THE ROLE OF OATP TRANSPORTERS IN THE ACTIVE UPTAKE OF DRUGS INTO HEPATOCYTES

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Tiivistelmä Referat Abstract

Active transport processes in the basolateral (sinusoidal) membrane of hepatocytes have an important role in the hepatic clearance and overall disposition for several types of drugs. Organic anion transporting polypeptides (OATPs) expressed in the sinusoidal membrane have been shown to mediate the sodium-independent hepatic uptake of broad range of drugs and they have been associated with clinically relevant drug-drug interactions (DDIs) and genetic polymorphisms. The literature review focuses on sinusoidal OATP transporters and on the pharmacokinetic effects of OATP-mediated hepatic uptake. In addition, current methods to investigate the interactions between drugs and transporters are discussed, with the emphasis on methods applicable to study uptake transporters.

The aim of the experimental part of the master's thesis was to determine if two clinically used drugs, entacapone and fluvastatin, are actively transported from blood into rat and human hepatocytes, and to assess the role of OATP transporters in the hepatic uptake of the drugs in comparison with known OATP substrates, estrone 3-sulfate (E3S) and taurocholic acid and broad OATP inhibitor rifamycin SV. The uptake kinetics of compounds of interest were determined in freshly isolated and cryopreserved rat hepatocytes and in cryopreserved human hepatocytes using the oil-spin method. Uptake clearances (CL_{uptake}) via active uptake (CL_{active}) and passive diffusion (P_{diff}) were calculated from the initial uptake data over a 1 - 200 µM and 1 - 50 µM concentration range for entacapone and fluvastatin, respectively. The half-maximal inhibitor concentration (IC50) of E3S uptake transport was determined for entacapone in a competitive uptake experiment over a 10 – 400 µM concentration range.

Fluvastatin uptake showed active saturable transport kinetics in rat hepatocytes with a K_m value of 6 µM, whereas entacapone uptake in rat hepatocytes was somewhat linear and did not inhibit E3S uptake at clinically significant concentrations, with an IC50 value of 240 µM. Significantly lower hepatic uptake of taurocholate and entacapone was observed between rat and human hepatocytes, indicating species differences in hepatic uptake processes, although cryopreservation may have had an effect on the noticed difference. The results suggest that murine Oatp transporters do not have a significant contribution to hepatic uptake of entacapone. However, this should be confirmed with future studies with more repetitions and a reliable quantification method.

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Hepatic uptake, OATP, fluvastatin, entacapone

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Tiivistelmä – Referat – Abstract

Maksasolujen basolateraalisen (sinusoidaalisen) solukalvon aktiivisilla kuljetusmekanismeilla on suuri vaikutus monien lääkeaineiden maksapuhdistumaan sekä laajemmin farmakokineettisiin jakautumis- ja eliminaatiovaiheisiin. Sinusoidaalikalvolla ilmentyvien orgaanisia anioneita kuljettavien polypeptidien (engl. organic anion transporting polypeptides, OATP) on huomattu kuljettavan maksasoluihin laajasti erityyppisiä lääkeaineita. Monien kliinisesti merkittävien lääke-lääkeyhteisvaikutusten on havaittu liittyvän OATP-kuljetinproteiinien estyneeseen tai rajoittuneeseen toimintaan. Samoin useat geneettiset polymorfismit vaikuttavat OATP-substraattien farmakokinetiikkaan, tehoon ja toksisuuteen. Kirjallisuuskatsauksessa tarkastellaan sinusoidaalisten OATP-kuljetinproteiinien vaikutusta lääkkeiden farmakokinetiikkaan sekä tarjolla olevia menetelmiä kuljetinproteiinien tutkimiseen lääkekehityksessä.

Erikoistyön tavoitteena oli selvittää, onko fluvastatiinin ja entakaponin soluunotto aktiivista rotan ja ihmisen maksasoluissa, sekä arvioida OATP-kuljetinproteiinien merkitystä näiden lääkkeiden maksasoluunotossa. Lääkeaineiden soluunottokinetiikkaa tutkittiin tuoreissa eristetyissä rotan maksasoluissa sekä pakastetuissa rotan ja ihmisen maksasoluissa käyttäen öljyn läpi -sentrifugointimenetelmää. Soluunoton alkunopeus määritettiin entakaponi-pitoisuuksilla 1 -200 µM ja fluvastatiinipitoisuuksilla 1 - 50 µM. Määritetyistä arvoista arvioitiin aktiivisen (CL_{active}) ja passiivisen (P_{diff}) mekanismin osuudet lääkeaineiden soluunotossa. Lisäksi rotan Oatp-kuljetinproteiinien merkitystä entakaponin soluunottoon tutkittiin pitoisuusalueella 10- 400 µM kilpailevassa soluunottokokeessa Oatp-substraatti estroni-3-sulfaatin kanssa.

Fluvastatiinin soluunotto rotan maksasoluihin oli saturoituvaa, ja laskennallinen K_m-arvo oli 6 µM. Sitä vastoin entakaponin soluunotto oli lineaarista tutkitulla pitoisuusalueella, eikä estroni-3-sulfaatin soluunotto estynyt kliinisesti merkittävillä entakaponipitoisuuksilla IC50-arvon (puolet maksimaalisesta inhibitiopitoisuudesta) ollessa 240 µM. Entakaponin soluunotto oli huomattavasti alhaisempaa ja hitaampaa ihmisen maksasoluissa verrattuna rotan maksasoluihin, mikä viittaa lajien välisiin eroihin soluuottomekanismeissa. Havainto voi kuitenkin selittyä tuoreiden ja pakastettujen solujen välisillä eroilla. Kokonaisuudessaan tulokset viittaavat siihen, että entakaponi ei ole fluvastatiinin tavoin rotan Oatp-kuljetinproteiinien substraatti. Tulosten luotettavuus tulisi kuitenkin vahvistaa uusissa kokeissa käyttäen rinnakkaisia määrityksiä ja luotettavaa menetelmää näytteiden lääkeainepitoisuuksien määrittämiseksi.

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ABBREVIATIONS

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
AUC	Area under the plasma concentration-time curve
BCRP	Breast cancer resistant protein
BSEP	Bile salt export pump
C_{max}	Maximun plasma concentration
CL_{int}	Intrinsic clearance for metabolism and/or biliary excretion
$CL_{int,all}$	Overall hepatic clearance
CL_{uptake}	Hepatic uptake clearance
COMT	Catechol *O*-methyltransferase
CYP	Cytochrome P450
DDI	Drug-drug interaction
EMA	European medicines agency
E3S	Estrone 3-sulfate
FDA	United States Food and drug administration
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
ICT	International transporter consortium
IC50	Half maximal inhibitory concentration
KHB	Krebs-Henseleit buffer
K_m	Michaelis-Menten constant
LLC-PK1	Pig kidney proximal tubule epithelial cell line
MATE	Multidrug and toxin extrusion transporter
MDCK	Madin-Darby canine kidney cell line
MRP	Multidrug resistance-associated protein
MS	Mass spectrometry
NTCP	Na^+-taurocholate co-transporting polypeptide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OST	Organic solute transporter

P_{diff}	Passive hepatic uptake clearance
P-gp	P-glycoprotein
QTOF	Quadrupole time-of-flight
SLC	Solute carrier
Tris base	Tris(hydroxymethyl)aminomethane
$t_{1/2}$	Elimination half-life
UPLC	Ultra performance liquid chromatography
V	Hepatic uptake rate
V_{max}	Maximum hepatic uptake rate

1 INTRODUCTION

Cell membrane transporters have a significant role in drug elimination by the liver. For several types of drugs, the active transport of the compound through the basolateral membrane of hepatocytes has been demonstrated to be not only an important determinant of the hepatic clearance of drugs but also the rate-limiting step for drug disposition as a whole (Simonson et al. 2004; Shitara et al. 2006; Maeda et al. 2011). Consequently, the inhibition or induction of hepatic transporters may cause a notable change in drug concentration in the liver and plasma and result in inefficacy or increased toxicity. Also drug-drug interactions and inter-individual variability in the expression and functionality of hepatic transporters may similarly affect safety and efficacy of drugs.

Given the importance of transporters on pharmacokinetics, efficacy and toxicity of drugs, there has been a lot of interest in developing effective models for evaluating drug-transporter interactions. Characterization of a compound as a transporter substrate or inhibitor early in the drug development process aids in the selection of dosing regimen and may lead to the design and conduct of drug-drug interaction studies, or evaluation of the outcomes of possible genetic polymorphisms (Giacomini et al. 2010).

The objective of the literature review of this master's thesis is to consider the pharmacokinetic effects of active transport on the basolateral membrane of hepatocytes, and to provide an overview of the available methods of studying drug-transporter interactions. The focus is on hepatic organic anion transporting polypeptide (OATP) transporters OATP1B1 and -1B3 which have shown to be involved in the hepatic uptake of an increasing number of clinically used drugs.

The hepatic uptake two drugs, fluvastatin and entacapone, is further examined in the experimental part. Hepatic uptake is a prerequisite for the pharmacologic effect of antihyperlipidemic HMG-CoA reductase inhibitors such as fluvastatin and the main route of elimination for most of statins including fluvastatin is via the bile after metabolism by the liver (Dansette et al. 2000; Schachter 2005). Thus, inhibition or

altered activity of active hepatic uptake of statins may be a major mechanism of reduced efficacy and toxic effects such as myopathy. Similar to fluvastatin, catechol *O*-methyltransferase (COMT) inhibitor entacapone used in the treatment of Parkinson's disease is considered a high clearance drug with significant first-pass metabolism and it appears to be eliminated mainly via biliary excretion (Wikberg et al. 1993; Heikkinen et al. 2001). OATPs have been identified to have a significant contribution for hepatic uptake of fluvastatin (Noe et al. 2007; Greupink et al. 2011). However, no published data about hepatic transport mechanisms of entacapone are available and role of hepatic uptake transporters on entacapone pharmacokinetics is unclear. In the present study, the role of OATPs in the sinusoidal uptake of entacapone is evaluated in comparison with fluvastatin and known substrates of OATP-mediated transport.

2 ACTIVE TRANSPORT IN THE LIVER

2.1 Liver Function and Drug Metabolism

The liver is responsible for the majority of drug metabolism in the body due to a very high concentration of most of the drug metabolizing enzymes and a unique blood supply from the digestive organs (Murray et al. 1988; de Waziers et al. 1990; Lakehal et al. 1999). Majority of the blood leaving the digestive system passes through the hepatic portal vein and is then delivered to the liver parenchyma where the contents of the blood are processed before being passed on to other parts of the body (Vollmar and Menger 2009). The functions of the liver include metabolism, storage and synthesis of glucose and lipids, synthesis and degradation of proteins and glycoproteins, as well as metabolism and degradation of several hormones and xenobiotics, such as drugs (Jungermann and Kietzmann 1996). Accordingly, the essential role of the liver is to regulate the entry of nutrients and xenobiotics into the body.

The purpose of hepatic drug metabolism is to facilitate the excretion of drugs in urine or bile by chemical modification to more water soluble forms. Hepatic metabolism process can be divided into three phases, where phase I consists of mainly oxidation, reduction

and hydrolysis reactions catalyzed by cytochrome P450 enzymes (CYPs), followed by further enzymatic conjugation reactions with charged species such as glutathione, sulfate, glycine, or glucuronic acid in phase II, and finally excretion of metabolites out of the cells in phase III (Ishikawa 1992; Roberts et al. 2002; Nakata et al. 2006).

2.2 Structure of the Liver Parenchyma

The liver is formed by parenchymal cells, i.e. hepatocytes, and nonparenchymal cells, including sinusoidal endothelial cells, phagocytic Kupffer cells, hepatic stellate cells, and intrahepatic lymphocytes (Ishibashi et al. 2009). Hepatocytes constitute almost 80 % of the total volume of the liver and perform the majority of the organ's metabolic functions (Blouin et al. 1977; Ishibashi et al. 2009). The parenchyma is composed of structural units referred to as lobules, roughly hexagonal prisms with portal tracts - each with a portal venule, a hepatic arteriole and a bile duct - at each of the corners (Figure 1) (Ishibashi et al. 2009). Blood from the portal tracts flows through small sinusoids lining single layers of hepatocytes, and into the central vein that forms the hub of the lobule. Bile flows the opposite way from small bile canaliculi running between hepatocytes to the interlobular bile ducts of the portal tracts. Each layer of hepatocytes is thus separated by either a sinusoid or a bile canaliculus. The interconnecting layers of hepatocytes radiate from the central vein to the periphery forming the six-sided shape of the lobule.

Hepatic elimination process involves the uptake of xenobiotics from the sinusoidal blood into hepatocytes where enzymatic metabolism occurs, followed by excretion either into bile or back into sinusoidal blood with subsequent elimination by other organs, such as kidney (Kusuhara and Sugiyama 2010). The specialized structure of hepatocytes enables the flux of substrates through the hepatobiliary system. Hepatocytes are highly polarized epithelial cells with three distinct membrane domains (Hubbard et al. 1983). The basal domain, characterized by irregular microvilli, is a large surface facing the space between porous sinusoidal endothelial cells and hepatocytes, known as the space of Disse or perisinusoidal space (Hubbard et al. 1983; Jungermann and Kietzmann 1996).

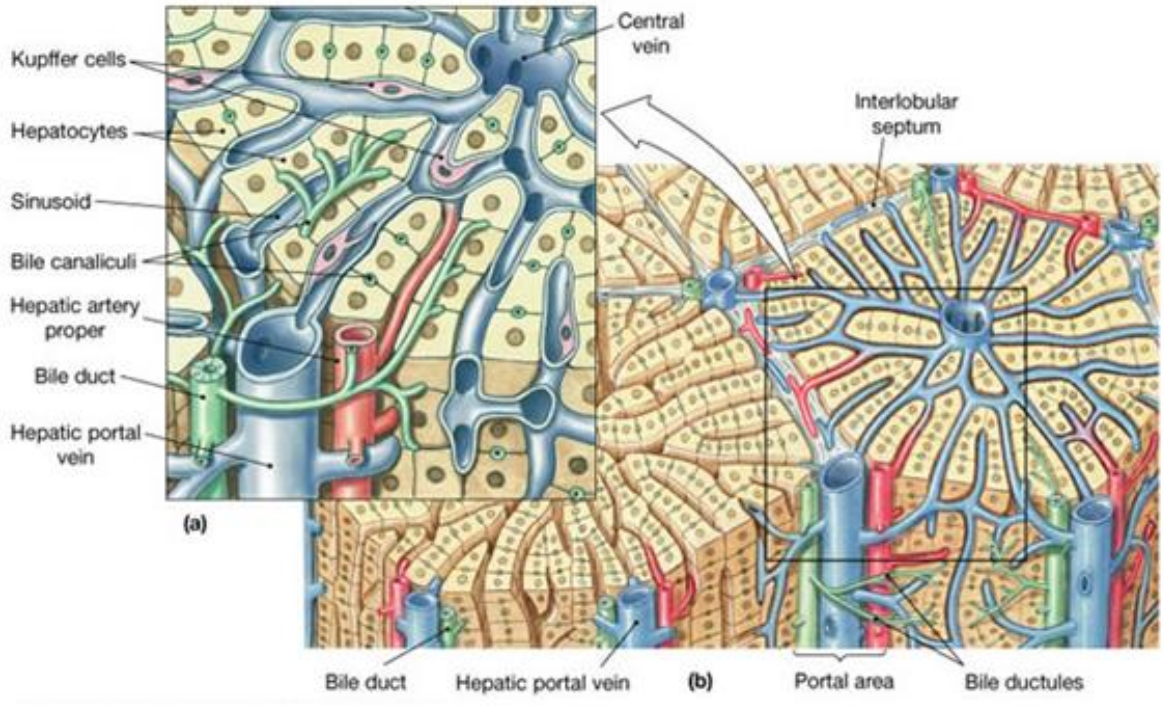


Figure 1. Lobular structure of the liver. The hepatocytes in a liver lobule form a series of irregular plates arranged in hexagonal prisms. (Martini and Nath 2011).

