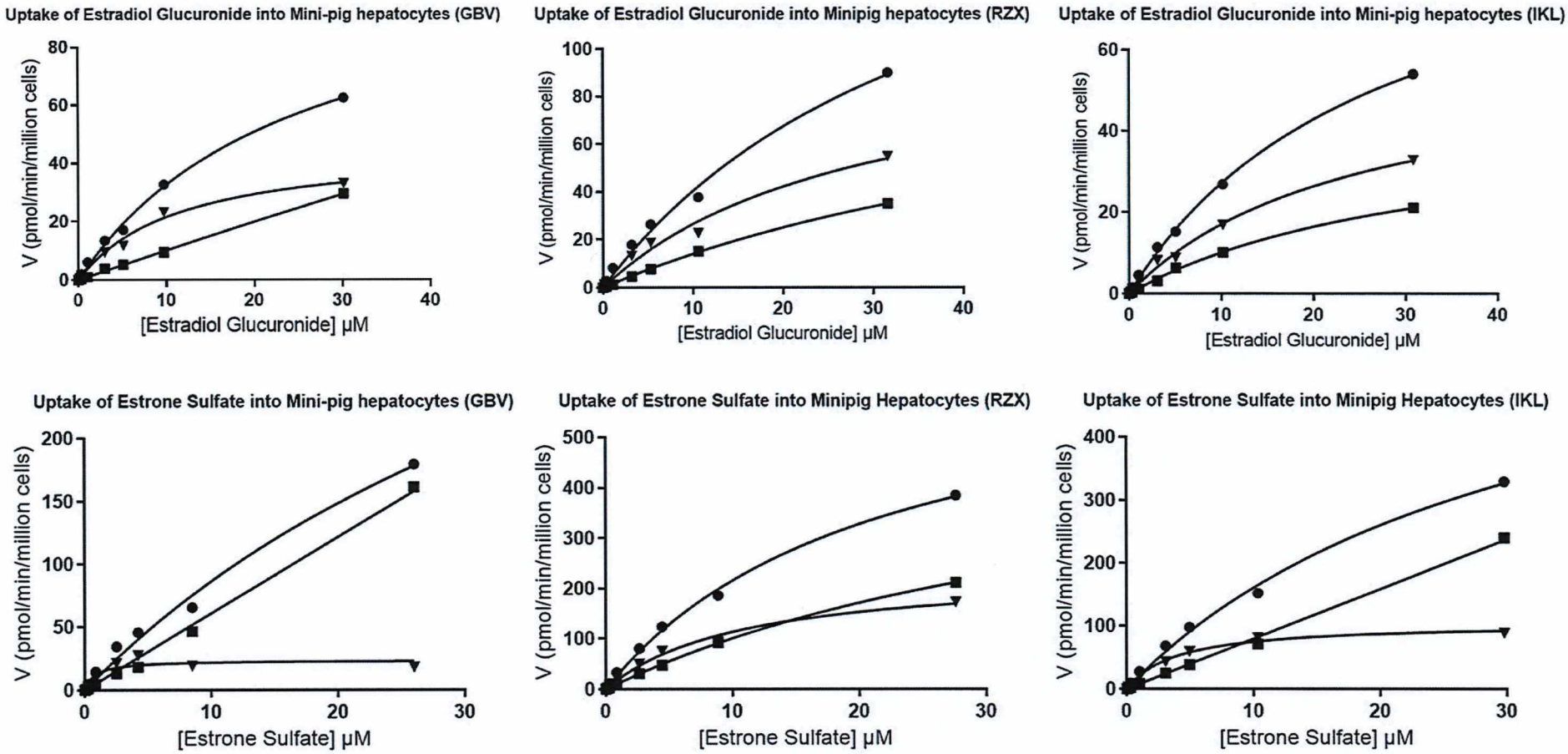

Figure 28: Concentration-dependent uptake of ES and EG in minipig hepatocytes. Key ●Total Uptake, ■Passive uptake, ▼Active uptake.

