Transport of statins by human ABC efflux transporters *in vitro*

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ABSTRACT

Transporters are cell membrane proteins, which mediate the cellular influx or efflux of compounds. Transporters expressed in tissues important for pharmacokinetics, can play a crucial role in drug absorption, distribution and excretion.

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are used to treat hypercholesterolemia and to prevent cardiovascular diseases. Statins are usually well-tolerated and most adverse effects are mild. Statin-induced muscle toxicity is generally dose- and concentration-dependent. The inhibition of statin transport can alter statin plasma concentrations and thus the risk of adverse effects.

The aim of the study was to characterize the transport of atorvastatin, fluvastatin, pitavastatin, pravastatin and rosuvastatin via ABC efflux transporters, including breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) 2-4 and 8 *in vitro*.

Vesicular transport assays were used to investigate statin transport through specific efflux transporters. The transport studies were divided into three parts: screening of statin transport, time-linearity of transport and concentration-dependent transport.

The results demonstrated that atorvastatin and pitavastatin are substrates of BCRP, P-gp and MRP3, fluvastatin is a substrate of BCRP, P-gp, MRP2, MRP3 and MRP4, pravastatin is a substrate of MRP3 and rosuvastatin is a substrate of BCRP, P-gp and MRP4 *in vitro*. Simvastatin acid was not transported by any of the studied transporters.

This thesis improves general understanding of pharmacokinetic properties of atorvastatin, fluvastatin, pitavastatin, pravastatin and rosuvastatin. These data are useful in predicting the effects of transporter-mediated drug-drug interactions and genetic variability in transporter function on the pharmacokinetics, efficacy and safety of statin therapy.

Keywords: Efflux transporter, statin, concentration-dependent transport

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1 INTRODUCTION

1.2 Transporters

Transporters are membrane proteins, which regulate the passage of endogenous and foreign compounds, such as drugs, in the body (Chen et al., 2001; Shin et al., 2017; Suzuki et al., 2003). Transporters have a crucial role in drug absorption, distribution, metabolism and elimination (ADME), resulting in an effect on drugs pharmacokinetics and pharmacodynamics (Benet et al., 1996; Greiner et al., 1999; Kawahara et al., 1999; Sparreboom et al., 1997). Alternations in transporter activity caused by other drugs or genetic variation can result in changes to drug pharmacokinetics (Keskitalo et al., 2009a; Maeda et al., 2006).

Transporters are divided into two major superfamilies: ATP-binding cassette (ABC) superfamily, consisting of 48 members, and solute carrier (SLC) superfamily, consisting of 300 members (Dean et al., 2001; Keogh, 2012; Muller, 2006). Both ABC and SLC superfamilies can be further subdivided into smaller subfamilies based on similarities in structures or sequences, and for example, ABC transporters are categorized into seven subfamilies (ABC-A to ABC-G) based on sequence identity (Vasiliou et al., 2009). Transporters from both superfamilies have a wide range of substrates including foreign compounds, small peptides, bile salts and hormones (Abe et al., 1998; van Edert, 1999; Kullak-Ublick et al., 2001).

ABC transporters vary structurally from each other, but they all share the same base structure including two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) (Higgins and Linton, 2004). TMDs are hydrophobic regions, which vary structurally between transporters, and play a major part in substrate recognition and form a binding pocket for the substrate. NBDs are conserved proteins, which bind to and hydrolyze adenosine triphosphate (ATP), producing energy for membrane protein to operate. (Johnson and Chen, 2017) As an example of the structural variety, the number of terminal membrane domains can vary with different transporters. Multidrug resistance-associated proteins (MRP) 1, 2, 3, 6 and 7 have a third membrane-spanning domain, but from the same family, MRP4, 5 and 8 do not (Belnsky et al., 1998; Hopper et al, 2001; Bera et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001). In addition, the structure of breast cancer resistance protein (BCRP), which is a so-called half-transporter, is composed of only one transmembrane domain and one nucleotide-binding domain (Xu et al., 2004). To form a functional BCRP complex, it requires two half-transporter subunits to combine into a homodimer (McDevitt et al., 2006; Xu et al., 2004).