Also called the sinusoidal membrane, the basal surface is specialized for exchange of solutes with plasma in the perisinusoidal space. The lateral domain is a smooth surface contiguous to a neighboring lateral membrane and it bears several types of junctional elements, such as tight junctions, desmosomes, and gap junctions (Hubbard et al. 1983). Separated from the lateral membrane by tight junctions, the apical membrane is a small microvillous area known as canalicular membrane. The canalicular membrane is specialized for bile secretion into bile canaliculi via numerous efflux transporters. The canalicular membranes of adjacent hepatocytes form the walls of the bile canaculi.

2.3 Hepatocyte Membrane Transporters

The highly fenestrated membrane of sinusoidal endothelial cells allows passage of substances carried in the blood into the perisinusoidal space, from where they can be taken up into hepatocytes through the sinusoidal membrane (Fraser et al. 1978; Braet and Wisse 2002; Geraud et al. 2012). Small lipophilic molecules may pass the

membrane by simple or facilitated diffusion (FDA 2000). However, the exchange of some lipophilic and various amphipathic and polar substances is actively facilitated against electrochemical potential gradient by several membrane bound transport proteins. The majority of basolateral uptake transporters belong to the gene superfamily of solute carriers (*SLC*), including the Na^+-taurocholate co-transporting polypeptide (NTCP), organic anion transporting polypeptides (OATPs), and organic anion and cation transporters (OATs, OCTs) (Figure 2) (Faber et al. 2003; Chandra and Brouwer 2004).

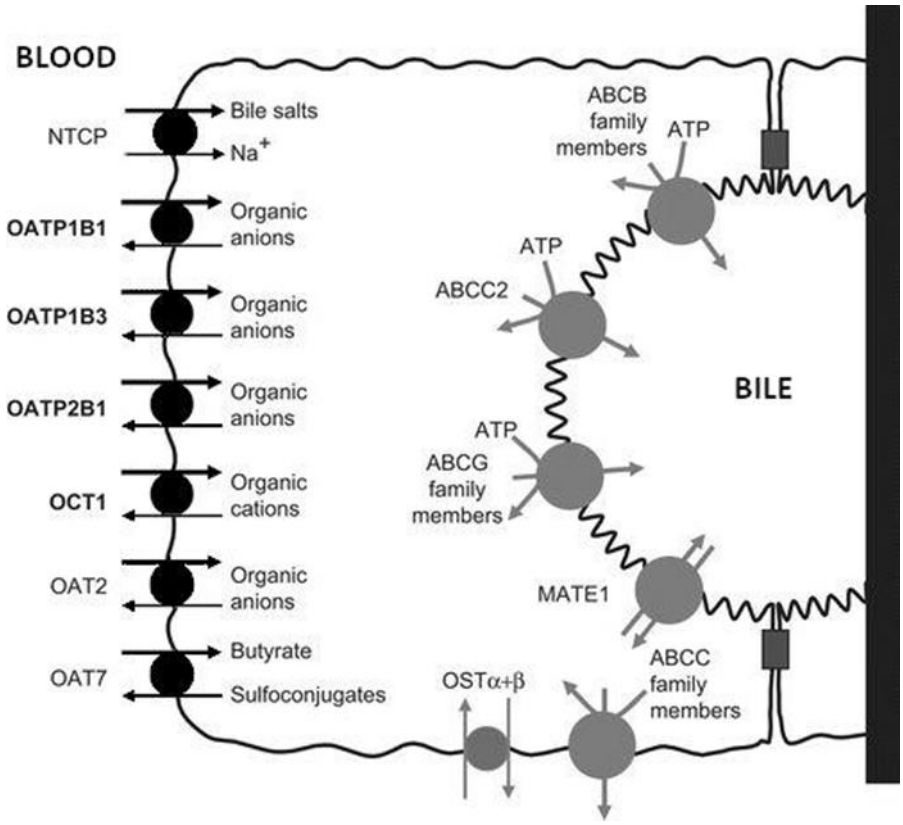


Figure 2. Membrane transporters in human hepatocytes. Basolateral influx transporters (black) transport drugs from sinusoidal blood into hepatocytes where they are metabolized. Efflux transporters (grey) pump the metabolites either into bile or back into blood (Fahrmayr et al. 2010).

Efflux transporters belonging to the adenosine triphosphate (ATP) binding cassette (*ABC*) superfamily are mainly expressed on the canalicular membrane, including *ABCB* family members P-glycoprotein (P-gp), and bile salt export pump (BSEP), the *ABCC* family member multidrug resistance associated protein 2 (MRP2), and the *ABCG* family member breast cancer resistant protein (BCRP) (Figure 2) (Faber et al. 2003; Chandra

and Brouwer 2004). Also multidrug and toxin extrusion transporter (MATE1), a non-*ABC* transporter that uses the proton gradient as a driving force, has been identified in the canalicular membrane (Otsuka et al. 2005). A variety of MRPs and a non-*ABC* transporter, heteromeric organic solute transporter (OST) OSTα-OSTβ, are also expressed in the basolateral membrane pumping metabolites back into sinusoidal blood (Faber et al. 2003; Ballatori et al. 2005). Consequently, the substrate specificity of efflux pumps in the canalicular and basolateral membrane direct the way drug metabolites are excreted, i.e. via the bile or via the urine.

2.4 Organic Anion Transporting Polypeptides

Human organic anion transporting polypeptides (OATPs) are 12 transmembrane domain proteins that mediate the sodium-independent uptake of a wide range of amphipathic organic compounds (Kullak-Ublick et al. 1995; Tamai et al. 2000; Adachi et al. 2003). OATPs belong to the gene superfamily *SLCO* (formerly *SLC21*) which is subdivided into 6 families (OATP1-OATP6) and 13 subfamilies (Hagenbuch and Meier 2004). Their transport mechanism has not yet been fully elucidated. The suggested mechanism is anion exchange, in which the uptake of organic anions is coupled with the efflux of neutralizing anions such as bicarbonate and glutathione (Satlin et al. 1997; Li et al. 2000). OATP-mediated transport appears to be pH-dependent but independent of sodium, chloride and potassium gradients, membrane potential and ATP levels (Kullak-Ublick et al. 1995; Kobayashi et al. 2003; Mahagita et al. 2007). Most OATPs have a wide substrate spectrum and tissue distribution, including brain, kidney, testis, intestine and liver (Hagenbuch and Meier 2004). However, the OATP1 subfamily members OATP1B1 and OATP1B3 are localized exclusively in the basolateral membrane of hepatocytes, except for tumor tissues (König et al. 2000a; König et al. 2000b). The third OATP family member expressed in the sinusoidal membrane is OATP2B1, which is also expressed in other tissues such as intestine, placenta and ciliary body (Kullak-Ublick et al. 2001; St-Pierre et al. 2002; Kraft et al. 2010). Also OATP1A2 is expressed in the liver among other tissues but the liver expression is localized specifically to epithelial cells of the bile duct instead of hepatocytes (Lee et al. 2005).

OATP1B1 and OATP1B3 share overlapping substrate specificity and are capable of transporting a large variety of endogenous compounds, including bile salts such as taurocholate, steroid hormones and their conjugates such as estrone-3-sulfate, as well as thyroid hormones and bilirubin (Tamai et al. 2000; König et al. 2000b; Kullak-Ublick et al. 2001; Cui et al. 2001). In addition, numerous drugs have been identified as their substrates, including antihyperlipidemic drugs (ezetimibe, pravastatin, rosuvastatin, pitavastatin, fluvastatin), anti-histamines (fexofenadine), anticancer drugs (methotrexate) and antidiabetic drugs (repaglinide). (Kopplow et al. 2005; Shimizu et al. 2005; Seithel et al. 2007; Niemi et al. 2011). Three HMG-CoA inhibitors, fluvastatin, atorvastatin and rosuvastatin, are also substrates for OATP2B1 (Kopplow et al. 2005; Grube et al. 2006). Also two structurally related antibiotics, rifamycin SV and rifampicin, have been shown to interact with OATP-mediated transport. Rifampicin mainly inhibits OATP1B3, while rifamycin SV is a potent inhibitor of all human liver OATPs (Vavricka et al. 2002). The broad and partially overlapping substrate specificity of hepatic OATP transporters indicates that they play an important role in hepatic elimination process of many drugs together with other transporters with wide substrate spectrum, such as P-glycoprotein and MRPs.

There are species differences in the protein structure, functionality, substrate specificity and tissue distribution of transporters belonging to the *SLCO* gene superfamily. In rodents, 14 members of OATP/*SLCO* family (Oatps in rodents) have been identified, some of which do not exist in humans (Hagenbuch and Meier 2004). Three Oatp transporters, Oatp1a1, Oatp1a4 and Oatp1b2, are expressed in rat hepatocytes (Bergwerk et al. 1996; Reichel et al. 1999; Li et al. 2002). Direct human orthologues of these transporters have not been identified. Similar to OATP1A2, the tissue expression of Oatp1a1 and Oatp1a4 is not limited to liver but they are expressed also in other tissues such as kidney and brain, respectively (Li et al. 2002). They also share similar substrates with OATP1A2, including conjugated bile acids, sulfated steroids, β-lactam antibiotics and fexofenadine (Cvetkovic et al. 1999; Cattori et al. 2001). Oatp1b2 resembles OATP1B1 and -1B3 in substrate specificity and predominant expression in the sinusoidal membrane (Li et al. 2002). Members of OATP/*SLCO* family expressed in

the basolateral membrane of human and rat hepatocytes are summarized in Table 1, together with their tissue distribution and selected drug substrates.

Table 1. Summary of members of OATP/*SLCO* superfamily expressed in human and rat hepatocytes, their tissue distribution and selected substrates. Capital symbols stand for human genes and gene products while lower case symbols denote rodent genes and gene products (Kouzuki et al. 1999; Eckhardt et al. 1999; Cattori et al. 2001; Kullak-Ublick et al. 2001; Noe et al. 2007; Fahrmayr et al. 2010; Greupink et al. 2011; Varma et al. 2011).

Gene symbol	**Protein name**	**Substrates/Inhibitors**	**Tissue Distribution**
Slco1a1	Oatp1a1	Estrone 3-sulfate, taurocholate, rifamycin SV, pravastatin	Liver, kidney, brain
Slco1a4	Oatp1a4	Estrone 3-sulfate, taurocholate, rifamycin SV, rifampicin, atorvastatin, pitavastatin, pravastatin, rosuvastatin	Liver, brain, ciliary body, retina
SLCO1B1	OATP1B1	Estrone 3-sulfate, taurocholate, rifamycin SV, fluvastatin, atorvastatin, cerivastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin	Liver
Slco1b2	Oatp1b2	Estrone 3-sulfate, taurocholate, rifampicin, fluvastatin, lovastatin, pravastatin	Liver, ciliary body
SLCO1B3	OATP1B3	Estrone 3-sulfate, taurocholate, rifamycin SV, rifampicin, fluvastatin, pitavastatin, pravastatin, rosuvastatin	Liver, cancer cell lines
SLCO2B1	OATP2B1	Estrone 3-sulfate, rifamycin SV, fluvastatin, atorvastatin, rosuvastatin	Intestine, liver, placenta, ciliary body

3 PHARMACOKINETIC EFFECTS OF HEPATIC OATP TRANSPORTERS

3.1 Saturable Transport and Hepatic Drug Clearance

The transport capacity of the membrane transporters is limited. The transport process is saturable when the rate of transport ceases to increase in direct proportion to substrate concentration and approaches an upper limit (Rowland and Tozer 2011). Active, saturable transport follows Michaelis-Menten kinetics, where the rate of transport approaches a maximum, V_{max}, by the relationship:

$$V = \frac{V_{max} \times S}{K_m + S} \qquad \text{Equation 1}$$

in which K_m is the Michaelis-Menten constant and S is the substrate concentration at the transporter binding site. At substrate concentrations well below $K_{m,}$ rate of transport and substrate concentration vary in direct proportion and the transport kinetics is linear. However, at concentrations above K_m, the kinetics become nonlinear and show saturability as the transport rate approaches the value of V_{max}. Nonlinearity of saturable transport processes in drug elimination can be problematic since it may cause unwanted drug accumulation with increasing doses.

Cooperation of uptake and efflux transporters in transcellular transport of drugs and their metabolites has an essential role in the hepatic elimination process and may have a major impact on the systemic clearance and exposure of many drugs (Zamek-Gliszczynski et al. 2006; Nies et al. 2008). In addition to transporter-mediated pathways, drug transport across the hepatic membranes occurs also by passive diffusion. The importance of transporters in the hepatic clearance depends on the contribution of both the transporter-mediated pathways and the passive processes. The permeation clearance of unbound drugs for influx across sinusoidal membrane is comprised of transporter-mediated uptake clearance ($PS_{inf,act}$) and passive diffusional clearance (PS_{diff}) (Shitara et al. 2013). The overall hepatic clearance ($CL_{int,all}$) consists of permeation clearances of unbound drugs for uptake and efflux across the sinusoidal membrane (PS_{inf} and PS_{eff},

respectively) and the intrinsic clearance for metabolism and/or biliary excretion (CL_{int}) (Equation 2).

$$CL_{int,all} = \frac{PS_{inf,act} + PS_{diff}}{PS_{eff} + PS_{diff} + CL_{int}} \times CL_{int} \qquad \text{Equation 2}$$

For highly permeable drugs, neither active uptake nor efflux is the rate-limiting step in the overall hepatic clearance (Giacomini et al. 2010). However, most anionic drugs, as well as some hydrophilic organic cationic and zwitterionic drugs have poor membrane permeability (FDA 2000). For these drugs, membrane permeation is mainly dependent on active processes and uptake or efflux clearances can be rate-determining in some cases (Giacomini et al. 2010). Active hepatic uptake becomes the rate-determining process of the overall hepatic clearance when sinusoidal efflux clearance is negligible and CL_{int} is much larger than passive diffusional clearance (Shitara et al. 2013).