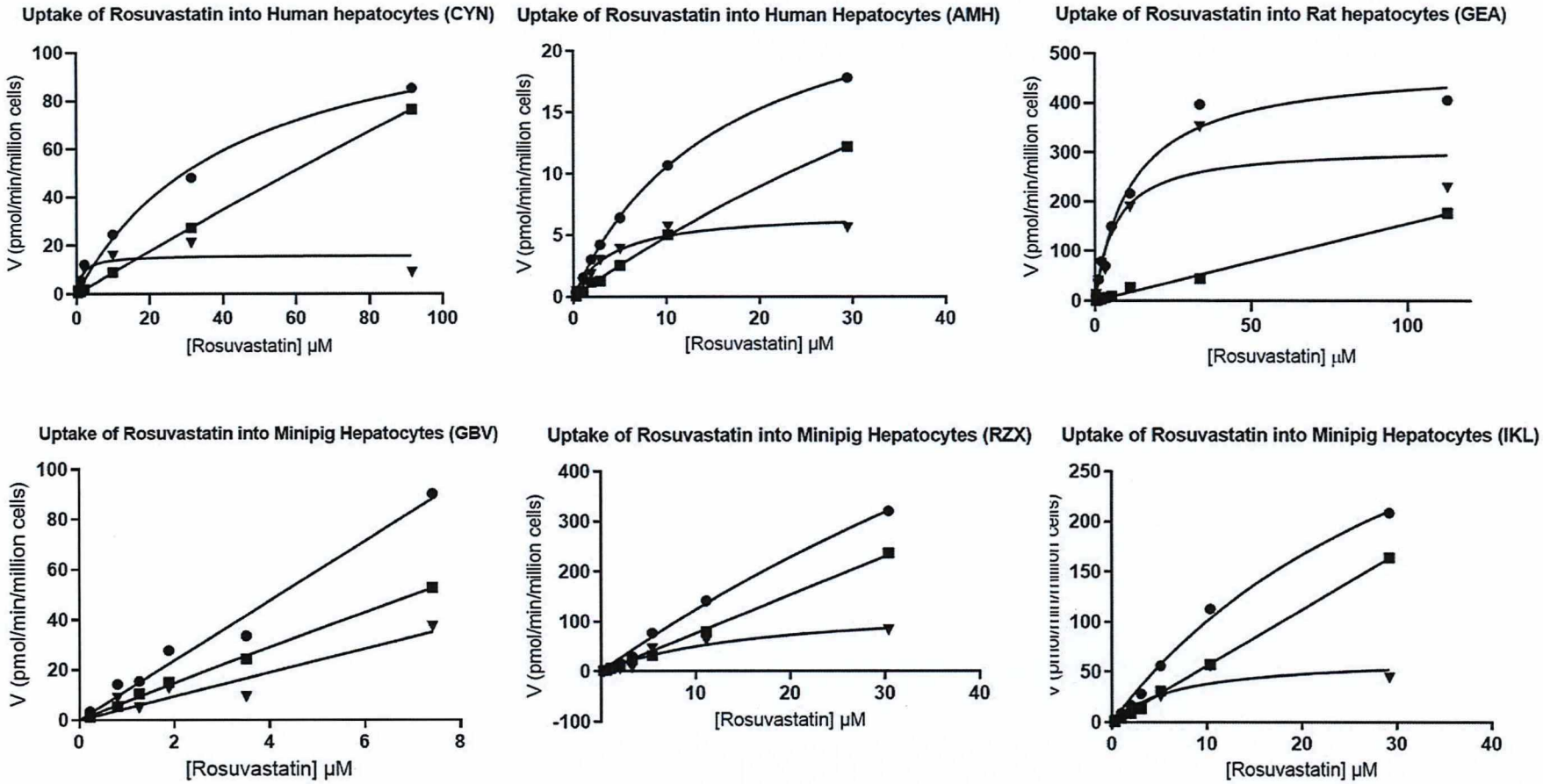

Figure 29: Concentration-dependent uptake of rosuvastatin in human, rat and minipig hepatocytes. . Due to cell health minipig hepatocytes (GBV) were only tested up to 7µM, saturation was not observed therefore kinetic parameters could not be determined. Key ● Total Uptake, ■ Passive uptake, ▼ Active uptake.

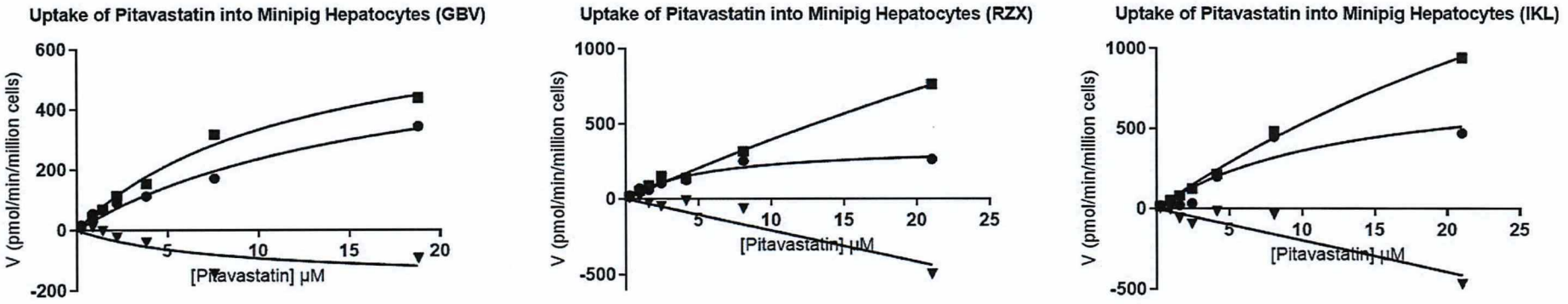

Figure 30: Concentration-dependent uptake of pitavastatin in minipig hepatocytes. Key ●Total Uptake, ■Passive uptake, ▼ Active uptake.

Table 9: Kinetics parameters of the uptake of known OATP1B1/OATP1B3 substrates into cryopreserved Rat, Minipig and Human hepatocytes.

Substrate	Species (lot no)	Km (µM)	Vmax (pmol/min/million cells)	Intrinsic clearance (µl/min/million cells)	Intrinsic clearance (mL/min/gtissue)	Intrinsic clearance (ml/min/Kg)
Estrone Sulfate	Rat (GEA)	11.68	393.90	33.72	3.64	131.12
	Minipig GBV	0.78	23.39	29.99	3.60	60.09
	Minipig RZX	10.76	235.00	21.84	2.62	43.77
	Minipig IKL	3.99	104.30	26.14	3.14	52.39
	Human CYN	5.01	101.70	20.30	2.39	58.44
	Human AMH	0.7	24.04	34.34	4.04	98.86
Estradiol Glucuronide	Rat (GEA)	17.86	367.40	20.57	2.22	79.98
	Minipig GBV	11.41	46.07	4.04	0.48	8.09
	Minipig RZX	29.31	104.00	3.55	0.43	7.11
	Minipig IKL	24.65	58.80	2.39	0.29	4.78
	Human CYN	24.51	48.44	1.98	0.23	5.69
	Human AMH	1.218	4.459	3.66	0.43	10.54
Rosuvastatin	Rat (GEA)	6.80	312.00	45.88	4.96	178.4
	Minipig GBV	ND	ND	NA	NA	NA
	Minipig RZX	16.69	134.5	8.06	0.97	16.15
	Minipig IKL	7.012	64.42	9.19	1.10	18.41
	Human CYN	1.58	15.98	10.11	1.19	29.12
	Human AMH	4.01	6.8	1.70	0.20	4.88
Pitavastatin	Minipig GBV	ND	ND	NA	NA	NA
	Minipig RZX	ND	ND	NA	NA	NA
	Minipig IKL	ND	ND	NA	NA	NA

ND = Not Determined, NA = Not Applicable

Note: Due to the high passive uptake kinetic parameters (Km and Vmax) could not be determined for Minipig hepatocyte batch GBV treated with rosuvastatin and all minipig hepatocyte batches treated with pitavastatin. Therefore clearance values could not be calculated.