A transporter's binding pocket formed by TMDs varies between outward- and inward-facing conformations based on binding of substrate and ATP, followed by ATP hydrolysis, conformational changes and different exposure of binding pocket (Jardetzky, 1966; Johnson and Chen, 2017). When an ABC-transporter's binding pocket translocate from inward- to outward-facing, substrate affinity decreases and substrate is able to pass across the membrane (Ward et al., 2013). The steps, which are included in ABC transporters' cycle (binding of substrate, binding of ATP, NBD dimerization, transmembrane domain conformational switch, ATP hydrolysis, releasing the substrate in the other side and returning from the cycle ready to the next activation) are known but the specific order of these steps is still unclear (Verhalen and Wilkens, 2011; Ward et al., 2013; Zarrabi et al., 2014).

The most convincing evidence indicating a role for transporters in drug pharmacokinetics in humans comes from pharmacogenetic studies. For example, a decreased function single nucleotide variant in the *SLCO1B1* gene encoding the organic anion transporting polypeptide 1B1 (OATP1B1) influx transporter, is associated with a markedly increased systemic exposure to many statins and an increased risk of simvastatin-induced muscle toxicity (Pasanen et al., 2006; SEARCH Collaborative Group et al., 2008; Niemi et al., 2011). Moreover, decreased function variants in the *ABCG2* gene encoding the BCRP efflux transporter are associated with a markedly increased exposure and enhanced cholesterol-lowering efficacy of rosuvastatin (Ghasman et al., 2012; Keskitalo et al., 2009a). Moreover, transporters have been implicated in several drug-drug interactions. For example, the P-glycoprotein (P-gp) -inhibiting antifungal itraconazole has been shown to increase the systemic exposure to the oral anticoagulant dabigatran (Pureksaritanont et al., 2017).

1.21 P-glycoprotein

P-gp efflux transporter was found in 1976 by Juliano and Ling and it is the first discovered multidrug resistance protein. P-gp is also known as multidrug resistance protein 1 (MDR1). P-gp transporter protein is expressed on the apical membrane of small intestinal epithelial cells (Drozdzik et al., 2019; Thiebaut et al., 1987), capillary endothelial cells in the brain (blood-brain barrier) (Virgintino et al., 2002), hepatocytes in the liver (Ng et al., 2000), proximal tubule epithelial cells in the kidney (Afrouzian et al., 2018) and in the pancreas (Thiebaut et al., 1987) (table 1). The P-gp protein abundance is high in the brain microvascular endothelial cells, but protein levels are still lower than those of for example BCRP (Shawahna et al., 2011). P-gp can transport a large selection of compounds, but

good P-gp substrates are often lipophilic, positively charged and their molecular weight is greater than 300 (Sharom et al., 1998; Sharom, 2014). For example, estradiol (Barnes et al., 1996) and cortisol (Uedas et al., 1992) are good endogenous substrates for P-gp. In addition, P-gp transports multiple drugs, such as dabigatran etexilate (Ishiguro et al., 2004), digoxin and paclitaxel (Collett et al. 2004).

1.22 MRP1

MRP is a second type of transporter discovered in multidrug resistant cancer cells (Cole et al., 1992). The first discovered MRP was later named MRP1. The MRP1 protein is mainly found in different cancer cells, but it has also been detected for example in the placental trophoblast (Nagashige et al., 2003), blood-testis barrier, oral epithelium and kidney urinary collecting duct tubules (Wijnholds et al., 1998). MRP1 transports multiple different compounds including endogenous substrates such as bilirubin (Jedlitschky et al., 1997), glutathione conjugates, and glucuronide conjugates (Jedlitschky et al., 1996), and exogenous compounds like antivirals (Srinivas et al., 1998).

1.23 MRP2

MRP2 was originally named as canalicular multispecific organic anion transporter (cMOAT) and it has been studied for several decades. The MRP2 protein is expressed on the apical membrane of hepatocyte in the liver (Nies et al., 2001), in the proximal tubules of kidney (Schaub et al., 1999), on the apical membrane of epithelial cells in the small intestine (Fromm et al., 2000), in the colon (Sandusky et al., 2002), gallbladder (St-pierre et al., 2000), bronchi (Sandusky et al., 2002) and placental syncytiotrophoblasts (St-pierre et al., 2000) (table 1). MRP2 is therefore expressed throughout the human body. Harwood et al (2019) have concluded that the MRP2 protein is most abundant in jejunum and second abundant in ileum when compared with MRP3, BCRP and P-gp. In the liver, the abundance of MRP2 is similar to P-gp, but compared with BCRP, it is five times more abundant and around twice as abundant as MRP3, while OATP1B1 and OATP1B3 are approximately ten times more abundant than MRP2 (Burt et al 2016). MRP2 can transport a broad range of compounds and it shares many substrates with MRP1. MRP2 substrates include, for example, bilirubin glucuronide, glutathione and sulfate conjugates (Jedlitschky et al., 1996, 1997). Because of its ability to transport a wide range of compounds and its expression in pharmacokinetically important tissues, MRP2 appears to play an important role in the elimination of bile acids, conjugated drug metabolites and a variety foreign compounds (Shin et al., 2017).