3.2 Drug-Drug Interactions

Due to the wide substrate specificity of OATP transporters, many simultaneously administered drugs compete of the same transporter binding sites causing saturation of transport capacity and inhibition of drug uptake. In addition, not all OATP inhibitors are OATP substrates but inhibit the uptake via other mechanisms. OATP-mediated drug-drug interactions (DDIs) may be clinically significant when OATP-mediated uptake is the rate-determining process of the overall hepatic clearance. This has been reported to be the case for many statins, such as atorvastatin, pravastatin and rosuvastatin. In a clinical study by Maeda et al. (2011), the oral coadministration of OATP1 inhibitor rifampicin increased the AUC (area under the plasma concentration-time curve) of atorvastatin, while coadministration of CYP3A4 inhibitor itraconazole did not change the plasma exposure of atorvastatin, although atorvastatin is metabolized by CYP3A4. The effects were similar for pravastatin, which is only minimally metabolized in the liver. Simonson et al. (2004) have published similar results for rosuvastatin when coadministered with immunosupressant cyclosporine, another OATP1 inhibitor. The AUC and maximum plasma concentration (C_{max}) values for rosuvastatin were increased significantly in heart transplant recipients on an antirejection regimen including

cyclosporine. In addition to rifampicin and cyclosporine, also antidiabetic drug repaglinide and macrolide antibiotics clarithromycin, erythromycin and roxithromycin have been shown to inhibit the OATP1B1 and -1B3-mediated uptake of pravastatin in *in vitro* studies (Jacobson 2004; Seithel et al. 2007; Bachmakov et al. 2008). Antidiabetic drug rosiglitazone, on the other hand, has been found to stimulate pravastatin uptake *in vitro* (Bachmakov et al. 2008). This finding suggests that also drug-induced stimulation of OATP1 mediated transport could cause alterations in drug plasma concentrations. Elevated statin plasma concentrations caused by OATP-mediated DDIs increase the risk for statin-induced side effects, such as myopathy and rhabdomyolysis (Thompson et al. 2003; Generaux et al. 2011). Moreover, the uptake of statins into hepatocytes is essential for their inhibitory action of microsomal HMG-CoA reductase (Dansette et al. 2000). Thus, reduced hepatic uptake of statins may also lead to decreased drug efficacy.

Knowledge on the drug-drug interactions of the third basolateral *SLCO* transporter OATP2B1 is much more partial than for OATP1B1 and -1B3 (Fahrmayr et al. 2010). Only a few drugs have been identified as OATP2B1 substrates compared to OATP1B1 and -1B3 (Tamai et al. 2000; Kopplow et al. 2005; Grube et al. 2006). One *in vitro* study by Noe et al. (2007) showed that gemfibrozil, fibrate used to treat hypercholesterolemia, inhibited OATP2B1-mediated uptake at high concentrations.

Overall, inhibition of OATP-mediated hepatic uptake may have a substantial effect on drug disposition, safety and efficacy together with other causes of drug interactions, such as inhibition or induction of metabolizing enzymes. The clinical relevance of OATP1 inhibition depends on the contribution of OATPs to the total hepatic clearance and overall drug disposition of substrate drugs (Shitara et al. 2013). OATP1 substrates can be also substrates of other transporters and transporters may have multiple binding sites, which makes the estimation of the transporter contribution more complex.

3.3 Pharmacogenetics and Interindividual Variability

Apart from drug-drug interactions, also genetic polymorphisms in OATPs affect the pharmacokinetics of substrate drugs causing interindividual variability in drug response

and safety. Similar to DDIs, altered activity of OATP transporters caused by genetic variations can result in undesirable systemic exposure of substrate drugs which may lead to drug accumulation and organ toxicity (Giacomini et al. 2013). Over 40 single-nucleotide polymorphisms (SNPs) have been identified in the *SLCO1B1* gene coding OATP1B1 (Tirona et al. 2001; Fahrmayr et al. 2010). The effect of genetic variations on the OATP1B1-mediated transport of HMG-CoA reductase inhibitors has been investigated in numerous studies both *in vitro* and *in vivo* (Kameyama et al. 2005; Pasanen et al. 2006; Deng et al. 2008; Rodrigues et al. 2011). Significant interindividual differences in plasma concentrations have been reported for statins such as atorvastatin, simvastatin and pravastatin (Niemi et al. 2004; Pasanen et al. 2006; Niemi et al. 2006; Deng et al. 2008). Niemi et al. demonstrated also that reduced uptake of pravastatin in patients expressing mutated OATP1B1 proteins caused significantly lower pharmacological effect on cholesterol synthesis (Niemi et al. 2005). Decreased hepatic uptake and elevated plasma concentrations caused by OATP1B1 polymorphisms have been reported also for several other drugs than statins, such as ezetimibe, repaglinide, lopinavir and methotrexate (Oswald et al. 2008; Kalliokoski et al. 2008; Hartkoorn et al. 2010; Ramsey et al. 2012).

Polymorphisms in the genes coding OATP1B3 and OATP2B1 have not been as comprehensively studied (Fahrmayr et al. 2010; Schwarz et al. 2011). Recent study by Nies et al. (2013) suggests that the expression of OATP1B3 and OATP2B1 is not significantly influenced by genetic variants but interindividual variability of these transporters is caused by non-genetic factors. In addition to genotype, number of other factors such as gender, age, disease state or the regulation of transporter expression may be causes of interindividual variability in transporter action. In the study by Nies et al. (2013), liver condition cholestasis resulted in reduced OATP1B1 and -1B3 expression levels in liver. Also a gender-related difference in pharmacokinetics of pravastatin has been reported in a study that compared the effects of OATP1B1 polymorphism on pharmacokinetics of pravastatin (Niemi et al. 2006). However, the current data about non-genetic factors in interindividual variability of OATP transporters is scarce and further studies are needed. Also the significance of polymorphisms in *SLCO1B3* and *SLCO2B1* genes remains unclear and needs to be further elucidated.

4 METHODS FOR EVALUATION OF DRUG INTERACTIONS WITH UPTAKE TRANSPORTERS

4.1 OATP Transporters in Drug Development

Screening for drugs for their ability to be transporter substrates and/or inhibitors is becoming more and more common in drug development process due to the growing knowledge on the impact of transporters on pharmacokinetics and unpredictable variation in drug efficacy and toxicity. In addition to predicting interindividual variability caused by polymorphisms and DDIs, characterization of transporter substrate drugs helps to understand the contribution of saturable transport processes in the overall drug disposition (Mizuno et al. 2003). In preclinical drug development, this can aid in the selection of dosing regimen.

Recently, the International Transporter Consortium (ITC) and both European Medicines Agency (EMA) and United States Food and Drug Administration (FDA) have recommended investigating for potential OATP1B1 and -1B3 substrates and inhibitors in drug development when hepatic clearance of the drug is significant, e.g. hepatic elimination is more than or equal to 25 % of the total clearance (Giacomini et al. 2010; FDA 2012; EMA 2012). Currently available methods for screening of drugs for their ability to be substrates or inhibitors of transporters vary from membrane based *in vitro* assays to *in vivo* genetic knockout animal models (Table 2). Also computational models have been developed for screening of large databases. Methods have been developed for both efflux and uptake transporter studies. The methods applicable for studying uptake transporters such as OATPs will be discussed in more detail in following sections.

Table 2. Methods used for evaluation of drug-transporter interactions.

Method Type	Application in Drug Development
***In vitro* methods**	
Membrane based assays	
Membrane vesicular transport assay	Screening of substrates/inhibitors of uptake/efflux transporters
ATPase assay	Evaluation of transport function of efflux transporters
Photolabeling assay	Evaluation of binding sites and affinities of efflux transporters
Cell based assays	
Uptake assay	Screening of substrates/inhibitors of uptake/efflux transporters
Cytotoxicity assay	Screening of inhibitors of uptake/efflux transporters
Transcellular transport assay	Evaluation of vectorial transport function of efflux and uptake transporters, permeation studies
***In silico* methods**	
Computational models	Prediction of substrate and inhibitor binding to uptake/efflux transporters
Pharmacokinetic models	Calculation of kinetic parameters, *in vitro/in vivo* extrapolation of pharmacokinetic data
***Ex situ* methods**	
Isolated organ models	Prediction of *in vivo* transporter binding and contribution to drug disposition
***In vivo* methods**	
Genetic/chemical knockout models	Evaluation of physiological function of transporters and contribution to drug disposition
Imaging	Visualization of transporter function *in vivo*, qualitative and quantitative evaluation of transporter function

4.2 Membrane Based Assays

Membrane based assays have been mainly used to characterize substrates and inhibitors of ATP-dependent *ABC* efflux pumps (Jedlitschky et al. 1997; Kis et al. 2009; Heredi-Szabo et al. 2013). Only one membrane based assay, the membrane vesicular transport assay, can be used to identify substrates and inhibitors of uptake transporters. In the assay, membrane vesicles expressing transporters of interest with substrate binding site facing outwards are incubated in a buffer mixture containing the drug (Meier et al. 1984). Substrates of the transporter are taken up into the vesicles and rapid filtration is used to separate the vesicles from the incubation solution. The test compound trapped inside the vesicles is retained on the filter and the transported molecules can be quantified by liquid chromatography / mass spectrometry (LC/MS), fluorescence detector or liquid scintillation counting. The membrane vesicles can be prepared from different sources such as transporter transfected or over-expressed cells and different tissue samples. NTCP function and substrates have been studied using rat liver sinusoidal membrane vesicles (Meier et al. 1984; Koopen et al. 1997). Since NTCP is a sodium dependent transporter, the assessment of NTCP activity is based on the difference of drug accumulation in the presence and absence of sodium. OATPs, however, are Na^+-independent transporters which makes the method inapplicable for screening of OATP substrates.

The membrane vesicular transport assay has the advantage of being relatively easy to conduct and feasible for high-throughput screening (Giacomini et al. 2010). It is an effective method to determine transporter functions and to define detailed kinetic analyses for substrate or inhibitor interaction. Its disadvantage is that highly lipophilic compounds may have non-specific binding to lipid membranes or high passive diffusion which may cause false negative results (Xia et al. 2007). In addition, membrane vesicles obtained from tissue samples may be problematic if they are not purified and contain many varied transporters. This may limit their usefulness for evaluating the interaction of a particular transporter with a given compound.

4.3 Cell Based Assays

Cell based assays are often more labor intensive and time consuming than membrane based assays but they may provide more comprehensive information about drug-transporter interactions due to intact cell structure and function. Cell based assay systems can be used for transporter substrate and inhibitor screening, assessment of transport mechanisms and transporter-mediated DDIs, and for determining the rate-limiting step in trans-epithelial transport.

4.3.1 Common Cell Models

Array of cell based systems used in the transporter interaction studies include primary cells, sandwich-cultured primary hepatocytes, polarized cell lines without recombinant transporters, single- and multiple transfected cell lines and *Xenopus laevis* oocytes. Primary cells isolated from intact tissue express the full spectrum of transporters present in a particular tissue. They are useful in investigation of the mechanistic interplay between uptake and efflux transporters and metabolism, as well as clinically relevant drug interactions (Soars et al. 2009). However, the polarization of the cells is quickly lost after isolation and the expression and localization of transporters may change over time in cell cultures (LeCluyse et al. 1996; Yang et al. 2012). The polarity of primary hepatocytes can be regenerated when cells are cultured in a sandwich configuration between two layers of collagen gel (LeCluyse et al. 1994; Liu et al. 1999). Sandwich-cultured hepatocytes can be used to estimate both hepatic uptake and efflux of drugs and *in vivo* biliary clearance.

Immortalized polarized cell lines are used to study vectorial transport and they enable the measuring of flux of substrates in two directions (apical to basolateral and vice versa) (Giacomini et al. 2010). They are standard methods to evaluate intestinal absorption and blood-brain barrier permeation. Some of the most used cell lines are human colon epithelial cancer cell line Caco-2, Madin-Darby canine kidney cell line (MDCK) and pig kidney proximal tubule epithelial cell line (LLC-PK1). Caco-2 cells express a large variety of transporters specific for intestinal epithelium while endogenous expression

level of transporters is low in MDCK and LLC-PK1 cells (Goh et al. 2002; Ahlin et al. 2009). MDCK and LLC-PK1 cells are often transfected with a single or multiple recombinant transporters. Recombinant transporter cell lines can be used for quantitative drug transport studies and to investigate transporter polymorphisms and species differences in transporter function. Several recombinant MDCK cell lines expressing OATP transporters have been developed, including OATP1B1 and MRP2 double transfected cells and OATP1B1, OATP1B3, OATP2B1 and MRP2 quadruple-transfected cells (Kopplow et al. 2005; Matsushima et al. 2005). Although the endogenous expression level of MDCK and LLC-PK1 cells is low, the contribution of the endogenous transporters should also be taken into account when using transfected cell lines (Goh et al. 2002).

Xenopus laevis oocytes are also widely used for transporter studies (Xia et al. 2007). Transporter mRNA or cDNA can be microinjected into oocytes and the cells perform the translation and post-translational modification which leads to appropriate transporter expression on the cell membrane (Sigel and Minier 2005). Oocytes have low endogenous transporter expression which makes them a good system for characterizing transporter substrates and inhibitors and determining kinetic analyses for drug-transporter interactions (Sobczak et al. 2010). Similar to single- or multiple transfected mammalian cells, they can also be used to study transporter polymorphisms and species differences of transporters (Xia et al. 2007). However, disadvantage of oocytes is a rather short expression turnaround time of a transporter. In addition, oocyte systems are only suitable for medium throughput screening due to the arduous microinjection method.

4.3.2 Uptake Assays

The involvement of a given influx transporter in drug uptake can be assessed using uptake assays. In uptake assays, the amount of test compound accumulated in the transporter-expressing cell is measured and the relative contribution of transporter is assessed comparing the uptake into control cells (Xia et al. 2007; Soars et al. 2009). Either isolated cells in suspension or cells seeded in 96-well tissue culture plates can be

used. In a standard uptake assay, cells are incubated in a substrate buffer, followed by the removal of the buffer and washing of the cells. The cells are lysed and the amount of test compound accumulated in the cells is quantified. Homogenous samples of the incubation mixture are taken over time and the concentration-time data can be used to calculate kinetic parameters such as K_m, V_{max} and uptake clearance (CL_{uptake}). Identification of substrates for a particular transporter can also be done by manipulating the ionic composition or pH of the incubation buffer if the transporter function is dependent on ion gradients or a given pH. This is a useful method for studying for example NTCP-mediated transport (Kouzuki et al. 1999). Uptake of the test compound is measured both in the presence and absence of Na^+ and the difference of the uptake in the two buffers is attributed to NTCP-mediated uptake. As for inhibition studies, the inhibitory ability of the test compound can be determined by co-incubating the test compound with a fluorescent or radiolabeled probe substrate and observing changes in the cell accumulation of the probe substrate.

Variations of the standard uptake assay are the oil-spin assay and the media-loss assay (Petzinger and Fückel 1992; Soars et al. 2007) (Figure 3). The oil-spin assay is similar to the standard assay with the exception that the sample taken from the incubation buffer is immediately centrifuged through an inert oil layer (Petzinger and Fückel 1992). The density of the oil layer is lower than that of the cells but higher than that of the medium. Thus, centrifugal filtration of the cells through the oil leads to efficient cell separation with very little extracellular fluid adhering to the cells. The test compound or probe substrate concentration is then measured as in the conventional assay. In the media-loss assay, the decreasing concentration of drug in the media over time is measured from the supernatant after centrifugation (Soars et al. 2007). Combining the media-loss assay with either the standard uptake assay or the oil-spin assay can provide even more accurate prediction of *in vivo* drug clearance.