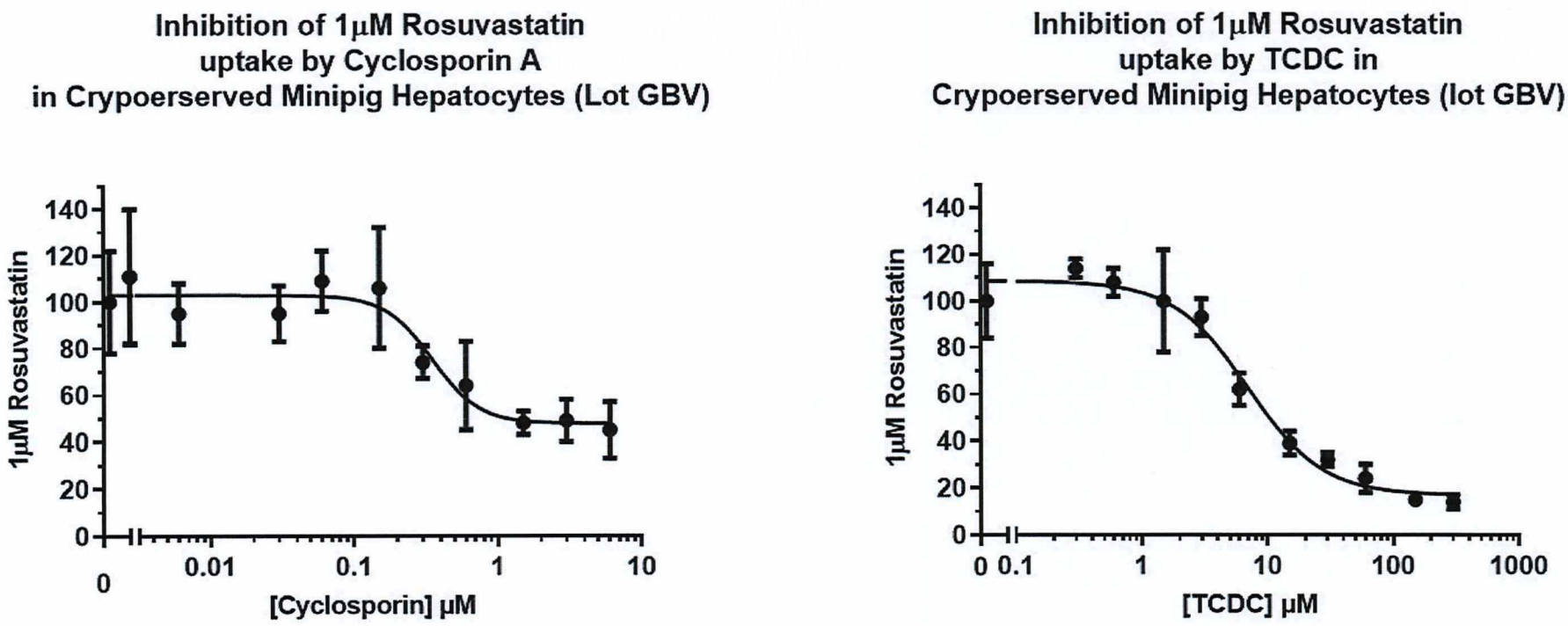

Figure 31: Inhibition of Rosuvastatin uptake by Cyclosporin A (IC50 0.35μM) and TCDC (IC50 7.0μM) in minipig hepatocytes.

5 DISCUSSION

The experimental studies described within this thesis were used to identify whether minipig hepatic uptake transporters, in particular OATPs, are representative of human hepatic uptake transporters. As hepatic transporters are involved in the clearance of xenobiotics from the body, which can be a rate limiting step. This characterisation will improve the prediction of human pharmacokinetics from minipig *in vitro* and *in vivo* experiments.

The functionality of the minipig hepatic uptake transporters and interspecies differences were determined. *In vitro* OATP uptake experiments (time-dependent and concentration dependent) were successfully completed using resuscitated cryo-preserved rat, human and minipig hepatocytes. This was conducted using known hepatic OATP probe substrate compounds estrone sulfate (ES), estradiol glucuronide (EG), rosuvastatin and pitavastatin. Total and passive uptake were compared for each species substrate combination in both a time dependent and concentration dependent manner. Passive uptake was assessed via the addition of a cocktail of inhibitors (rifamycin SV and imipramine). Where possible the active transport of each probe substrate was determined.

The overall results show all three species have an affinity for three probe substrates EG, ES and rosuvastatin (Figures 19, 20 and 21). Furthermore, rat hepatocytes showed the highest intrinsic clearance for all three probe substrates (Table 9).

Rosuvastatin uptake was lower in minipig and human hepatocytes in comparison to ES or EG (Figure 19, 20 and 21). One batch of minipig hepatocytes (batch RZX) treated with rosuvastatin did not meet the criteria set by the FDA (Food and Drug Administration, 2017), whereby a decrease in uptake of at least 2-fold in the presence of a cocktail of inhibitors (rifamycin SV and imipramine) should be observed (Table 8), but met the criteria for the other two probe substrates.

The uptake of all three probe substrates has previously been well characterised for rat and human hepatocytes (Niemi *et al.*, 2011, Liao *et al.*, 2019, Badolo *et al.*, 2011, Shitara *et al.*, 2003a, Hassen *et al.*, 1996, Han *et al.*, 2010). It is observed in published data the affinity of hepatic hepatic uptake transporters for Published literature data rosuvastatin and EG especially in the rat and human. This is also observed in GSK

internally derived data, produced independently of this project (unpublished data) (Table 10) and data from this study for human and rat hepatocytes.

Table 10: Fold change values of rosuvastatin GSK unpublished data compared to project data. (*) Average of 3 independent fold change values from study.