1.24 MRP3

MRP3 has a similar selection of substrates as MRP1 and MRP2, and it is able to transport monoanionic bile acids, (Soroka et al., 2001), and glucuronide (Bodó et al., 2003) and glutathione conjugates (Zeng et al., 2000). The MRP3 protein is expressed on the basolateral membrane of epithelial cells in the intestine (Li et al., 2019), in the cortex of adrenal gland (Scheffer et al., 2002), in the liver (Nies et al., 2001), kidney (Li et al., 2019), pancreas and gallbladder (König et al., 2005) (table 1). In addition, MRP3 mRNA is expressed in lower levels in the lung, bladder, spleen, stomach and tonsils (Kool et al., 1997). The abundance of MRP3 transporter protein is highest in the adrenal glands and in the kidney, but in the liver the protein expression levels are typically low (Scheffer et al., 2002). Still, mRNA levels are usually reported to be high in the liver (Kool et al., 1997). The protein abundance in the jejunum is lower than that of MRP2 but almost double when compared to BCRP and in the colon MRP3 is significantly more abundant than P-gp, BCRP, MRP2 or MRP4 (Harwood et al., 2019). MRP3 expression can be induced in different circumstances (Aleksunes et al., 2008; Le Vée et al., 2019).

1.25 MRP4

In contrast to MRP3, MRP4 mRNA levels are reported to be minor in the liver (Knauer et al., 2010). Highest MRP4 mRNA levels occur in the kidney and small intestine, and lower expression occurs in the skeletal muscle (Knauer et al., 2010). MRP4 protein is expressed on the basolateral membrane of hepatocytes (Rius et al., 2003), apical membrane of proximal tubules of kidney (Van Aubel et al., 2002), in the intestine, brain (Nies et al., 2004), erythrocytes (Klokouzast et al., 2003), platelets (Jedlitschky et al., 2004), pancreas (König et al., 2005) and adrenal gland (Zelcer et al., 2003) (table 1). Drozdzik et al (2019) have recently studied protein abundance of transporters in the human liver and intestine, finding that in the colon the protein abundance of MRP4 is very similar to MRP2 and almost double compared to P-gp or BCRP, but lower than MRP3. In the duodenum, the relative abundance of MRP4 is comparable to the colon, but in the rest of the small intestine, the proportion of MRP4 of all efflux transporters is diminished to about half. In the study of Drozdzik et al (2019), MRP4 protein was not detected at all in the liver. MRP4 transports bile acids, like MRP2 and MRP3, and other substrates which include compounds such as dehydroepiandrosterone-3-sulfate (Zelcer et al., 2003), and conjugated steroid hormones (Chen et al., 2001). Like other transporters, the substrate specificity of MRP4 consists of a wide variety of compounds, but the actual method how it can recognize, bind and transport both hydrophobic and hydrophilic compounds, is still poorly understood (Hardy et al., 2019).

1.26 MRP8

The *ABCC11* gene encoding for MRP8 and the mRNA were first identified by Bera et al (2001), who also found that it is highly expressed at the mRNA level in breast cancer. Since then, MRP8 protein is found in the gut (Matsumoto et al., 2014), liver (Magdy et al., 2013), testis (Klein et al., 2014) and brain (Bortfeld et al., 2006) (table 1). MRP8 mRNA is also found in the prostate and placenta (Bera et al., 2001). MRP8 shares similar substrate specificity with MRP4 and is able to transports nucleotide analogs, lipophilic anions and monoanionic bile acids (König et al., 2013). Like MRP4, MRP8 does not have N-terminal membrane domain, which separates these two transporters from MRP2 and MRP3 (Guo et al., 2003). A genetic variant in the *ABCC11* gene determines whether the earwax is dry or wet (Yoshiura et al., 2006).