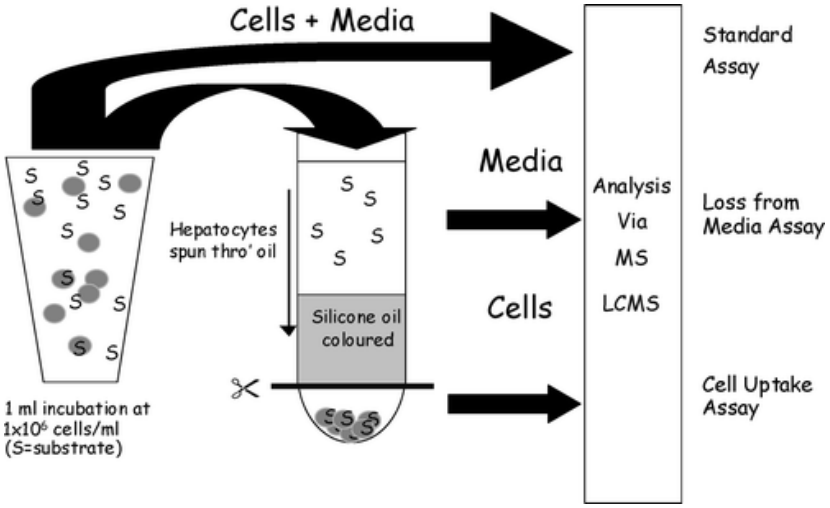


Figure 3. Different uptake assay formats. Media-loss assay and oil-spin assay differ from the standard assay in a way cells are separated from the incubation buffer. Sampling is followed by rapid centrifugation and the formed supernatant and cell pellets are further analyzed (Soars et al. 2009).

Human cryopreserved hepatocytes are a good cell model to study hepatic drug uptake since they express all the endogenous hepatic transporters. There can, however, be large interbatch differences in the uptake activities of cryopreserved hepatocytes (Shitara et al. 2003). Another downside is their high price. For this reason, also cryopreserved rat hepatocytes are widely used in uptake studies, together with transporter expressing cells such as MDCK cells, human embryonic kidney 293 (HEK293) cells and *Xenopus laevis* oocytes.

Uptake assays require multiple steps for washing or centrifuging, sample collection, transferring and processing. Thus, uptake assays have rather low throughput, especially if no fluorescent or radiolabeled substrates are used. One way to enhance the throughput is to use scintillation proximity assay (SPA) technology where transporter expressing cells are directly grown in Cytostar-T scintillating microplates (Bonge et al. 2000). Uptake of radiolabeled transporter substrates is measured in real time in a microplate scintillation counter as radioisotopes are transported into proximity with the scintillation plate base. The method has been used for transporter inhibitor identification using radiolabeled substrates of for example OATP1B1, OAT3 and OCT1 (Lohmann et al. 2007).

4.3.3 Cytotoxicity Assay

Another method for identifying transporter substrates and inhibitors is to measure the accumulation of cytotoxic compounds in cells. In the cytotoxicity assay, cytotoxic transporter substrates can be identified by comparing the IC_{50} (the concentration that inhibits the cell growth by 50 %) in control cells and cells expressing the transporter (Xia et al. 2007). Inhibitors of uptake transporters can be indirectly identified by their ability to decrease the cytotoxic effects of a known substrate, while inhibitors of efflux pumps can be identified by observing potential increase in the cytotoxicity (Xia et al. 2007; Kis et al. 2009; Ding et al. 2011). Cytotoxicity assays are applicable for high throughput screening of transporter substrates but it is only usable for cytotoxic compounds. Inhibitor studies only give indirect results and the results are best to confirm with other methods.

4.3.4 Transcellular Transport Assays

The coordination of uptake and efflux transporters in vectorial transport across membranes can be studied using cell lines that co-express uptake and efflux transporters (Matsushima et al. 2005; Kopplow et al. 2005). Cell monolayers are cultured on a Transwell insert membrane until they are differentiated and form a confluent monolayer of polarized cells. The transport assay system consists of a cell monolayer on a porous film which separates two fluid compartments representing the apical and basolateral sides of the cell (Figure 4). The substrate solution containing the test compound can be added either to the apical (upper) chamber or the basolateral (lower) chamber depending on the interest in apical-to-basolateral (A-to-B) or basolateral-to-apical (B-to-A) transport. Samples are taken from the receiving side and the cumulative amount of drug is plotted as function of time. If only passive diffusion affects permeation, the flux is assumed to be identical in both directions, but the participation of transporters results in saturable flux rate with increasing concentrations.

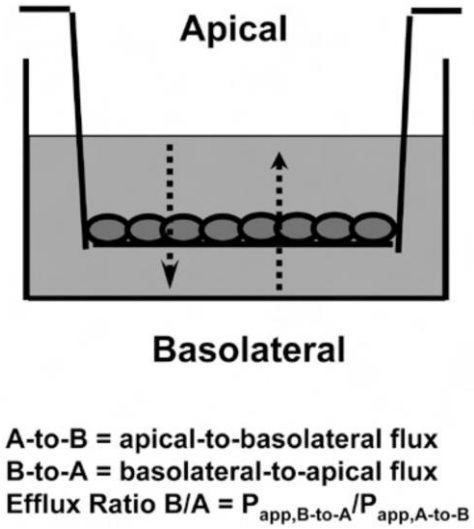


Figure 4. Transwell system used in transport assays. Upper chamber represents the apical side and lower chamber represents the basolateral side (Xia et al. 2007).

Double or multiple transfected cell lines can be used to demonstrate the contribution of an individual transporter to hepatic excretion. For example, the vectorial transport of OATP1B1 and OATP1B3 substrate olmesartan was studied using three different doubly- transfected MDCKII cell lines (Matsushima et al. 2005). The efflux transporter MRP2 responsible for the hepatic excretion of olmesartan was identified conducting transport assays in OATP1B3/MRP2, OATP1B3/P-gp and OATP1B3/BCRP double transfectants. Caco-2 cell monolayers are also used to investigate uptake and efflux transporter function using specific transporter inhibitors or probe substrates. However, transporter studies using Caco-2 cells may be non-specific due to the expression of multiple transporters in Caco-2 cells (Ahlin et al. 2009). There is also heterogeneity in transporter expression between different Caco-2 cell lines (Hayeshi et al. 2008). A novel approach to study the function of a particular transporter is to silence the expression of transporter genes by chemically synthesized RNA interference molecules (Yue et al. 2009). This method may enable the selective knock-down of endogenous transporter genes in mammalian cell lines.

Transport studies with transfected cell lines may not be good for predicting *in vivo* drug disposition and clearance due to the lack of endogenous variety of uptake and efflux transporters and metabolizing enzymes. Drugs may be substrates of multiple uptake and efflux transporters in intact organs and therefore transfected cells might not provide

knowledge on the complete mechanism for trans-cellular transport (Bartholome et al. 2007). Even Caco-2 cells that express multiple intestinal transporters vary in the transporter expression profile compared with primary intestinal epithelial cells (Taipalensuu et al. 2001). However, transport studies with Caco-2 cells provide a rather good method for prediction of drug intestinal permeability.

4.4 *In Silico* Methods

In silico modeling can be used in the early drug discovery and development phases as high-throughput methods to screen lead molecules for their potential to be transporter inhibitors and substrates. Together with *in vitro* methods, they aid in choosing compounds that have favorable characteristics for further development. In the preclinical phase, pharmacokinetic modeling is used to predict pharmacokinetics in humans and relationships between saturable active and passive transport can be examined in detail.

4.4.1 Computational Modeling

Computational modeling methods fill the gap between *in vitro* transporter properties and the knowledge on structural mechanisms of transporters on atomic level. They offer tools to predict ligand- and inhibitor-binding to transporters and to screen large databases for possible transporter substrates. *In silico* methods can be divided into structure-based approaches and substrate-based techniques (Chang and Swaan 2006). Structure-based models, such as homology or comparative modeling, are used to generate three-dimensional models of transporters based on available crystallographic data of appropriate template proteins. Homology and comparative models are based on the mutual sequence similarity of a template protein and the target protein. The model is usually constructed by applying the structural information of a template protein in the generation of a three dimensional structure of the target protein.

Substrate-based models, such as pharmacophore and 3D-QSAR (quantitative structure-activity relationship) modeling, are used to describe transporter protein’s structural

requirements for substrate or inhibitor interaction by correlating the biological activity of substrates and inhibitors with their molecular characteristics (Chang and Swaan 2006). Pharmacophore models can give information about the binding or inhibition process and help in designing more compounds interacting with transporters. They are also useful for large database screening to identify new transporter ligands. 3D-QSAR models can be used to assist the design of more potent transporter inhibitors or substrates with higher affinity. Pharmacophore and 3D-QSAR models have been generated for transporters such as P-gp, OCTs, OATs and PEPT1 (Biegel et al. 2005; Diao et al. 2010; Broccatelli et al. 2011; Duan et al. 2012). High throughput screening based on computational models is cost-effective and may help to reduce experimental efforts in the early phases of drug development. Downside of computational models is that most models can only be used to screen inhibitors, not substrates.

4.4.2 Pharmacokinetic Models

Pharmacokinetic models are widely used tools to describe and predict the processes of drug disposition *in vivo* using data gathered from *in vitro* studies. Models can be divided into empirical and mechanistic based on their approach (Yu and Wilson 2010). Empirical models describe relationships between drug exposure and effect without consideration of the underlying mechanisms. Mechanistic models, on the other hand, are based on these physiological mechanisms and they can be used to describe the interplay of multiple processes that affect drug disposition in different tissues. A model describing hepatic clearance can be used for simultaneous assessment of uptake, passive diffusion, intracellular binding, and metabolism using data gathered from experimental *in vitro* assays. Simple two-compartment models have been generated to study the hepatic uptake kinetics and metabolism of OATP substrates (Poirier et al. 2008; Menochet et al. 2012a). They enable the analysis of the whole *in vitro* data set in one step and can be used to quantify time-dependent nonlinearities of transporter uptake that are important in determining kinetic parameters of transporter substrates. A two-compartment model has also been used to study interspecies and interindividual differences between uptake of OATP substrates in rat and human hepatocytes (Menochet et al. 2012b).

Physiologically based pharmacokinetic (PBPK) modeling is a mechanistically based approach that integrates drug-dependent and human physiology-dependent parameters (Yu and Wilson 2010). PBPK models describe the body by incorporating parameters corresponding to tissue and organ volumes, blood-flow rates, drug transporters and metabolizing enzymes. Drug-dependent parameters include molecular weight, solubility, particle size, pKa, logP and plasma protein binding. The *in vivo* pharmacokinetic profile of a compound can be simulated for different species by using species dependent parameters available in the literature. In addition to providing predictions of human pharmacokinetics, PBPK models are also useful tools for predicting the relative importance of uptake and efflux transporters in the drug disposition, as well as transporter-mediated drug-drug interactions *in vivo*. For example, the effects of changes in OATP1B1 activity on systemic and hepatic exposure of pravastatin has been simulated using a PBPK model that includes blood, liver and peripheral organs (Watanabe et al. 2009). Also a PBPK model to predict DDIs of pravastatin caused by OATP1B1 inhibitors, cyclosporine, gemfibrozil and rifampicin has been recently established (Varma et al. 2012).

Overall, PBPK modeling has been shown to be a powerful tool to extrapolate animal pharmacokinetic data to humans. Compared to allometric approach, in which body weight of different animal species is used to extrapolate pharmacokinetic parameters to humans, PBPK modeling has proven to be produce more precise predictions of human pharmacokinetics (Kirman et al. 2003; Strougo et al. 2012). Both EMA and FDA recommend the use of PBPK models in the prediction of transporter-mediated drug-drug interactions (FDA 2012; EMA 2012). The limitation of PBPK modeling is that it requires intense resources to generate the data on the various parameters described in the models.

4.5 Intact Organ and *in Vivo* Models

Models utilizing intact organs or live animals provide information about physiological functions and effects of transporters and are used as tools to predict pharmacokinetics of drugs in humans. Compared to *in vitro* methods, isolated organs or *in vivo* models allow

a more accurate determination of transporter contribution in absorption, hepatic elimination, renal excretion and brain penetration. Isolated and perfused organs are a good tool to study transporter function in a given tissue because the concentration of the drug in the organ can be controlled and the effect of other organs is eliminated (Xia et al. 2007). By using specific transporter inhibitors, contribution of a given transporter to the pharmacokinetic profile can be assessed. For example, contribution of Oatp transporters in hepatic clearance of digoxin and atorvastatin has been examined in rat liver perfusion studies using rifampicin and rifamycin as selective Oatp inhibitors (Lau et al. 2004; Lau et al. 2006; Weiss et al. 2008). In live animals, the conventional method to study drug pharmacokinetics and distribution is to collect blood, urine and tissue samples after the administration of radiolabeled or non-labeled drug (Jaisue et al. 2010; van de Steeg et al. 2011). The amount of accumulated drug in the samples can then be analyzed. In order to get a pharmacokinetic profile, samples are taken or animals are sacrificed at different time points.

4.5.1 Genetic and Chemical Knockout Models

Genetic knockout mice and naturally-occurring transporter-deficient animals are widely used to investigate the physiological function of a specific targeted transporter. Genetic knockout mice are generated by disrupting the endogenous transporter gene while natural mutant animals have a spontaneous mutation in a transporter gene. Spontaneous mutations of P-gp and Mrp2 genes have been identified in subpopulations of mice, rats and dogs (Buchler et al. 1996; Lankas et al. 1997; Mizukami et al. 2012). Genetic knockout of numerous different uptake and efflux transporter genes has been used to investigate the physiological contribution of transporters on drug absorption, tissue distribution and elimination (Xia et al. 2007; Giacomini et al. 2010). Recently, studies on Oatp1 (Slco1a/1b-/-) knockout mice have been used to investigate the impact of Oatp1 transporters on in vivo disposition of anticancer drugs paclitaxel and methotrexate and antihistamine fexofenadine (van de Steeg et al. 2010; van de Steeg et al. 2011). Also humanized transgenic mice expressing human OATP1B1, OATP1B3 and OATP1A2 transporters instead of endogenous Oatp1a and -1b transporters have been generated (van de Steeg et al. 2009). Van de Steeg et al. have studied the

pharmacokinetics of methotrexate and paclitaxel also in the humanized transgenic mice (van de Steeg et al. 2013).

Limitations of knockout and mutant models include species, strain, sex, diet and housing condition differences which have to be taken into consideration when interpreting data and extrapolating findings across species (Giacomini et al. 2010). Strain and gender differences in transporter expression have been reported and there are definitive species differences in transporter expression, function, substrate affinity and interplay between transporters and metabolizing enzymes (Merino et al. 2005; Li et al. 2008). Humanized transporter animal models can at least partly help in translation of preclinical *in vivo* data to the clinical phase. Another problem in both knockout models and humanized transporter animal models is that deletion or addition of one transporter may cause changes in the expression of other transporters or enzymes and in the physiology of the animal (Lam et al. 2005; van de Steeg et al. 2010). These potential changes have to be taken into consideration when pharmacokinetics or toxic effects of drugs are assessed.