Compound	Source Data	Rat	Human
Rosuvastatin	GSK unpublished data (a)	37	8.1
	GSK unpublished data (b)	50.2	4.28
	Project data	24	3 (batch CYN)
			3.2 (batch AMH)
Estradiol Glucornide	GSK unpublished data (a)	53	24
	GSK unpublished data (c)	18.3*	3.5
	Project data	31	4.1 (batch CYN)
			4.8 (batch AMH)

The minipig data for rosuvastatin is further supported by another GSK study which was used to determine the uptake of a new chemical entity (NCE) in rat, human and minipig hepatocytes (batch IKL) to assess the IVIVC of the NCE using rosuvastatin a clinically know OATP probe substrate as a control. The minipig hepatocyte data were determined to be inconclusive in this unpublished GSK data as the fold change of the control rosuvastatin was reported to be 1.9, therefore not meeting the criteria of >2fold; a similar observation for batch RZX in this present study. Minipig hepatocytes batch IKL has been used in the current project and a fold change of 2.1 for rosuvastatin was observed which is just above the threshold criteria. Overall, the fold change observed with rosuvastatin in minipig hepatocytes has just been over 2-fold, although the criteria is met it does question the suitability of rosuvastatin as a probe for minipig hepatocytes and the study design. The time dependent linearity was carried out across three batches of minipig hepatocytes with each batch containing a pool of three animals, therefore a total 9 minipigs have been assessed, only achieving a fold change of just above 2 for two of the batches treated with rosuvastatin does not show robustness in the current assay design. Furthermore the dynamic range of the fold change in particular for rosuvastatin is very low, and this would also pose the question as to whether current design does not give a reliable assay for minipig hepatocytes if 1 in 3 of the assays would fail due to the criteria.

The uptake of rosuvastatin in human cryo-preserved hepatocytes was previously reported by Menochet *et al.*,2012 to be variable between three donors, where one donor showed a 6.8-fold greater activity in the uptake of rosuvastatin than the other

two donors. However, the K_m was consistent across all three human donors, which were estimated using a mechanistic two compartment model (Ménochet *et al.*, 2012b). In comparison, the K_m values for rosuvastatin were lower in this project where the Michaelis Menten equation was used. The Michaelis Menten equation has two parameters; the V_{max}, which is the maximum reaction rate, and the Km, which is the Michaelis constant. The Km describes the rate of an enzymatic reaction by relating this to the concentration of the substrate ([S]). Therefore, the Km is the substrate concentration at half V_{max}, Km and V_{max} are constants for a given temperature and pH and are used to characterise enzymes. The higher the Km the lower the enzyme's affinity for its substrate, and a high Km is an indicator that a larger amount of substrate is required before the enzyme gets to half V_{max} (Cho and Lim, 2018). Therefore, the Km is an important intrinsic parameter of enzyme-catalysed reactions and gives a good indication of the substrates binding affinity. The mechanistic two-compartment model developed by Ménochet, allows simultaneous fitting of all concentration-time points during the experiment and relies only on measurements made at 37°C. It also allows the assessment of multiple processes, active uptake of drugs into the hepatocytes, bidirectional passive diffusion, and intracellular binding (Ménochet *et al.*, 2012a). Therefore, the mechanistic two-compartment model describes all of the processes occurring during the *in vitro* uptake experiments unlike the Michaelis Menten model.

Passive uptake in the experiments carried out, was assessed via the addition of a cocktail of inhibitors, rifamycin and imipramine. Rifamycin SV has been shown to be a potent inhibitor of OATP1B1, OATP1B3 and OATP2B1; and a moderate inhibitor of Na+ taurocholate co-transporting polypeptide (NCTP) at administered concentrations of 100μM and 200μM in human and cynomolgus hepatocytes (Bi *et al.*, 2017, Zhang *et al.*, 2019), Whereas imipramine inhibits OCT1 and OATP1B1 and OATP1B3 (Patel *et al.*, 2018, Morse *et al.*, 2020). This method of assessment of passive uptake is supported in the literature and prevents any concerns of reduced passive diffusion via increased bilayer rigidity at cold temperatures (Bi *et al.*, 2017). Other inhibitors such as cyclosporin A and rifampicin (Kalliokoski and Niemi, 2009) are also known to inhibit SLC transporters and may inhibit the uptake of probe substrates to a greater or lesser extent. Rifampicin is a potent inhibitor of OATP1B1, OATP1B3 and to lesser degree OATP2B1 and NTCP (Zhang *et al.*, 2019). The inhibitory potency of inhibitors being used should ideally be assessed, to allow an appropriate concentration and pre-incubation time to be selected for an inhibitor as

these conditions may differ for different species as well as substrates. A study was carried out by Taguchi *et al.,*(2019) looking at the effects of pre-incubation with cyclosporin and rifampicin over 1 to 60 minutes in HEK293 cells transfected with human OATP1B1. The IC_{50} values determined for cyclosporin were consistent with time whereas rifampicin showed no real difference in the IC_{50} with or without pre-incubation (Taguchi *et al.*, 2019). Various pre-incubation scenarios with cyclosporin, rifampicin and also the probe were assessed such as co-incubation, pre-incubation alone, and combination of pre- and co-incubation (Taguchi *et al.*, 2019). The authors observed that selection of the inhibitor is just as important as is assessing the pre-incubation step in an assay (Taguchi *et al.*, 2019). Variabilities in the methods across labs have always existed to determine the uptake of drugs such as in the pre-incubation of the hepatocytes, or the presence of an inhibitor. Whilst other publications have reported a 60 minute pre-incubation (Ufuk *et al.*, 2018, Farasyn *et al.*, 2021) and a 20 minute pre-incubation (Menochet et al., 2012), in this present study a pre-incubation time of 30 minutes was used, as this aligned with in-house uptake validated methods in rat, dog and human hepatocytes. The inhibition of rosuvastatin by cyclosporin in this study (Figure 31) determined an IC50 of 0.35μM which was in between the reported IC50 values without pre-incubation 0.72μM and 0.10μM with pre-incubation (Ufuk *et al.*, 2018). There will always be a difference in IC50 values across different species and labs but Ufuk's data showed a shift in the IC50 by pre-incubating for up to an hour. A <20 % inhibition (Ufuk *et al.*, 2018) was observed with cyclosporin, whereas with the minipig hepatocytes approximately 50% maximal inhibition (Figure 31) was observed with cyclosporin. The observation in the present study could be due to the lesser pre-incubation time of 30 minutes being used or just a species difference. It has been hypothesised by Shitara and Sugiyama 2017, that cyclosporin inhibits OATP1B1 not only from the outside (cis-inhibition) but also from the inside (trans-inhibition) of the cells (Shitara and Sugiyama, 2017). Following washing the cells, a long lasting inhibition of cyclosporin can still be observed for up to 18 hours on OATP1B1(Shitara and Sugiyama, 2017) . This effect with cyclosporin has also been observed *in vivo* (Taguchi *et al.*, 2019). The FDA draft guideline recommends pre-incubation for a minimum of 30 minutes for the determination of IC50 values of an NCE (In Vitro Drug Interaction Studies — Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions Guidance for Industry, 2020).