1.27 BCRP

BCRP was originally found in 1998 from breast cancer cells by Doyle et al (1998). The origin breast cancer cells gave the transporter also its name. BCRP protein is located on the apical membrane of epithelium of the small intestine and colon, on the canalicular membrane of hepatocytes, in the placental syncytiotrophoblasts, mammary glands (ducts and lobules of breast) (Maliepaard et al., 2001), on the apical membrane of proximal tubule cells in kidney (Huls et al., 2008), and in the endothelial cells of blood-brain barrier (Cooray et al., 2002) (table 1). BCRP mRNA is expressed in lower amount in the prostate, testis and in the skeletal muscle (Doyle et al., 1998; Knauer et al., 2010). Like P-gp, the protein abundance of BCRP is high in the microvessels, with slightly higher amounts than P-gp (Uchida et al., 2011). BCRP protein is quite evenly distributed along the small intestine, in a manner similar to MRP2 (Harwood et al., 2019). BCRP is known to transport antibiotics, antivirals, calcium channel blockers (Shin et al., 2017), mitoxantrone (Özvegy et al., 2001), sulfasalazine (Jani et al., 2009) and topotecan (Li H et al., 2008). Its endogenous substrates include dehydroepiandrosterone sulfate (Suzuki et al., 2003), protoporphyrin (Jonker et al., 2002) and uric acid (Hosomi et al., 2012).

Table 1. Localizations of selected ABC transporters in human tissues.

Transporter	Gene	Localization
P-gp	*ABCB1*	Liver, kidney, gut, brain and pancreas
MRP2	*ABCC2*	Liver, kidney, gut, gallbladder, bronchi and placenta
MRP3	*ABCC3*	Liver, kidney, gut, pancreas, gallbladder and adrenals glands
MRP4	*ABCC4*	Liver, kidney, gut, brain, pancreas, blood cells and adrenal glands
MRP8	*ABCC11*	Liver, testis, gut and brain
BCRP	*ABCG2*	Liver, kidney, gut, brain, placenta and mammary glands.

1.3 Statins

Cardiovascular diseases are among the most common causes of death globally (Roth et al., 2015; Wilkins et al., 2017). Atherosclerosis is a major factor causing coronary heart disease and stroke, and the risk of developing atherosclerosis is directly linked with increased total serum cholesterol (Bonora et al., 2003; Kannel et al., 1964; The Lipid Research Clinics Coronary Primary Prevention Trial results, 1984). High plasma low-density lipoprotein (LDL) cholesterol concentration is associated with endothelial dysfunction, inflammation, foam cell formation and unstable plaques, which are underlying causes of LDL's role in atherosclerosis (Ehara et al., 2001; Grosheva et al., 2009; Kinlay et al., 2001; Mohty et al., 2008; Reddy et al., 1994; Singh et al., 2017). Development of atherosclerosis is a sum of multiple factors, including LDL, but also interplay between metabolic and inflammatory processes (Graham et al., 2017; Lubos et al., 2007; Yuan et al., 2001).

Cholesterol is synthesized in the liver via a pathway also known as the "mevalonate pathway". In this synthesis, the rate limiting step is when HMG-CoA is transformed to mevalonate by HMG-CoA-reductase (Goldstein and Brown, 1990). When this critical step is inhibited, LDL receptors are upregulated in the liver, LDL is cleared from blood circulation and the total and LDL cholesterol concentrations in the blood are decreased (Parini et al., 2008).

Statins are HMG-CoA reductase inhibiting drugs that are used to treat hypercholesterolemia and to prevent cardiovascular diseases (Sacks et al., 1996; Scandinavian Simvastatin Survival Study Group, 1994). Statins have been shown to reduce morbidity and mortality associated with cardiovascular diseases and the effect is

shown for patients with and without coronary heart disease (Cholesterol Treatment Trialists, 2012; Horwich et al., 2004). In addition to decreasing cholesterol levels, statins also decrease triglyceride levels (Jones P et al., 2003). The statins' mechanism of action is competitive and reversible, which means statin medication is usually lifelong (Endo et al., 1976).