Also chemical knockout of transporters using transporter inhibitors can be used to evaluate transporter function in drug disposition. Mice or rats are treated with a selective transporter inhibitor and the pharmacokinetics of test compounds are investigated in the presence of the inhibitor. Selective uptake inhibitors are available for P-gp and BCRP but so far no specific inhibitors for uptake transporters have been identified (Allen et al. 2002; Shepard et al. 2003). Chemical and genetic knockout models can also be combined. The selection of appropriate dose of the inhibitor is important in order to achieve inhibitory effect *in vivo*. The specificity of the transporter inhibitor is also important and the limitation of chemical knockout models is that it may be hard to find inhibitors that inhibit specifically only one type of transporters.

4.5.2 Imaging

Imaging techniques are used to visualize transporter functions *in vivo* and to qualitatively and quantitatively evaluate the role of transporters in humans. Positron

emission tomography (PET) and single photon emission computed tomography (SPECT) are non-invasive methods that can be used for accurate measurement of pharmacokinetic end points in animals and humans by detecting radioactivity emitted by radiolabeled tracers (Fischman et al. 2002). Utilization of genetic knockout mice and specific transporter inhibitors in imaging studies can help to evaluate the impact of a given transporter. For example, PET has been used to evaluate hepatobiliary transport of OATP1B1 and OATP1B3 substrates in humans with or without OATP1 inhibitor rifampicin administration (Takashima et al. 2012). Using rifampicin-treated mice and genetic knockout mice unable to express the uptake transporters Oatp1a/1b or Mrp2, the hepatic uptake and biliary efflux of was recently studied using SPECT imaging (Neyt et al. 2013). The study showed that the hepatic uptake and efflux of (99m)Tc-mebrofenin was impaired in *Slco1a/1b* and *Abcc2* knockout mice, respectively. Rifampicin administration had similar effect on hepatic uptake and efflux. The results demonstrated that hepatic uptake and biliary efflux can be quantified simultaneously *in vivo* using imaging techniques. Also species differences in drug transport can be studied using imaging techniques. The whole-body distribution and OATP1B3/Oatp1 mediated hepatic transport of antihypertensive drug telmisartan in humans and rats have been studied by Shimizu et al (2012).

The drawbacks of imaging techniques include the lack of useful probes for in vivo functional characterization of many transporters and the inability to distinguish the parent drug from its metabolites (Fischman et al. 2002; Takashima et al. 2012). Many imaging techniques can also be rather expensive and specialized experts of organic radiochemistry are required for PET and SPECT imaging. However, molecular imaging methods can significantly accelerate the drug development process by substituting time-consuming dissection and tissue analysis (Rudin and Weissleder 2003). Also, multiple physiological or functional parameters can be obtained in a single study and longitudinal studies can be conducted using the same animal. In addition to decreasing the number of animals needed, this increases the statistical relevance of a study as each animal serves as its own control. The pharmacokinetic data collected in preclinical imaging supports clinical dose decisions by establishing evidence of the in vivo biological activity and safety profile of the lead compounds.

5 AIM OF THE STUDY

The aim of the experimental part of the master's thesis was to determine if entacapone and fluvastatin are actively transported from blood into rat and human hepatocytes through the sinusoidal membrane, and to assess the role of OATP transporters in the hepatic uptake of the drugs of interest.

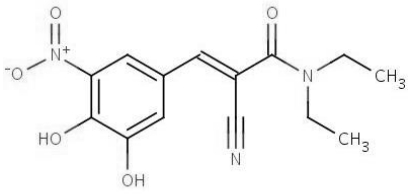
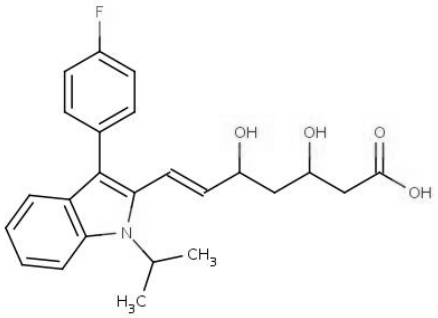
6 MATERIALS AND METHODS

6.1 Drugs and Chemicals

6.1.1 Entacapone and Fluvastatin

Sodium salt of fluvastatin was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Entacapone was provided by Orion Pharma (Espoo, Finland). Properties of entacapone and fluvastatin are summarized in Table 3.

Table 3. Properties of entacapone and fluvastatin (Noe et al. 2007; Varma et al. 2011; Greupink et al. 2011).

	Entacapone	**Fluvastatin**
Structure		
MW	305,29	411,47
Solubility	Lipophilic	Lipophilic
Hepatic transporter binding	No published data	OATP1B1, OATP1B3, OATP2B1, NTCP

Uptake studies with entacapone were performed with concentration range of 1 - 400 µM. Fluvastatin was used in the uptake studies with a concentration range of 1 - 50 µM. Substrate solutions were prepared in Krebs-Henseleit buffer (KHB), unless Na^+ -free choline buffer was used in the experiment, in which case corresponding buffer was used in substrate solutions.

7.1.2 Model Substrates

Taurocholic acid (taurocholate) and estrone 3-sulfate (E3S) were used as model substrates of Oatp1 and Ntcp transporters. For quantification purposes, model substrate solutions contained 1 % (V/V) of ^{3}H-labeled model substrate. E3S was used in competitive studies together with non-radiolabeled compounds. Estrone 3-sulfate sodium salt and taurocholic acid sodium salt hydrate were purchased from Sigma-Aldrich (Buchs, Switzerland), whereas [^{3}H]-estrone-3-sulfate ammonium salt with a specific activity of 57,3 Ci/mmol and [^{3}H]-taurocholic acid with a specific activity of 5 Ci/mmol were purchased from Perkin Elmer (Waltham, MA, USA).

7.1.3 Control Compounds

Sodium salt of rifamycin SV was used in the experiments as a broad Oatp1 inhibitor in order to rule out the effect of Oatp1-mediated active transport on the uptake of the investigated compounds. Highly lipophilic β-blocker (±)-propranolol hydrochloride was used as a control compound for passive transcellular permeation. [^{3}H]-inulin (specific activity 0,25 µCi/ml) was used as a non-permeable control compound. As a polysaccharide with high molecular weight, inulin does not pass through most cell membranes (Middleton 1977; Duff et al. 2002). Rifamycin, propranolol hydrochloride and [^{3}H]-inulin were purchased from Sigma-Aldrich (Buchs, Switzerland).

7.1.4 Hepatocyte Medium

Krebs-Henseleit buffer (KHB) was used to maintain hepatocyte viability during the uptake experiments. KHB was prepared in Milli-Q water, and it consisted of 142 mM

sodium chloride, 1,20 mM anhydrous magnesium sulfate, 0,96 mM potassium phosphate monobasic, 4,83 mM potassium chloride, 5 mM D-glucose, 1,53 mM calcium chloride dehydrate and 12,5 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), all from Sigma-Aldrich (Buchs, Switzerland), and 23,8 mM sodium bicarbonate (J.T. Baker, Phillipsburg, NJ, USA). pH was adjusted to 7,4 by using 2 M sodium hydroxide (Reagena, Toivala, Finland). Solution was sterilized by filtration using a Nalgene bottle top filter with 0,2 µm pore size (Apogent Technologies, Rochester, NY, USA).

Na^+ -free choline buffer was used instead of KHB in one experiment in order to rule out the effect of Na^+ -dependent active transport. The composition of choline buffer was the same as that of Krebs-Henseleit buffer except that sodium chloride and sodium bicarbonate were replaced by choline chloride (Fluka Biochemika, Buchs, Switzerland) and tris(hydroxymethyl)aminomethane (Tris base) (Sigma-Aldrich, Buchs, Switzerland), respectively.

7.2 Hepatocytes

To evaluate the difference in uptake kinetics between murine and human hepatocytes, both rat and human hepatocytes were used in the study. Due to limited availability of freshly isolated hepatocytes, part of the experiments with rat hepatocytes were conducted using cryopreserved cells, as were all of the experiments with human hepatocytes.

The cryopreserved cells used in the study were pooled male Wistar rat hepatocytes and human hepatocytes from one female donor. Both rat and human hepatocytes were purchased from Celsis IVT (Baltimore, MD, USA). Before the experiments, cryopreserved cells were thawed in 37 °C water bath long enough to melt the solution and mixed with pre-warmed thawing medium (InVitroGRO™ HT, Celsis IVT, USA). After centrifugation at 600 rpm for 5 minutes (Heraeus Megafuge 1.0, Thermo Fisher Scientific, USA) and removal of the supernatant, hepatocytes were diluted to pre-warmed KHB to a density of 2×10^6 cells/ml. Hepatocytes were calculated and viability

was determined microscopically (Olympus CK40-F200, Tokyo, Japan) by eosin Y staining using a hemocytometer. Cell suspension was stored on ice until needed, and the uptake studies were conducted in a time period no longer than 2 hours after the hepatocytes were thawed.

Fresh rat hepatocytes were isolated from eight-week-old male Wistar rats (HanTac:WH strain, Taconic Europe, Denmark) by a two-step *in situ* collagenase perfusion method (Berry and Friend 1969). Cell viability was determined by eosin Y staining. Liver perfusion and cell isolation of the freshly isolated hepatocytes was conducted by qualified personnel. Isolated cells were diluted to a density of 2 x 10^6 cells/ml with cold KHB and stored on ice until needed. Uptake studies were conducted no longer than 4 hours after the liver perfusions.

7.3 Cellular Uptake Experiments

To characterize the uptake kinetics of the compounds of interest, uptake experiments were performed using the oil-spin method (Yabe et al. 2011). Substrate associated with the hepatocytes was measured after separation from free substrate by a rapid centrifugation of the substrate solution over an inert silicone/mineral oil layer covering an alkaline solution layer (Figure 5).

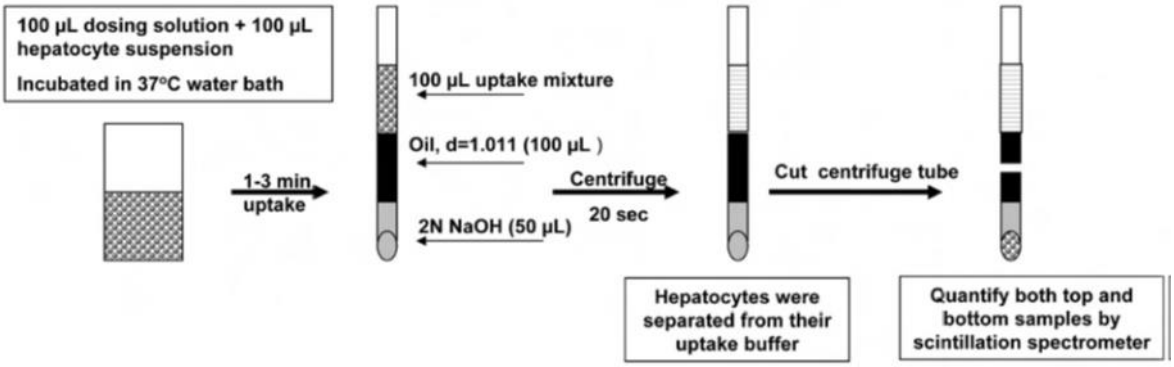


Figure 5. Flow chart of hepatocyte uptake assay using the oil-spin method (Xia et al. 2007).

Prior to the start of the assays, the hepatocyte solutions were preincubated on an orbital shaker (Heidolph Titramax 100, Schwabach, Germany) at 37 °C for 15 minutes. The uptake assay was initiated by adding equal volume of hepatocyte solution to prewarmed substrate solution. The reaction mixture was incubated at 37 °C on a thermoblock (700 rpm) (Eppendorf Thermomixer Comfort, Hamburg, Germany). In each experiment, incubations were carried out in triplicate. To determine intracellular and extracellular concentrations, 100 µl aliquots of the reaction mixture were pipetted into microcentrifuge tubes containing 100 µl 2 M NaOH covered by 100 µl of silicone-mineral oil (BD Gentest, San Jose, CA, USA). At designated time points (30, 90, and 180 s), the reaction was terminated by centrifuging the samples at 14 000 rpm for 30 seconds in a tabletop microcentrifuge (Eppendorf MiniSpin Plus, Hamburg, Germany). After separation of cells from the media, the tubes were kept on ice before freezing at -20 ºC. For normalization of the uptake activity, 100 µl of each reaction mixture was added to 100 µl of NaOH without oil filtration in order to measure the total radioactivity in 100 µl of each reaction mixture. Samples were stored at -20 ºC before sample preparation.

Uptake experiments for the non-radiolabelled substrate solutions of fluvastatin and entacapone were performed as described for radiolabelled compounds, with the exception that 5 M ammonium acetate solution was used as the bottom layer instead of sodium hydroxide.

7.4 Sample Preparation

7.4.1 Sample Preparation for Liquid Scintillation Analysis

Frozen microcentrifuge tubes were cut in the center of the oil layer and the bottom halves containing the cell pellets were placed into scintillation vials. After cutting, cell samples were allowed to sit in room temperature for 1-2 hours in order to let NaOH dissolve the hepatocytes, followed by the addition of 100 µl of 2 M hydrogen chloride (Reagena, Toivala, Finland) to neutralize the samples. Scintillation cocktail (Ecoscint A, National Diagnostics, Atlanta, GA, USA) was then added to the vials and cell

suspension was pipetted out of the microcentrifuge tubes and mixed with the scintillation cocktail by vortex and manual pipetting.

For measurement of extracellular substrate concentration, top halves of the microcentrifuge tubes containing the supernatant were collected into scintillation vials. Supernatant samples were pipetted out of microcentrifuge tubes inside the vials and mixed with scintillation cocktail.

7.4.2 Sample Preparation for UPLC/MS Analysis

Non-radiolabelled samples of fluvastatin and entacapone were prepared for ultra performace liquid chromatography / mass spectrometry (UPLC/MS) analysis. Aliquots were pipetted from the upper supernatant samples, pipetted into eppendorfs and diluted with methanol. Microcentrifuge tubes were then cut as previously described, after which bottom samples were pipetted into eppendorfs. Samples were precipitated with 100 µl of methanol (J.T. Baker, Phillipsburg, NJ, USA) and centrifuged at 14 000 rpm for 5 minutes. The supernatants were pipetted into UPLC vials.

7.5 Quantification and Metabolite Screening

7.5.1 Quantification of Radiolabeled Samples

Radiolabelled samples were analyzed using a liquid scintillation counter (Perkin Elmer Wallac 1414, Waltham, MA, USA). Quantification was performed by first calculating the average radioactivity (decays per minute, DPM) per pmole of model substrate in 100 µl of reaction mixture, and comparing this value to the radioactivity of each sample.