In our study the total uptake of rosuvastatin in minipig hepatocytes was lower than in human or rat hepatocytes, although it was relatively consistent across all three minipig hepatocyte batches. The fold difference (2.3, 1.5 and 2.1) in the uptake of rosuvastatin was lower in minipig than the other two species due to the high passive element (Figures 19, 20 and 21). To investigate this further, a temperature dependent experiment was conducted to compare uptake of rosuvastatin at 4°C and 37°C (Figure 22) into minipig hepatocytes. This was only conducted in batch GBV as there was a shortage in supply of the other two minipig batches . Transporter mediated uptake is temperature dependent especially for compounds that have a low passive diffusion. Transporter proteins are expected to have minimal or no activity at 4°C, therefore minimal diffusion or uptake would be observed at this temperature. Therefore, all transport occurring at this temperature is considered passive (Hewitt *et al.*, 2007), except if passive diffusion is temperature dependent, then a compound that is highly passively diffused it may be difficult to determine active from passive uptake. A 5.4-fold difference between total (37^0C) and passive (4^0C) uptake was observed (Figure 22), this is greater than the fold difference detected in the time dependent assay carried out at 37°C in the presence of the cocktail inhibitors. The level of total uptake was comparable across the both time dependent experiments. This may suggest that the minipig cryopreserved hepatocytes, have an additional active transport mechanism, previously not identified, that is contributing to the transport of rosuvastatin. Other reasons to consider could be that there are differences in either uptake transporter and homologue; or transporter expression between the minipig and other species; or variation within batches could also contribute (Table 9).

The difference in fold change in the uptake of rosuvastatin observed following incubation at 4°C could be caused by the passive diffusion being underestimated in the minipig hepatocytes due to increased bilayer rigidity or the concentration of the inhibitors being too low (Bi *et al.*, 2017). It has been reported that transporters in pigs and dogs are comparable to those in humans with amino acid sequences >72% identical and >91% for monkey, however the OATP1B1 human orthologue is not present in pigs or dogs, (Dalgaard, 2015, Bleasby *et al.*, 2006). Therefore, OATP1B3 may be responsible for the uptake of rosuvastatin in minipig hepatocytes, but another transporter such as NTCP may also play a role in the uptake of rosuvastatin. NTCP is a sodium (Na+)-dependent transporter expressed on the sinusoidal membrane of hepatocytes, and it has an important role in maintaining bile acid homeostasis (Bi *et*

al., 2013), and is involved in the uptake of bile acids but also of other drugs such as statins. The contribution that NTCP plays in the uptake of statins or other drugs can be further assessed by using Na+ containing buffer in comparison to Na free buffer. Human NTCP accounts for about 35 % of total rosuvastatin uptake into isolated human hepatocytes (Stieger, 2011). Comparing the cynomolgus monkey hepatocyte uptake data of rosuvastatin (Ufuk *et al.*, 2018) with minipig hepatocyte data generated in this project, there appears to be a species difference with the K_m and clearance values, as two of the batches of minipig hepatocytes have higher K_m value than the cynomolgus monkey data. The third batch of minipig hepatocytes failed to generate any kinetic data. The V_{max} is lower in the minipig hepatocytes compared to the cynomolgus monkey hepatocytes. This may be due to the potential lack of OATP1B1 gene in the minipig hepatocytes (Dalgaard, 2015, Vamathevan et al., 2013) hence there is a reduced uptake of rosuvastatin, and the contribution that is observed is dependent upon both OATP1B1 and NTCP. To confirm this, the assay would need to be carried out with and without Na+ containing-buffers or in cell-lines transfected with minipig NTCP. In the cynomolgus monkey hepatocytes, all three transporters are expressed, they have an affinity to rosuvastatin, and the V_{max} is higher. In this study an experiment was carried out to assess the role NTCP plays in the uptake of rosuvastatin in minipig hepatocytes, by using two different inhibitors, cyclosporin A, a broad spectrum inhibitor known to inhibit both OATP and NTCP transport (Jamei *et al.*, 2014, Dong *et al.*, 2013), and taurochenodeoxycholate (TCDC) a bile acid which has been shown to reduce NTCP at the plasma membrane by retrieval into intracellular compartments (Mühlfeld *et al.*, 2012). The homeostasis of bile acids is regulated by negative feedback inhibition of genes that are involved in the uptake and synthesis of bile acids, which down regulate cholesterol 7a-hydroxylase (cyp7a), via bile acid receptor (fxr) activation of an inhibitory nuclear receptor (shp). Denson *et al* concluded negative feedback regulation of NTCP by bile acid activated fxr via induction of shp (Denson *et al.*, 2001). Additionally, inhibition of bile acid synthesis in cultured pig hepatocytes has been demonstrated by suppression of cholesterol 7a-hydroxylase activity (Kwekkeboom *et al.*, 1990).

In this present study it was shown that cyclosporin A and TCDC inhibited rosuvastatin uptake in minipig hepatocytes with an IC50 of 0.35μM and 7.0μM, respectively. The potent IC50 of cyclosporin is an indication that all the SLC transporters have been

inhibited. TCDC also inhibited the uptake of rosuvastatin indicating that NTCP may play a role in the uptake of rosuvastatin in minipig hepatocytes. Further investigation is required to confirm this by assessing the prevalence of NTCP and other SLC transporters in minipig hepatocytes.

The calculated kinetic parameters for human and rat hepatocytes (Table 9) are consistent with the literature (Li *et al.*, 2013b, Ménochet *et al.*, 2012b, Hassen *et al.*, 1996, Shitara *et al.*, 2003b, Han *et al.*, 2010, Badolo *et al.*, 2011, Niemi *et al.*, 2011, Liao *et al.*, 2019). However, there is variability observed in the literature and in internal unpublished GSK data. This variability can be attributed to several factors, such as methodology and laboratory differences. Inter-individual variation from different donors may also contribute (De Bruyn *et al.*, 2011). The pooled human hepatocytes and pre-clinical hepatocytes used in this project only have CYP characterisation data, no transporter data is provided. One of the reasons why pooled donors are used is that this method negates the use of multiple single donor batches in experiments, in order to address inter-experimental variability. Also, by pooling hepatocytes this provides a larger batch available and there can be consistency between laboratories and experiments. One disadvantage of pooled donors could be lack of consistent plating from one occasion to another as certain donors may be more prevalent than others in the donor pool therefore leading to variation in uptake from experiment to experiment. In an uptake experiment a difference in substrate uptake was observed between single donor hepatocytes and when these cells were in a pool with other donors (De Bruyn *et al.*, 2011).