Statins have been suggested to have additional beneficial effects independent of the lipid-lowering mechanism. These are called pleiotropic effects. Pleiotropic features are most likely a consequence of reduction of systemic inflammation, endothelial dysfunction, and platelet hyper-reactivity (Anderson et al., 1995; Bickel et al., 2002; Huhle et al., 1999; Treasure et al., 1995). These effects include, for example, reduced concentration of thrombotic factors (for example D-dimer) (Kaba et al., 2004) improvement of endothelial function (Treasure et al., 1995), modification of inflammatory reactions (Patti et al., 2006), antioxidant effects (Wassmann et al., 2001) antithrombotic effects, prevention of platelet aggregation (Ali et al., 2009) and stabilization of plaques (Sato et al., 2010). Even though pleiotropic effects are recognized, their mechanisms are still mostly unclear. It has been suggested that pleiotropic effects involve endothelial-derived nitric oxide (NO) (Laufs and Liao, 1998). Hypercholesterolemia causes poor bioactivity and impaired formation of endothelial-derived NO (Tamai et al., 1997; Tanner et al., 1991). Endothelial-leukocyte interactions (Jädert et al., 2012), vascular smooth muscle proliferation (Janssens et al., 1998), vasodilation (Vallance et al., 1989), and platelet aggregation (Radomski et al., 1987), all of these are dependent of endothelial NO. Statins increase endothelial NO synthase and by that endothelial NO level, which could be a mediator of pleiotropic effects (Laufs and Liao, 1998).

Statins are strongly associated with a lower risk of Alzheimer disease (Haag et al., 2009), but also with improvement of other conditions, such as dementia (Jick et al., 2000) and chronic obstructive pulmonary disease (COPD) (Soyseth et al., 2006). COPD is usually a progressive disease where the airflow is chronically limited and COPD patients in many cases develop ischemic heart disease (IHD) (de Lucas-Ramos et al., 2012). Statins have been demonstrated to improve prognosis after acute coronary events and to decrease the mortality rate of COPD patients (Cannon et al., 2004; Soyseth et al., 2006).

Statins are usually well-tolerated drugs and most adverse events are mild (Bellosta et al., 2004). Common mild adverse events are abdominal pain (Siedlik et al., 1999), headache (Jones P et al., 2003), nausea (Cilla et al., 1996), and rare adverse events include, myalgia, myopathy (Pasternak et al., 2002) and insomnia (Tuccori et al., 2008). Myopathy is a

condition whose symptoms are skeletal muscle pain or weakness and increased creatine kinase levels (Tobert, 1988). In the worst case, myopathy develops into life-threatening rhabdomyolysis (Joy and Hegele, 2009). Rhabdomyolysis is condition where skeletal muscles have severe injuries and lysis, resulting in the release of muscle components into the circulation (Armitage, 2007). In rhabdomyolysis creatine kinase levels are approximately 40 times greater than the upper limit of normal, while in myopathy this level is up to ten times greater (Armitage, 2007; Silva et al., 2007). Rhabdomyolysis can also lead to acute renal failure, which may be fatal (Grossman et al., 1974).

In addition to adverse events, statins have been associated with increased risk of other conditions, such as type 2 diabetes (Sattar et al., 2010). This association with diabetes has noted in some cases to depend on the dose and potency of statin (Dormuth et al., 2014). The mechanism is not well understood, but a few explanations have been suggested. Statins (for example atorvastatin) can cause reduced expression of insulin sensitive glucose transporter type 4 (GLUT4), which transports glucose into the cells (Jiang et al., 2016). Since GLUT4 plays a crucial role in glucose metabolism, its disturbance might have severe outcomes. Sarcopenia is also one of the adverse effect of statins, which is more common in elderly patients, and includes progressive loss of skeletal muscle mass and weaker muscle function (Delmonico et al., 2007; Goodpaster et al., 2006; Herghelegiu et al., 2018). This is important in the case of diabetes, because sarcopenia can cause insulin resistance and contribute to the development of diabetes (Srikanthan et al., 2010). Statins have multiple ways to increase risk for diabetes and statins' unfavorable effect to mitochondrial function and dysfunction in skeletal muscle (Jones S et al., 2003), adipose tissue (Abe et al., 2008) and pancreatic beta cell (Xia et al., 2008) has been studied. These are associated with insulin resistance and so with diabetes (Stienstra et al., 2014).