7.5.2 Quantification of Non-Radiolabeled Samples

Non-radiolabeled samples were analyzed using Waters Acquity UPLC system (Milford, MA, USA) coupled to Sciex API 4000 triple quadrupole MS detector (Foster City, CA, USA). For entacapone samples, separation was accomplished with a 2,1 x 50 mm

UPLC BEH C18 column (Waters, Milford, MA, USA). For fluvastatin samples, separation was done with a 2,1 x 100 mm Purospher Star Hibar C18 column (Merck, Darmstadt, Germany). Chromatographic conditions for both compounds are summarized in Table 5.

Table 5. Chromatographic conditions for entacapone and fluvastatin.

	Entacapone	**Fluvastatin**
Mobile Phase A	0,1 % Formic acid, pH 3	0,1 % Formic acid, pH 3
Mobile Phase B	Acetronitrile	Acetonitrile/Methanol 50:50 (V/V)
Column Temperature (°C)	55	50
Flow Rate (µl/min)	800	400
Gradient Run	95-10 % B in 3 min, hold 0,5 min, then to 95 % B over 0,1 min	30 % A 70 % B

The quantification of the test compounds in the samples was performed by comparing the samples' peak areas to a calibration curve plotted with a set of external standards. Fluvastatin standard curve ranged from 10 nM to 10 µM. Entacapone standard curve ranged from 10 nM to 50 µM.

7.5.3 Fluvastatin Metabolite Screening

The presence of main metabolites of fluvastatin in cell and supernatant samples was investigated in UPLC/MS/MS analysis using the same devices and chromatographic conditions as for fluvastatin quantification. The concentration of metabolites in the samples was not quantified.

7.6 Data Analysis

The initial uptake velocity (V) of test compounds was determined from the slope of the plot of substrate amount in hepatocytes versus time. Initial uptake velocity values were used to calculate hepatic uptake clearance (CL_{uptake}) by dividing by substrate

concentration in the reaction mixture. Passive uptake rate (P_{diff}), Michaelis-Menten constant (K_m) and maximum uptake rate (V_{max}) were calculated by simultaneous fitting of all time and concentration points using nonlinear regression in GraphPad Prism (GraphPad Sofware, San Diego, CA, USA) by using the following equation:

$$V = \frac{V_{max} \times S}{K_m + S} + P_{diff} \times S \qquad \text{Equation 3}$$

where S is the substrate concentration in the reaction mixture. Total uptake clearance (CL_{uptake}) included both the active (CL_{active}) and passive component (P_{diff}). The saturable active hepatic uptake was estimated by V_{max}/K_m and the contribution of saturable uptake of the total uptake by using equation 4:

$$\text{The contribution of active hepatic uptake (\%)} = \frac{V_{max} / K_m}{V_{max} / K_m + P_{diff}} \times 100 \qquad \text{Equation 4}$$

The inhibitor concentration that corresponds to the 50% of the maximum inhibition of E3S uptake transport (IC50) was determined for rifamycin SV and entacapone by fitting the uptake clearance data to the following equation (5) using nonlinear regression in GraphPad Prism.

$$Y = bottom + (top - bottom) \times \left(\frac{IC50}{X + IC50}\right) \qquad \text{Equation 5}$$

7 RESULTS

7.1 Cell Viability and Non-Specific Binding

The viability of cell suspensions used in the experiments ranged from 93 % to 79 % for freshly isolated rat hepatocytes, from 82 % to 68 % for cryopreserved rat hepatocytes and from 82 % to 72 % for cryopreserved human hepatocytes. After the experiments,

the viability of the cells had decreased to an average of 54 % with no significant difference between freshly isolated or cryopreserved hepatocytes.

The integrity of the cells and non-specific binding to cell membranes was investigated with an oil-spin assay using cryopreserved rat hepatocytes and 1 µl/ml [^{3}H]-inulin. Low amount of radioactivity was detected in the samples but the amount did not significantly change over time which indicates that inulin seemed to bind to the cells to a small degree without accumulation into the cells (Table 6).

Table 6. Radiolabeled inulin in cell samples expressed as a percentage of the radioactivity in the reaction mixture. Cryopreserved rat hepatocytes were incubated with 1 µl/ml [^{3}H]-inulin at 3 different time points under 3 minutes.

Time (s)	**Percentage of inulin in cell samples**			
	Sample 1	Sample 2	Sample 3	avg ± std
30	0,03	0,18	0,03	0,08 ± 0,09
90	0,03	0,04	0,20	0,09 ± 0,10
180	0,14	0,03	0,03	0,07 ± 0,07

7.2 Kinetic Evaluation of Taurocholate, Fluvastatin and Entacapone Uptake

A time-dependent increase in intracellular concentration and a concentration-dependent increase in uptake rate was observed for all the compounds investigated. The uptake rates of the investigated compounds mainly showed a decrease after 90 second time point, as is shown for taurocholate in Table 8.

Table 8. Uptake rate of sodium taurocholate in freshly isolated rat hepatocytes calculated using time intervals from 30 seconds up to either 90 or 180 seconds. Values are represented as means of triplicate samples ± standard deviation. N = 1

Taurocholate concentration (µM)	**V (pmol/min/10^6 cells), 30 s - 90 s**	**V (pmol/min/10^6 cells), 30 s - 180 s**
1	109 ± 42,7	30,0 ± 10,1
10	501 ± 247	299 ± 102
25	888 ± 720	496 ± 200
50	923 ± 1110	932 ± 497
100	1470 ± 1240	1660 ± 961

Thus, the kinetic parameters were calculated using 30 and 90 second time points since this time interval seemed to represent the linear initial uptake velocity better than the time interval up to 180 seconds.

In each experiment with multiple substrate concentrations, extracellular radioactivity was determined in supernatant samples of one concentration of taurocholate or estrone 3-sulfate. Taurocholate and E3S amount in supernatant samples decreased in proportion to cellular uptake and the sum of radioactivity (dpm) detected in the cell and supernatant samples did not differ significantly from the radioactivity in reaction mixtures in any of the experiments, as is shown for 10 µM taurocholate in Table 7.

Table 7. Combined radioactivity in supernatant and cell samples expressed as a percentage of the radioactivity in the reaction mixture. Freshly isolated rat hepatocytes were incubated with 10 µM [^{3}H]-sodium taurocholate at 3 different time points under 3 minutes.

Time (s)	**Percentage of combined radioactivity in cell and supernatant samples**			
	Sample 1	Sample 2	Sample 3	avg ± std
30	104	101	99,9	102 ± 2,1
90	103	104	100	103 ± 2,0
180	104	101	100	102 ± 2,1

Concentration-dependent uptake rates of taurocholate, fluvastatin, and entacapone in freshly isolated rat hepatocytes are represented in Figure 6, together with time curves of uptake. For entacapone, the UPLC method had a significant carryover effect. Consequently, the results for samples with lowest concentrations were not reliable and were not used in the calculation of uptake rate and clearance. Moreover, the quantified concentrations of entacapone and fluvastatin reaction mixtures were lower than they were prepared to be. The quantified concentrations were used to calculate the kinetic parameters of the drugs.

Uptake rates of taurocholate and fluvastatin showed saturability with increasing concentrations, whereas entacapone uptake was linear and showed no saturability (Figure 6). At fluvastatin concentration 25 µM, V and CL_{uptake} were negative in the time

interval up to 90 seconds. This data point was removed from nonlinear regression analysis in order to get reliable results. The uptake rates and clearances of taurocholate, fluvastatin and entacapone at different concentrations are summarized in Table 9.

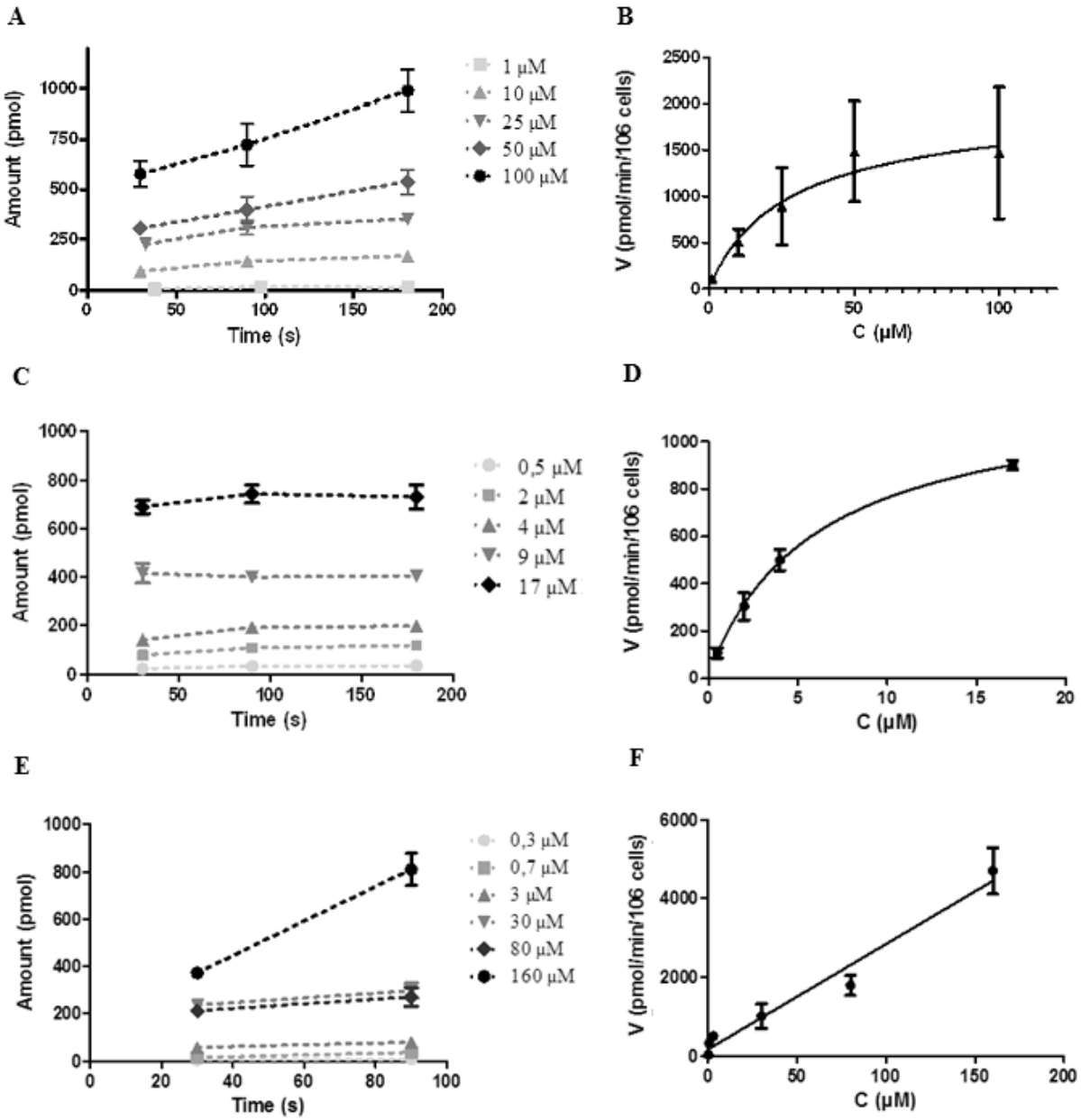


Figure 6. The uptake of sodium taurocholate (A,B), fluvastatin (C,D) and entacapone (E,F) into freshly isolated rat hepatocytes. The initial uptake rates (B,D,F) are calculated using 30 second and 90 second time points. Values are represented as means of triplicate samples ± standard deviation. N = 1

Table 9. Initial uptake rate and uptake clearance of taurocholate, fluvastatin and entacapone in freshly isolated rat hepatocytes. Values are represented as means of triplicate samples ± standard deviation. The quantified concentrations of entacapone and fluvastatin solutions are presented in parentheses. N = 1

C *(µM)*	**V** *(pmol/min/10^6 cells)*	**CL_{uptake}** *(µl/min/10^6 cells)*
Taurocholate		
1	109 ± 42,7	109 ± 42,7
10	501 ± 247	50,1 ± 24,7
25	888 ± 720	35,5 ± 20,6
50	923 ± 1110	18,5 ± 22,0
100	1470 ± 1240	14,7 ± 12,4
Fluvastatin		
1 (0,5)	101 ± 37,0	217 ± 79,2
5 (2)	305 ± 100	176 ± 57,8
10 (4)	500 ± 76,9	128 ± 19,6
25 (9)	-173 ± 326	-19,1 ± 35,8
50 (17)	533 ± 635	30,8 ± 36,7
Entacapone		
1 (0,3)	< LOQ	< LOQ
3 (0,7)	< LOQ	< LOQ
10 (3)	546 ± 280	172 ± 88,2
50 (30)	620 ± 990	24,7 ± 39,4
100 (80)	1060 ± 578	17,5 ± 9,49
200 (160)	4340 ± 1500	26,8 ± 9,25

The uptake clearance of taurocholate differed in freshly isolated and cryopreserved rat hepatocytes. Uptake clearance was lower in cryopreserved hepatocytes with every concentration tested, while the relative decrease in clearance with increasing concentrations was larger in freshly isolated hepatocytes (Table 10). The uptake of taurocholate and entacapone was also investigated in cryopreserved human hepatocytes. The uptake clearance of both compounds was lower in human hepatocytes compared to freshly isolated rat hepatocytes (Table 10). For entacapone, the difference was more drastic. In rat hepatocytes, entacapone uptake clearance decreased somewhat linearly

with increasing concentrations, while in human hepatocytes uptake clearance seemed to stay at a constantly low level in the concentration range of 10 – 100 µM.

Table 10. Uptake clearance of taurocholate and entacapone in freshly isolated and cryopreserved rat hepatocytes and cryopreserved human hepatocytes. Values are represented as means of triplicate samples ± standard deviation. N = 1, *Quantified C = 3 µM, 30 µM, 80 µM **Quantified C = 5 µM, 26 µM, 70 µM

	CL_{uptake} in freshly isolated rat hepatocytes *(µl/min/1e6 cells)*	**CL_{uptake} in cryopreserved rat hepatocytes** *(µl/min/1e6 cells)*	**CL_{uptake} in cryopreserved human hepatocytes** *(µl/min/1e6 cells)*
Taurocholate			
1 µM	109 ± 42,7	27,9 ± 0,29	64,6 ± 8,74
10 µM	50,1 ± 24,7	23,9 ± 25,8	12,7 ± 8,91
25 µM	35,5 ± 20,6	17,9 ± 3,83	--
50 µM	18,5 ± 22,0	--	4,01 ± 7,83
100 µM	14,7 ± 12,4	1,32 ± 0,44	--
Entacapone			
10 µM	172 ± 88,2*	--	5,08 ± 5,67**
50 µM	24,7 ± 39,4*	--	3,11 ± 1,61**
100 µM	17,5 ± 9,49*	--	2,21 ± 1,75**

The Michaelis-Menten constant (K_m) was higher for taurocholate compared to fluvastatin, being 24 µM and 6 µM, respectively (Table 11). Also maximum uptake rate (V_{max}) and passive uptake rate (P_{diff}) were lower for fluvastatin. The saturable components for taurocholate and fluvastatin uptake were 82 % and 94 % of the total uptake, respectively.