Reviewing the Km data (Table 9) in detail it was observed, minipig hepatocytes batch GBV and human hepatocytes batch AMH showed the highest affinity to ES with Km values of 0.78 and 0.70 respectively. Minipig hepatocyte batch GBV showed the lowest Km value (11.4) for EG out of the three batches assessed. The Km values for minipig hepatocytes batches IKL and RZX were similar to human hepatocyte batch CYN in the range 25 to 30, but the human hepatocyte batch AMH again had the lowest Km value for EG of 1.2, therefore indicating that this batch of hepatocytes has good affinity for both ES and EG. Regarding rosuvastatin, no kinetic data was generated for minipig batch GBV (Figures 25 and 29). The other two minipig batches (RZX and IKL) gave different Km values of 16 and 7; human hepatocyte batch CYN had a lower Km value for rosuvastatin than batch AMH. This shows the differences in species and

their affinity for different substrates but also within the same species different batches have different affinities for substrates.

There are published data on the uptake of rosuvastatin in rat and cynomolgus monkey (Ufuk *et al.*, 2018) hepatocytes, but currently a paucity of publications are available for minipig hepatocytes. Proteomic characterisation of drug metabolising enzymes and transporters was determined by Elomsi *et al,* (2020) where 16 CYP enzymes, 5 UGT enzymes and 11 transporters were quantified in addition to 20 phase I and 14 Phase II enzymes which were characterised (Elmorsi *et al.*, 2020). The passive element for rosuvastatin compared to EG and ES uptake in minipig hepatocytes is very high for all three batches highlighting that there may be another transporter or specificity differences. To assess if this phenomenon was particular to rosuvastatin or to statins in general, uptake of pitavastatin was assessed in the same minipig hepatocytes batches as used in this project. However, pitavastatin kinetics could not be determined in minipig hepatocytes. The cause of this phenomenon with rosuvastatin and pitavastatin is not known, however, it highlights the differences in active uptake between the minipig hepatocytes and other species. This further suggest that there is variability in specificity and expression of transporters between the species. As conducted previously for rosuvastatin, one could assess this by increasing the inhibitor cocktail concentration or by carrying out the uptake of pitavastatin at 4°C. This also highlights differences in uptake between the probe substrates, which shows the importance of using multiple probe substrates in *in vitro* assays.

Overall, the data show that rat hepatocytes have the highest intrinsic clearance for all three substrates when taking bodyweight into consideration, drug transporters are highly expressed in rodents compared to other species. Minipig hepatocyte batches GBV and IKL had lower Km values for ES compared to EG. The Intrinsic clearance (ml/min/Kg) determined for minipig and human hepatocytes was similar across both species for EG (4.78 to 10.54 ml/min/Kg). All three minipig hepatocytes had a similar intrinsic clearance to one batch of human hepatocytes (CYN). This difference in uptake between rat and human hepatocytes was also observed for rosuvastatin and has also been shown in literature (Ménochet *et al.*, 2010). A study investigating the uptake of 7 known OATP probe substrates into rat and human hepatocytes, showed cellular intrinsic clearance being on average 7.3 lower in human than rat cells (Ménochet *et al.*, 2012b, Ménochet *et al.*, 2010). One factor that may contribute to this difference,

is that the overall abundance of transporters has been shown to be higher in rat hepatocytes (Wang *et al.*, 2015b).

The prediction of human pharmacokinetics relies on extrapolation from *in vitro* and *in vivo* data, and therefore remains challenging (Suenderhauf and Parrott, 2013). However, several approaches have been developed. Allometric predictions take body and/or organ weight into account, e.g. scaled intrinsic clearance. However, failure of this method to predict clearance has been observed for drugs relying on transporter or metabolic enzyme mediated clearance. This is due to the large interspecies differences in their activity and specificity (Rowland and Dedrick, 2012). Therefore, empirical scaling factors have been used in previous studies (Gardiner and Paine, 2011). A physiological method, such as the Well-stirred liver model, can then be applied to the scaled intrinsic clearance values. This model takes liver blood flow into account, but not active transport processes (Ito and Houston, 2004). Another method is physiologically based pharmacokinetic (PBPK) modelling, in which kinetic parameters Km and Vmax, alongside a physiological framework, are used to predict pre-clinical in vivo data. The predicted values can be compared to actual in vivo values, to give mechanistic understanding and inform human PBPK models (Suenderhauf and Parrott, 2013). However, for minipig, although commercial models are available, further refinement is needed (Lignet *et al.*, 2016). Therefore, it is important to know the characterisation of minipig uptake transporters, and the interspecies differences, and these can therefore can be accounted for, where new chemical entities are substrates of hepatic uptake transporters. For example, this will enable the improvement of minipig PBPK models, allow better prediction of human pharmacokinetics from pre-clinical *in vivo* and *in vitro* minipig studies, and improve dose prediction.

As with any *in vitro* system, attempting to replicate and translate to the dynamic *in vivo* whole organ system there are limitations and assumptions, including the potential for OATP substrate dependent inhibition (Zamek-Gliszczynski *et al.*, 2013).

In this present investigation plated cryo-preserved hepatocytes were utilised. However, there are many *in vitro* systems that can be used to evaluate how transporters are involved in the uptake and clearance of compounds. The use of the correct *in vitro* system is vital and by using a battery of assays it provides an overall picture of the molecule and its ADME. The International Transporter Consortium (ITC) white paper

investigated *in vitro* methods used to support the evaluation of transporters in drug discovery and development (Brouwer *et al.*, 2013). The efflux transport of compounds can be assessed by using a simple system such as vesicles which have been transfected with the efflux transporter e.g. P-gp, or BCRP. The efflux of molecules is mediated by ATP-dependent unidirectional pumps where the molecule is transported uphill from the hepatocyte into the bile. Recombinant cell lines such as HEK293 and MDCKII are stably transfected with single or in some cases double transporters from specific species such as rat, human or monkey and used to assess the inhibition and uptake of compounds, mock cells and the use of control inhibitor serve as controls (Brouwer *et al.*, 2013). Cellular systems such as vesicles and recombinant cells are used to assess the DDI risk of a molecule and also to provide quantitative data which can be used alongside hepatocyte data for modelling. Currently there are no commercial cell-lines or vesicles available that have been transfected with minipig transporters to be able to assess the DDI risk or the uptake of compounds regarding the minipig.