Table 11. Kinetic parameters for the hepatic uptake of taurocholate and fluvastatin in freshly isolated rat hepatocytes. Values represent the mean of triplicate samples and with standard error presented in parentheses. N = 1

	K_m *µM*	**V_{max}** *pmol/min/ 10^6 cells*	**P_{diff}** *µl/min/ 10^6 cells*	**V_{max}/K_m** *µl/min/ 10^6cells*
Taurocholate	24,49 (39,60)	1778 (793,7)	15,92 (446,5)	72,60
Fluvastatin	5,82 (2,006)	1171 (114,6)	11,77 (57,84)	201,3

Goodness of fit and constraints for nonlinear regression analysis used in the calculation of parameters are summarized in Appendix 1. Respective kinetic parameters of entacapone could not be calculated due to the lack of saturability in the uptake data.

7.3 Fluvastatin Metabolism

In order to investigate the effect of metabolism on intracellular concentration of fluvastatin, cryopreserved rat hepatocytes were incubated in 10 µM fluvastatin solution and fluvastatin concentration in cell and supernatant samples was determined at six different time points during 15 minute time interval. Fluvastatin concentration in cell samples increased until 5 minute time point, being an average of 905 nM (Figure 7). At 15 minute time point, fluvastatin concentration in cell samples had decreased to an average of 624 nM. Extracellular fluvastatin concentration decreased in proportion to fluvastatin uptake, with more rapid decrease in concentration during first 3 minutes of incubation.

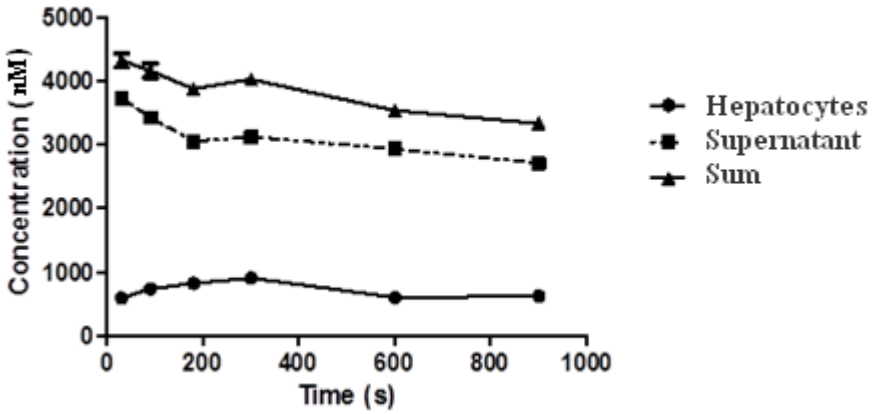


Figure 7. Comparison of fluvastatin concentration (µM) in the cell (●) and supernatant (■) samples during 15 minute incubation of cryopreserved rat hepatocytes in 10 µM sodium fluvastatin solution. Sum of fluvastatin (µM) in cell and supernatant samples (▲). Each symbol and vertical bar represent the mean ± STD of triplicate samples.

Only very minor peaks for hydroxylated fluvastatin were detected in the LC/MS analysis of cell samples (Appendix 3). Slightly larger peak areas for hydroxylated fluvastatin were detected in supernatant samples, with larger peaks towards 15 minute time point. Also minor peaks for fluvastatin glucuronide were detected in the samples.

Peak areas of fluvastatin glucuronide were significantly lower in the cell samples compared to supernatant samples and peaks areas in either sample type did not increase towards the end of the incubation time. No peaks were detected for des-isopropyl-fluvastatin and des-isopropyl-dihydro-fluvastatin tetranor.

7.4 Inhibition Studies

The net uptake, V and CL_{uptake} of 1 µM [^{3}H]-taurocholate in rat hepatocytes decreased in an inhibition study with 100 µM rifamycin. The sodium dependency of taurocholate uptake was observed when Na+ free choline buffer was used instead of KHB buffer. The net uptake and uptake rate of taurocholate slowed down in choline buffer compared to incubation in KHB buffer with or without rifamycin (Figure 8). A more prominent decline in uptake rate was detected when incubating taurocholate in choline buffer together with 100 µM rifamycin. The uptake clearance of 1 µM taurocholate decreased to 2,16 µl/10^6 cells/min in the presence of Oatp1 inhibitor rifamycin and in the absence of sodium.

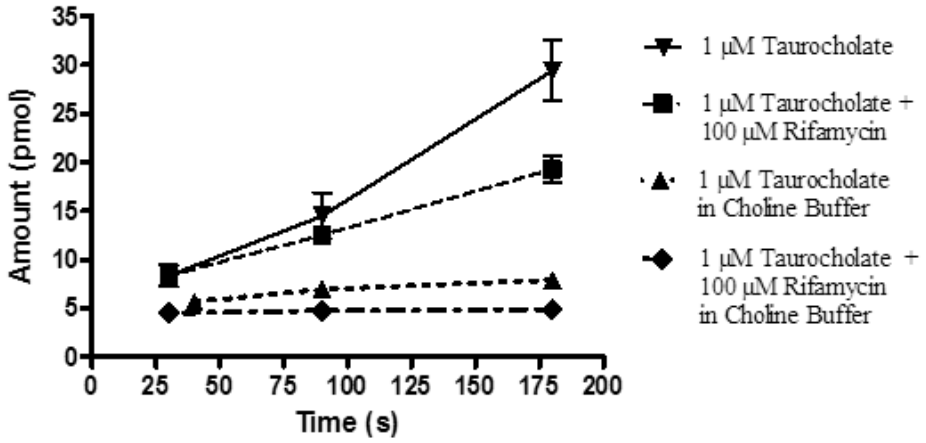


Figure 8. The uptake of 1 µM [^{3}H]-taurocholate in freshly isolated rat hepatocytes when incubated in KHB buffer (▼), in choline buffer (▲), in KHB buffer with 100 µM rifamycin (■), and in choline buffer with 100 µM rifamycin (♦). N = 1

The net uptake of 1µM [^{3}H]-estrone 3-sulfate into rat hepatocytes was decreased when incubated with 50 µM propranolol or 50 µM entacapone but V and CL_{uptake} did not change significantly (Figure 9). Similar to the inhibition study with taurocholate, incubation with rifamycin reduced the net uptake, V and CL_{uptake} of E3S.

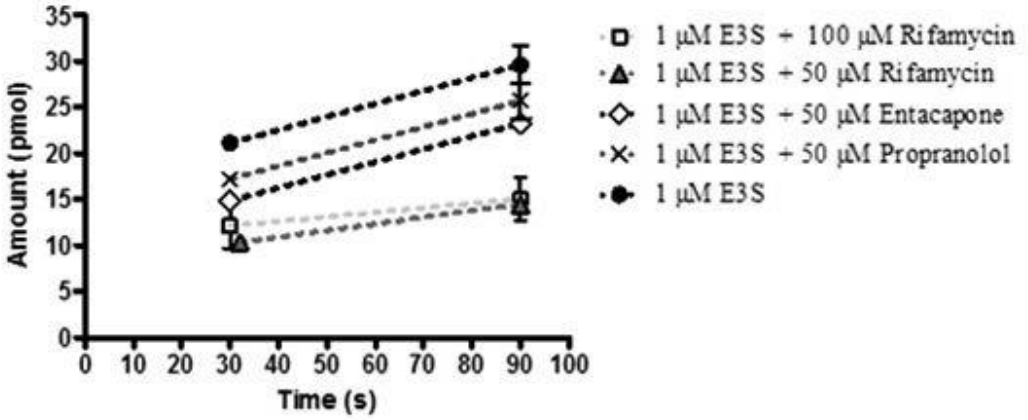


Figure 9. Effect of propranol, entacapone and rifamycin on estrone 3-sulfate (E3S) uptake in freshly isolated rat hepatocytes. 1 µM E3S uptake was investigated with separate incubations with 50 and 100 µM rifamycin, 50 µM propranolol and 50 µM entacapone in rat hepatocytes. Values are represented as means of triplicate samples ± STD. N = 1

The inhibitory effect of rifamycin on 1µM [^{3}H]-E3S uptake was investigated with a concentration range of 1-100 µM in both freshly isolated and cryopreserved rat hepatocytes. Contrary to the experiments with taurocholate, E3S uptake kinetics did not significantly differ in cryopreserved and freshly isolated hepatocytes. Rifamycin concentrations of 1 µM and 10 µM did not have a notable effect on E3S uptake (Table 9). However, CL_{uptake} of E3S was reduced from an average of 95 µl/min/10^6 cells to 42 and 34 µl/min/10^6 cells, when incubated with 50 µM and 100 µM rifamycin, respectively.

Table 9. Uptake clearance of 1 µM estrone 3-sulfate with rifamycin or entacapone in isolated rat hepatocytes. Values are represented as means of triplicate samples and individual experiments (N) ± STD, rifamycin N = 3, entacapone N = 1. *N = 1

Inhibitor concentration	**CL_{uptake}**	
µM	*µl/min/10^6 cells*	
	Rifamycin	**Entacapone**
0	95,07 ± 13,15	88,87 ± 14,36
1	87,06 ± 8,20	--
10	97,17 ± 5,52	95,94 ± 9,32
50	42,28 *	81,18 ± 14,17
100	33,59 ± 5,90	61,61 ± 10,35
200	--	55,15 ± 3,31
400	--	32,26 ± 3,14

Entacapone inhibited the net uptake of 1µM [^{3}H]-E3S into freshly isolated rat hepatocytes in the concentration range of 10-400 µM (Figure 10). V and CL_{uptake} of E3S were inhibited with entacapone concentrations ranging from 100 to 400 µM (Table 9).

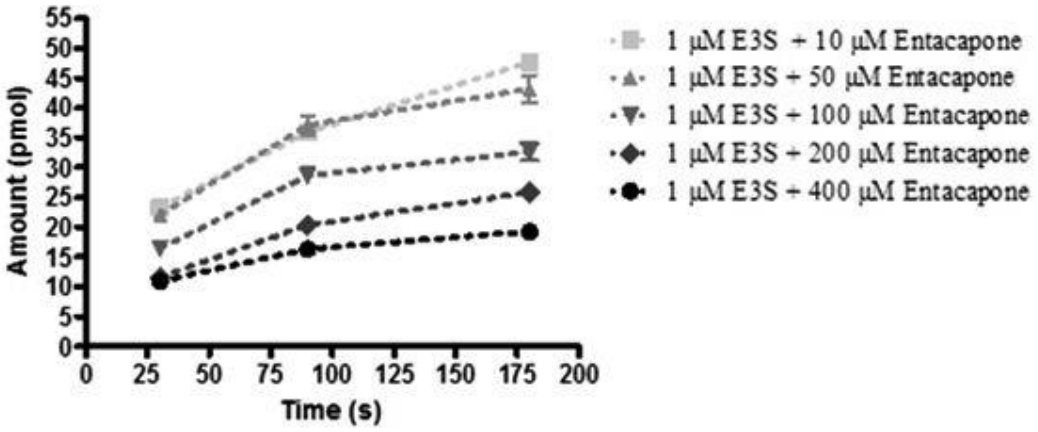


Figure 10. Effect of on entacapone estrone 3-sulfate (E3S) uptake in freshly isolated rat hepatocytes. 1 µM E3S uptake was investigated with entacapone concentrations ranging from 10 µM to 400 µM. Values are represented as means of triplicate samples ± STD. N = 1

The 50% inhibitory concentration (IC50) values of rifamycin and entacapone for uptake of [^{3}H]-estrone 3-sulfate in rat hepatocytes were 62 and 237 µM, respectively. Goodness of fit and constraints for nonlinear regression analysis used in the calculation of IC50 values are summarized in Appendix 2. The inhibitory effects of rifamycin and entacapone on saturable uptake clearance of E3S in rat hepatocytes are shown in Figure 11.

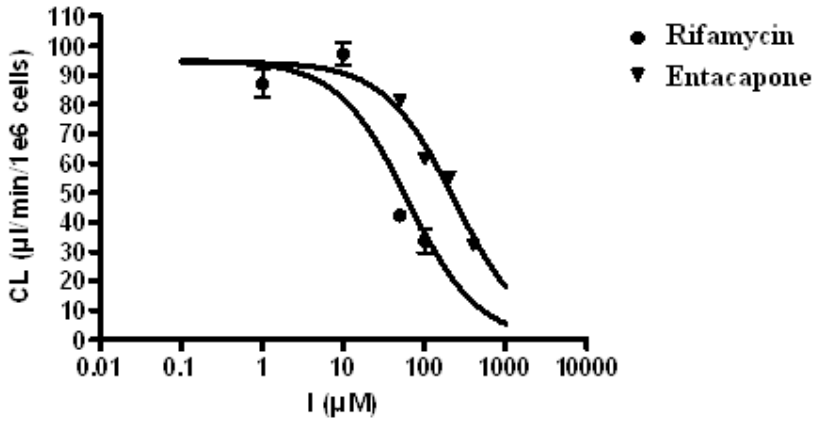


Figure 11. Comparison of the inhibition of 1 µM [^{3}H]-estrone 3-sulfate uptake clearance by rifamycin (lower curve) and entacapone (upper curve) in isolated rat hepatocytes. Rifamycin concentration range was 1 to 100 µM and entacapone concentration range was 10 to 400 µM. Each symbol and vertical bar represent the mean ± STD of triplicate samples and individual experiments (N) ± STD, rifamycin N = 3, entacapone N = 1

8 DISCUSSION

8.1 Uptake Kinetics of Taurocholate and Estrone 3-Sulfate

Taurocholate uptake kinetics observed in the study is in concord with the knowledge of the active transporter-mediated hepatic uptake process of taurocholate. The decrease in uptake rate after 90 second time point is similar to the observation of a previous study using the oil-spin method where uptake rate of taurocholate was determined at 15 second intervals through the initial 120 seconds of incubation (Edmondson et al. 1985). Hepatic uptake of taurocholate has been shown to be mediated by sodium-dependent

manner by murine Ntcp and human NTCP transporters, as well as sodium-independent manner by murine and human organic anion transporters including Oatp1a1, Oatp1a4, Oatp1b2, OATP1B1 and OATP1B3 (Kullak-Ublick et al. 1995; Kouzuki et al. 1999; Eckhardt et al. 1999; Cattori et al. 2001). Incubation in Na^+ free buffer appeared to cause a larger decrease in taurocholate uptake in rat hepatocytes compared to incubation with Oatp1 inhibitor rifamycin SV which indicates that sodium-dependent transport processes are mainly responsible of hepatic uptake of taurocholate. This finding is in accordance with previous data (Edmondson et al. 1985; Sandker et al. 1994).

The K_m value of 24 µM calculated for taurocholate is in good agreement to the results in other studies using freshly isolated and primary cultured rat hepatocytes in which K_m for taurocholate varied between 17 to 24 (Edmondson et al. 1985; Sandker et al. 1994; Kouzuki et al. 1998). The lower V and CL_{uptake} of taurocholate in cryopreserved rat and human hepatocytes compared to freshly isolated rat hepatocytes indicates that taurocholate uptake was less efficient in cryopreserved rat and human cells. Houle et al. (2003) have studied the effect of cryopreservation on taurocholate uptake in rat hepatocytes and found no significant difference in taurocholate uptake between freshly isolated and cryopreserved cells. The difference observed in the present study may be caused by slight difference in cell viability between freshly isolated and cryopreserved cells and by the fact that no Percoll centrifugation was performed in order to remove dead cells when cell viability of the cryopreserved cells was lower than 85 %, as was done in the study by Houle et al. (2003). The lower hepatic uptake of taurocholate in cryopreserved human hepatocytes is in accordance with previous studies using both freshly isolated rat and human hepatocytes and cryopreserved human hepatocytes, indicating that there are species differences in the active transport mechanisms of bile acids (Sandker et al. 1994; Kouzuki et al. 1998; Shitara et al. 2003).

The inhibitory effect of rifamycin SV on taurocholate and estrone 3-sulfate uptake in rat hepatocytes supports previous data about inhibition potency of rifamycin SV on murine Oatp and Ntcp transporters. High concentration (100 µM) of rifamycin SV has been reported to inhibit both sodium-dependent and sodium-independent taurocholate uptake in short-term cultured rat hepatocytes, whereas low concentration (10 µM) only

inhibited sodium independent taurocholate uptake (Fattinger et al. 2000). Rifamycin SV was shown to inhibit Ntcp, Oatp1a1 and Oatp1a4-mediated transport of taurocholate in *Xenopus laevis* oocytes. However, in a study using Oatp1b2 knockout mice, rifamycin SV did not exhibit significant liver uptake by Oatp1b2 (Chen et al. 2008). At rifamycin SV concentration of 100 µM, CL_{uptake} of taurocholate and estrone 3-sulfate were decreased by 32 % and 65 %, respectively. The relatively higher inhibition of E3S uptake by 100 µM rifamycin compared to that of taurocholate suggests that sodium-independent transport plays a more prominent role in hepatic uptake of E3S compared to that of taurocholate. This finding is in agreement with the knowledge that E3S is transported by all hepatic Oatp/OATP transporters but not Ntcp/NTCP (Cattori et al. 2001; Kullak-Ublick et al. 2001).

8.2 Fluvastatin Uptake and Metabolism

Previously, hepatic uptake of fluvastatin has been shown to be mediated by OATP2B1, OATP1B1, OATP1B3 and NTCP (Kopplow et al. 2005; Noe et al. 2007; Greupink et al. 2011; Varma et al. 2011). Unlike for simvastatin and pravastatin, genetic polymorphisms in the gene coding OATP1B1 have not been reported to result in decreased hepatic uptake of fluvastatin, suggesting that other transporters or passive diffusion are mainly responsible for the uptake (Niemi et al. 2006; Pasanen et al. 2006). This was confirmed in a study using double-transfected MDCKII cells expressing MRP2 together with OATP1B1, OATP1B3, or OATP2B1 (Kopplow et al. 2005). K_m value for fluvastatin in MRP2-OATP2B1-expressing cells was significantly lower compared to MRP2-OATP1B1 and MRP2-OATP1B1-expressing cells, being 0,7, 2,4 and 7,0, respectively. Lipophilic fluvastatin is also taken up into cells by passive diffusion but passive uptake has been reported to constitute only one tenth of the total uptake in primary cultured rat hepatocytes incubated at 4 °C and 37 °C (Ohtawa et al. 1999). Consistent with this data, hepatic uptake has been reported to be the rate-determining process in the overall hepatic elimination of fluvastatin in rats (Watanabe et al. 2010).

In the present study, fluvastatin uptake by murine transporters was investigated using freshly isolated rat hepatocytes. Saturable uptake kinetics with increasing concentration observed indicate active transport mechanism in hepatic uptake of fluvastatin in rats, which supports previous data by Ohtawa et al. (1999). Intracellular fluvastatin concentration seemed to reach a rather high value already at 30 second time point, after which uptake velocity appeared to slow down (Figure 6C). The apparent decrease in uptake rate might have been a result from rapid non-specific binding of lipophilic fluvastatin onto cell membranes but previous data about fluvastatin uptake kinetics do not support this hypothesis. In an uptake study using plated freshly isolated human hepatocytes, the uptake of fluvastatin was linear up to approximately 1 minute (Noe et al. 2007). The linearity of fluvastatin uptake up to 90 seconds of incubation in rat hepatocytes should be studied in future in order to confirm the reliability of the calculated kinetic parameters. The negative V and CL_{uptake} observed at fluvastatin concentration of 25 µM were most likely caused by error in pipetting. Otherwise, deviation in concentrations of triplicate samples was small.

The calculated K_m (5,8 µM) for fluvastatin is slightly higher compared to the K_m of fluvastatin (2,3 µM) in a study using MRP2-OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells and significantly lower compared to K_m of fluvastatin (37,6 µM) in primary cultured rat hepatocytes (Ohtawa et al. 1999; Kopplow et al. 2005). Passive diffusion comprised approximately 6 % of the total fluvastatin uptake which is less than in primary cultured rat hepatocytes. The higher K_m and P_{diff} for fluvastatin in primary cultured hepatocytes may have been caused by decreased expression of uptake transporters (Yang et al. 2012). Lower K_m for fluvastatin in transfected cells expressing human OATPs suggest that sodium-independent saturable transport plays a more prominent role in hepatic uptake of fluvastatin in humans compared to rats. However, transfected cell models cannot always predict the uptake behavior in freshly isolated hepatocytes as the full spectrum of endogenous transporters is not expressed (Noe et al. 2007). Further studies comparing fluvastatin uptake into isolated rat and human hepatocytes are needed to get a full view of inter-species differences in the hepatic uptake of fluvastatin. In addition, the K_m for fluvastatin was calculated based on data

from only one experiment. The reliability of the results should be confirmed by future research.

Fluvastatin is rapidly and completely metabolized after absorption primarily by CYP2C9 and to a lesser extent by CYP3A4, CYP2C8 and CYP2D6 in the liver, the elimination half-life ($t_{1/2}$) being 0,7 to 0,8 hours (Dain et al. 1993; Scripture and Pieper 2001). The main metabolites are two hydroxylated metabolites, 5-hydroxy-fluvastatin and 6-hydroxy-fluvastatin, and N-des-isopropyl-fluvastatin, which are excreted in the bile (Dain et al. 1993). Minor amounts of N-des-isopropyl-dihydro-fluvastatin tetranor, as well as glucuronide and sulfate conjugates of the main metabolites are also excreted in the urine. In the present study, the effect of rapid hepatic metabolism on the intracellular and extracellular concentration of fluvastatin in cryopreserved rat hepatocytes was investigated. The observed decrease in fluvastatin concentration in cell samples and in the sum of cell and supernatant samples indicates rather fast metabolism, which is in agreement with the human metabolism data. The maximum intracellular concentration decreased by 31 % in ten minutes. Such a high rate of metabolism has an effect on intracellular fluvastatin concentration already in 90 second incubation time used in the uptake experiments, which might lead to smaller perceived values of V and CL_{uptake}.

The presence of hydroxylated fluvastatin and fluvastatin glucuronide in cell and supernatant samples was confirmed in UPLC/MS analysis although the peak areas were very small compared to those of fluvastatin (Appendix 3). Direct comparison of metabolite and fluvastatin concentrations cannot be made using the peak areas. The lack of signal for major metabolite N-des-isopropyl-fluvastatin in the samples was surprising. It may be due to inter-species differences in fluvastatin metabolism or the used detection method of UPLC analysis. Quadrupole time-of-flight (QTOF) mass spectrometer would have enabled detection of metabolites with higher sensitivity and greater accuracy compared to of triple quadrupole MS (Zhang et al. 2000; Lacorte and Fernandez-Alba 2006).

8.3 Uptake Kinetics of Entacapone

The lack of saturability in the uptake kinetics of entacapone indicates that hepatic uptake of entacapone is not predominantly mediated by active transport mechanisms in the concentration range of 3 – 160 µM. Entacapone is slightly lipophilic at pH 7,5, with an apparent octanol-water partition coefficient ($logP_{app}$) value of 0,8 (Forsberg et al. 2005). The linear uptake kinetics is in agreement with lipophilicity, suggesting that entacapone is taken up into hepatocytes by passive diffusion. In a comparative experiment with passively permeating propranolol and Oatp inhibitor rifamycin, the net uptake and CL_{uptake} of E3S in freshly isolated rat hepatocytes was almost identical when incubated with 50 µM entacapone or 50 µM propranolol, whereas incubation with 50 µM rifamycin decreased the net uptake and CL_{uptake} of E3S significantly. Entacapone did not seem to inhibit E3S uptake in low concentrations, the half maximal inhibitory concentration (IC50) being 237 µM. Altogether, entacapone did not seem to share same active transport mechanisms with E3S in rat hepatocytes to a significant degree. The results suggest that Oatp transporters do not play a major role in the hepatic uptake of entacapone in rats, although some inhibition of E3S uptake was observed at higher concentrations.

However, the significant difference between the net uptake, V and CL_{uptake} of entacapone between freshly isolated rat and cryopreserved human hepatocytes indicates that either hepatic uptake is inefficient due to lesser active uptake or more potent efflux or intracellular metabolism is much more rapid in human hepatocytes. Similar to fluvastatin, entacapone is rapidly metabolized in the human liver, $t_{1/2}$ being only 0,4 - 0,7 hours (Heikkinen et al. 2001). Species difference in biotransformation of entacapone between rats and humans has been reported but no published data about intrinsic clearance for metabolism in rats is available (Wikberg et al. 1993). Also the effect of cryopreservation on transporter function may contribute to the observed difference in intracellular drug concentration if active transport is responsible of entacapone uptake. If the species difference in intracellular entacapone concentration results from hepatic uptake rather than metabolism, the contribution of passive diffusion to the total hepatic uptake of entacapone may be smaller than the results from uptake experiments in rat

hepatocytes suggest. The significant carryover effect in the UPLC method of entacapone prevented the reliable quantification of low intracellular concentrations, which limited the data used in K_m and IC50 calculations. Also more repetitions of the uptake assays would have been needed to improve the reliability of the results. The role of passive diffusion and active transport on hepatic uptake of entacapone should be further studied in the future using both 4 °C and 37 °C incubation conditions and more repetitions.

8.4 Use of Non-Radiolabeled Compounds in the Oil-Spin Method

Uptake experiments using the oil-spin method are usually performed using radio-labeled compounds in order to enhance the rather low throughput of the method (Soars et al. 2009). In the present study, radiolabeled fluvastatin and entacapone were not available and intracellular concentrations of the drugs was determined using the oil-spin method adapted to non-radiolabeled compounds developed by Paine et al. (2008). Compared to experiments with radiolabeled compounds, quantification of samples with UPLC/MS analysis added more steps to the sample preparation process as lysed cells had to be extracted from the UPLC samples. Since cell samples had to be pipetted out of the original microcentrifuge tubes and precipitated in methanol, it is possible that part of the drug remained in the original tubes or in the lower ammonium acetate phase after methanol precipitation. It is also possible that cells in the reaction mixture samples were not properly lysed with the addition of methanol. This could be the reason why the quantified concentrations of reaction mixtures were lower than they were prepared to be. If the quantified concentrations did not reflect the actual concentrations of the reaction mixtures, the calculated CL_{uptake} values for fluvastatin and entacapone should be lower. In addition to sample preparation, the finding of a good LC/MS method for quantification of samples can be problematic, as was seen for UPLC method of entacapone. Altogether, the oil-spin assay using non-radiolabeled substrates is a strenuous method that is clearly not useful in the early phases of drug discovery and development where higher throughput methods for screening of drug-transporter interactions are needed.

9 CONCLUSIONS

In conclusion, by comparing the uptake kinetics of fluvastatin and entacapone on the uptake of taurocholic acid and estrone 3-sulfate in freshly isolated rat hepatocytes and cryopreserved rat and human hepatocytes, fluvastatin uptake showed active saturable transport kinetics with rapid metabolism in rat hepatocytes, whereas entacapone uptake in rat hepatocytes appeared to be linear and did not seem to inhibit active transport mechanisms of E3S at clinically significant concentrations. Significantly lower hepatic uptake of taurocholate and entacapone was observed between rat and human hepatocytes, although cryopreservation may have had an effect on the noticed difference. The overall results of the study suggest that Oatp transporters do not play a significant role in hepatic uptake of entacapone. However, the reliability of the results is questionable due to lack of parallel experiments and problems in the quantification of samples by UPLC/MS analysis. Further studies are needed to elucidate the role of active and passive processes in the hepatic uptake of entacapone. Knowledge about the substrates of Oatp transporters helps to assess drug disposition in humans and to explain drug interactions and interindividual variability in drug pharmacokinetics, efficacy and toxicity.

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APPENDIX 1

Summary of Nonlinear Regression Analysis of Taurocholate and Fluvastatin Uptake

	Taurocholate	**Fluvastatin**
Uptake CL		
Best-fit values		
V_{max}	1778	1171
K_m	24,49	5,817
P_{diff}	15,92	11,77
Std. Error		
V_{max}	793,7	114,6
K_m	39,60	2,006
P_{diff}	446,5	57,84
95% Confidence Intervals		
V_{max}	31,08 to 3525	907.3 to 1436
K_m	-62,68 to 111.7	1.191 to 10.44
P_{diff}	-966,9 to 998.7	-121.6 to 145.1
Goodness of Fit		
Degrees of Freedom	11	8
R^2	0,4365	0,9577
Absolute Sum of Squares	4,939e+006	35812
Sy.x	670,1	66,91
Constraints		
P_{diff}	PPAS > 0.0	PPAS > 0.0
K_m	KM > 0.0	KM > 0.0

APPENDIX 2

Summary of Nonlinear Regression Analysis of E3S Inhibition by Rifamycin and Entacapone

	Rifamycin	**Entacapone**
Best-fit values		
Bottom	1,00 x 10^{-07}	1,00 x 10^{-07}
Top	95,14	94,43
IC50	61,84	237,1
Std. Error		
Bottom	54,84	29,01
Top	5,336	4,776
IC50	83,06	160,9
95% Confidence Intervals		
Bottom	0,0 to 126,5	0,0 to 92,32
Top	82,84 to 189,7	79,24 to 138,5
IC50	0,0 to 253,4	0,0 to 749,1
Goodness of Fit		
Degrees of Freedom	8	3
R^2	0,8341	0,9594
Absolute Sum of Squares	1263	116.3
Sy.x	12,57	6,227
Constraints		
BOTTOM	BOTTOM > 0.0	BOTTOM > 0.0
TOP	TOP > 1,500*BOTTOM	TOP > 1,500*BOTTOM
IC50	IC50 > 0.0	IC50 > 0.0

APPENDIX 3

Peak Areas of Fluvastatin and its Metabolites in UPLC/MS Analysis

	Analyte peak area					
Time (s)	**Fluvastatin**		**OH-fluvastatin**		**Fluvastatin glucuronide**	
	Cells	Supernatant	Cells	Supernatant	Cells	Supernatant
30	292000	1283333	0,0	16,3	2,8	2170
90	341333	1236667	0,9	27,9	2,8	1533
180	391667	1143333	1,4	1079	3,4	1923
300	420667	1053333	1066	2587	3,9	1890
600	307333	969333	9,8	7733	5,6	1653
900	300333	925667	1513	12400	3,5	1833