Hepatocytes can be used to determine the clearance of a molecule, and they can be used in suspension, plated or in a sandwich culture platform. Each platform has its limitations and should be selected based on what data are required. As previously mentioned the gold standard method to assess hepatic uptake is the oil spin method (Li *et al.*, 2013a). The transporter and enzyme activities are generally well retained therefore allowing transporter enzyme interplay, except that cell polarity is lost therefore efflux transporters such as P-gp or MRP2 cannot be assessed as they are expressed on the bile canicular side and their function is lost (Nozaki and Izumi, 2020). Suspended hepatocytes can be used to monitor media loss from buffer rather than uptake into cells (Harrison *et al.*, 2018), the oil spin method is very labour intensive and the media loss method does require enough uptake of the test compound into the cells to affect the media concentrations (Nozaki and Izumi, 2020). In hepatocyte sandwich cultures the hepatocytes are grown on collagen coated plates and have a Matrigel overlay. They are cultured over a period of days, allowing the hepatocytes to develop a functional canicular network that expresses efflux transporters such as BSEP, P-gp and MRP2, but also expresses hepatic uptake transporters such as OATP1B1, OATP1B3 and NTCP. Substrates of these hepatic efflux transporters (Pgp, BSEP and MRP2) accumulate in bile pockets which are formed between the hepatocytes. By disrupting the tight junctions that are formed between the hepatocytes

with Ca^{2+} free media the substrates are released from the bile pockets allowing the evaluation of biliary excretion (Nozaki and Izumi, 2020). With the Matrigel overlay on plated hepatocytes transporter activities decrease more quickly with time, however, the absence of the Matrigel does not allow the formation of the canicular network therefore efflux transporters are not expressed. Plated hepatocytes were used in this project and have their advantages in assessing the uptake of compounds over suspended hepatocytes and sandwich culture hepatocytes. Plated hepatocytes have a shorter culturing time in comparison to the sandwich culture and are less laborious to use in comparison to suspended hepatocytes (oil spin method) (Nozaki and Izumi, 2020).

Cyropreserved hepatocytes are a convenient tool used routinely to assess drug clearance and disposition. They are versatile and can be used as a suspension, plated as a monolayer or sandwich culture and also as spheroids. They can be co-cultured with other cells such as Kupfer cells to enhance liver-specific functions of hepatocytes. Cyporeservation of hepatocytes has aided in the convenience of when experiments can be carried out, they can also be repeated multiple times in-house or in other labs using the same batch of cells most. Multiple donor hepatocytes can be cryopreserved in one batch addressing donor variation in experiments. It has been shown cryopreservation can cause a reduction in the expression of enzymes and transporters and this could be a possible limitation to this study. Using immunofluoresece it has been observed a large portion of OATP1B1 and OATP1B3 transporters can become internalised during the cryopreservation process (Lundquist et al., 2014). Measuring the uptake of known OATPs, OCTs, and NTCP substrates showed decreased activity in cryopreserved hepatocytes compared with fresh hepatocytes in both rat and human (Lundquist et al., 2014). This lead to a decrease in the intrinsic clearance of probe substrates, with the uptake rate of rosuvastatin being 75% lower in human cryopreserved hepatocytes and 80% in rat cryopreserved hepatocytes compared to fresh hepatocytes (Lundquist *et al.*, 2014). A study investigating the effects of cryopreservation of human hepatocytes on the uptake EG showed cryporeserved human hepatocytes display carrier-mediated uptake of EG. While the affinity of EG was not affected by cryopreservation and the Km was unchanged, Vmax and CLuptake values decreased in average by 47%. The passive diffusion of EG decreased significantly after cryopreservation (Badolo et al., 2011). In literature cryopreservation has been shown to have a similar effect in both rat and

human hepatocytes, no information in regard to minipig has been published yet and further work would need to be carried out to assess the effects of cryporeservation on minipi hepatocytes.

Due to the limited availability of minipig hepatocytes another statin or a different class of compound could not be assessed. The timepoint for the concentration dependent assessment of pitavastatin was assumed to be the same as rosuvastatin as the additional time dependent experiments could not be carried out.

With no OATP1B1 homology currently determined for minipigs, further work needs to be carried out characterising the minipig system. Transporter expression in minipig hepatocytes needs to be carried out by proteomic analysis thus comparing transporter expression and functionality in minipig liver tissue and cryopreserved hepatocytes. For completion, uptake needs to be assessed in fresh hepatocytes and cryopreserved hepatocytes not only investigating statins but other classes of compounds too, as this phenomenon may be specific to certain compounds or classes of compounds. From carrying out a complete characterisation it will then be possible to confirm if minipig uptake transporters, in particular OATP are representative of those in human hepatocytes and if minipig can be used for the translation of IVIVC. Understanding the contribution of each transporter to overall hepatic uptake could improve the current practice in human prediction to assess the PK variability caused by drug–drug interactions and pharmacogenetics

In summary, minipig hepatocytes have a similar substrate affinity for EG and ES as human cells. Although both EG and ES can be used as positive substrate controls, they are not clinically relevant. Kinetics was determined in two out of the three minipig batches (RZX and IKL) for rosuvastatin and a fold change of >2 was only observed in the time linearity experiment for batches GBV and IKL which gave a fold change of just over 2, the third batch RZX gave a fold change of 1.5. There is not a huge difference in the fold changes for all three minipig hepatocytes and they are around the acceptance threshold. This could be due to the poor affinity of rosuvastatin for the uptake transporters present in minipig hepatocytes, as with pitavastatin no kinetics could be determined in minipig cells.

Regarding future work, the current experimental design needs to be refined but also additional parameter need to be considered. Work carried out by Ufuk and Menochet,

obtaining the unbound fraction of a substrate, would provide a more accurate picture as to the amount of free substrate that was available for active uptake. Carrying out initial experiments to assess the most appropriate concentration of inhibitors selected and the pre-incubation time would allow the best assessment of passive uptake. Determining these parameters or conditions will provide a more accurate picture of the actual active component, the passive component, and the fraction of substrate transported. Determining these components would allow a mechanistic compartmental model to be built thus providing a better understanding of the system. Although the homology and the prevalence of some drug transporters such P-gp, BCRP, OATP1B3, OAT1, OAT3, OCT1, and OCT2 ((Dalgaard, 2015) have been determined in minipigs there are still gaps e.g. for NTCP possibly further work needs to be carried out to determine the homology of OATP1B1. To determine the prevalence of transporters in the minipig, proteomics or immuno-histological staining needs to be carried out to assess what transporters are prevalent in the minipig liver, as well as fresh and cryopreserved minipig hepatocytes. This comparison would be advantageous giving an indication of any changes in transporters or metabolic enzymes, thus providing information on functionality of the hepatocytes and how similar they are to the intact liver, therefore providing data that equate more readily to the *in vivo* situation, or can be more easily extrapolated. Experiments need to be carried out to determine which transporter is predominantly involved in the uptake of rosuvastatin. This phenotypic assessment would need to be done in cell lines that have been transfected with specific minipig transporter genes such at OATP1B1, OATP1B3 and NTCP. Further investigation is also needed to determine the mechanism behind this observed difference in passive uptake of rosuvastatin and pitavastatin, to identify if this is a statin-specific or wider substrate phenomenon in minipigs. The pig is an appropriate species to use for the assessment of drug disposition as it does have a significant number of enzymes and transporters in common with humans (Dalgaard, 2015, Elmorsi *et al.*, 2020). Non-human primates are the closest to humans regarding genetic homology. Although dogs are easier to handle and have an extensive amount of background data available, they are susceptible to emesis although they are used in PK studies. The Göttingen minipig has proven to be a useful model due to its small size, handling and its well characterised genotype and homology to humans (Singh *et al.*, 2016). Therefore, if we propose to use minipigs in PK and disposition studies we need to characterise the ADME processes such as the rate limiting steps, the uptake of

molecules, or the inhibition of transporters or enzymes and their interplay to determine IVIVC. Also, for the future, the development of a minipig PBPK model could be advantageous in providing IVIVC, data on disposition of rosuvastatin and other molecules as demonstrated by Sjögren *et al*, assessing the pharmacokinetics and hepatic disposition of repaglinide (Sjögren *et al.*, 2012). Here in vitro data alongside a multiple sampling site model created a PBPK model could provide a mechanistic explanation of the liver disposition, *in vitro* based *in vivo* predictions, sensitivity analysis and estimations of individual pharmacokinetic parameters (Sjögren *et al.*, 2012).

In conclusion, this project demonstrated that minipig uptake transporters, in particular OATP are not fully representative of human hepatocytes based on the experimental observations. Further work as described would aid better understanding and characterisation of the minipig uptake assay for a clearer conclusion. No single animal can model all human parameters, therefore it is important to understand the species with which you are working. Alongside this, it is important to understand and characterise a NCE, its ADME properties, the clinical implications it will have, and the risks associated with other comedications such as statins which are both substrates and inhibitors of the OATPs.

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APPENDIX 1:

Table 1: Radio-HPCL conditions for the radiochemical purity determination of Estradiol Glucuronide

HPLC Pump:	Aquity Waters
Autosampler:	Aquity Waters
UV Detector:	Aquity Waters
Radiochemical Detector:	B-Ram (Model 4) by Lablogic
Column Oven:	Aquity Waters
Acquisition Software:	Laura (v4.1.14.96)
Column:	Zorbax ODS analytical 4.6x250mm 5-microns
Mobile Phase:	Solvent A: 1% (w/v) tetraethyl ammonium acetate tetrahydrate (TEAA) in deionized water, pH 4 Solvent B: 100% methanol
Gradient:	35% B for 5 min, 35% B to 70% B over 15 min, 70% B for 10 min, 70% B to 35% B over 5 min, 5 min equilibration.
Mobile phase Flow Rate:	1 ml/min
Column Temperature:	Room Temperature
UV Detection:	210 nm
Injection Volume:	30 μL
Scintillation Fluid Used:	FlowLogic
Scintillation Fluid Flow Rate:	3 mL/min

Table 2: Radio-HPCL conditions for the radiochemical purity determination of Estrone Sulfate.

HPLC Pump:	Aquity Waters
Autosampler:	Aquity Waters
UV Detector:	Aquity Waters
Radiochemical Detector:	B-Ram (Model 4) by Lablogic
Column Oven:	Aquity Waters
Acquisition Software:	Laura
Column:	Luna 3μm C-18 (150 x 4.6mm) from Phenomenex
Mobile Phase:	Solvent A: 1% Trifluoroacetic acid: Acetonitrile (70:30)
Gradient:	100% solvent A for 40 minutes
Mobile phase Flow Rate:	1 ml/min
Column Temperature:	Room Temperature
UV Detection:	282 nm
Injection Volume:	20 μL
Scintillation Fluid Used:	FlowLogic
Scintillation Fluid Flow Rate:	3 mL/min

APPENDIX 2:

Table 1: LC-MS/MS conditions for Rosuvastatin analysis

Column	Waters BEH Acuity 50 x 2mm 1.7 µm
Column Temperature (°C)	40
Eluent flow rate (mL/min)	0.8
Run Time (mins)	2
Injection volume	5 µL
Solvent A	5mM Ammonium Bicarbonate
Solvent B	Acetonitrile
Wash 1	20/80 (v/v) Acetonitrile / water + 0.1% Ammonia solution
Wash 2	40/30/30 Acetonitrile / IPA / 0.1% Formic acid
Compound	**Q1/Q3 Mass transition**
Rosuvastatin	480.2/418.1
GSK123	431.2/204.1
Gradient	5% B for 0.2 min, 5% to 95% over 1 min, 95% for 0.4 min, 95% to 5% over 0.01min and 0.39 min equilibration

APPENDIX 3:

Figure 1: Cell Health Data for Hepatocytes following Treatment with ES, EG and Rosuvastatin