Cholesterol is not the only product of mevalonate pathway (Parker et al., 1984). When statins inhibit the mevalonate pathway, the synthesis of other compounds may also be diminished. By this mechanism, statins inhibit the synthesis of isoprenoid intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Laufs and Liao, 1998). These compounds play a role for example in post-translational modifications of Ras and Rho (Adamson et al., 2003). This is notable, because Ras and Rho are involved in regulating cell proliferation, differentiation, apoptosis, and cytoskeletal changes (Chen et al., 1996; Haudek et al., 2009; Machesky et al., 1997; Mulcahy et al., 1985).

All statins share the same mechanism of action, but they differ markedly in pharmacokinetic properties. These differing properties may lead to interindividual differences in the cholesterol-lowering efficacy and risk of adverse effects of individual statins. Pharmacokinetic properties of atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin acid are summarized in the table 2.

1.31 Atorvastatin

Atorvastatin is currently one of the most commonly used statins (Li S et al., 2019). It is a synthetic drug, which is orally administered as a calcium salt of the active acid form (Cilla et al., 1996). Approximately 40% of the oral dose is absorbed from the gut (Black et al., 1998). Solubility of atorvastatin is high at the natural intestinal pH of 6 (Kearney et al., 1993). Atorvastatin undergoes major first-pass metabolism and partly as a result its oral bioavailability is around 14% (Amidon et al., 2014; Lennernäs, 2003). Atorvastatin binds strongly to plasma proteins, by more than 90 (Gibson et al., 1997). In the intestine, (requires low pH) or through an enzymatic acyl glucuronide intermediate pathway or coenzyme A intermediate pathway (Kearney et al., 1993; Li et al., 2006; Prueksaritanont et al., 2002). Atorvastatin is mainly metabolized by CYP3A4. Both atorvastatin and atorvastatin lactone are substrates for this metabolizing enzyme, but atorvastatin lactone has significantly higher affinity for CYP3A4. Atorvastatin lactone is also more lipophilic than the acid form. In addition to CYP3A4, also CYP2C8 is able to metabolize atorvastatin, but only at a low rate. (Jacobsen et al., 2000) Atorvastatin acid and 2-hydroxyatorvastatin acid actively inhibit HMG-CoA reductase, contributing to the efficacy of the drug (Lea and McTavish, 1997). 2-hydroxyatorvastatin and 4-hydroxyatorvastatin can be lactonized via formation of acyl glucuronide intermediates (Goosen et al., 2007). Reversely, lactones can be hydrolyzed back to acid forms spontaneously or by esterases and paraoxonases (Billecke et al., 2000; Vickers et al., 1990a). A major route for the elimination of atorvastatin is through biliary excretion and more than 98% of atorvastatin is eliminated via feces (Lennernäs, 2003). The remaining dose (less than 2%) is excreted into the urine (Stern et al., 1997). The half-life of atorvastatin is 14 hours (Kantola et al., 1998). The active HMG-CoA reductase inhibitory effect may be longer due to longer half-lives of active atorvastatin metabolites (Lins et al., 2003).

1.32 Fluvastatin

Fluvastatin was the first fully synthetic statin. Like most of the statins, it is orally administered in the active acid form. Fluvastatin is water soluble compound, which is rapidly and nearly completely (90-98%) absorbed from the small intestine (Tse et al., 1992). Plasma protein binding of fluvastatin is very high (over 99%) (Tse et al., 1993). Fluvastatin, like atorvastatin and simvastatin, undergoes significant first-pass metabolism, which results in a bioavailability of around 30% (Tse et al., 1992). Like most other statins, fluvastatin is almost completely metabolized (Dain et al., 1993). Fluvastatin has six metabolic pathways which include hydroxylation, loss of the 1-isopopyl group, beta-oxidation, lactone formation, threo-isomer formation and conjugation (Dain et al., 1993). The primary metabolizing enzyme for fluvastatin is CYP2C9, which is highly expressed in the gut and liver (De Waziers et al., 1990; Läpple et al., 2003). The three main metabolites are 5-hydroxy-fluvastatin, 6-hydroxy-fluvastatin and N-desisopropyl-fluvastatin (Fischer et al., 1999). 6-hydroxy-fluvastatin and N-desisopropyl-fluvastatin are formed by CYP2C9, while the formation of 5-hydroxy-fluvastatin is also catalyzed by CYP3A4, CYP2C8 and CYP2D6 (Fischer et al., 1999). Around 60% of an oral dose of fluvastatin is converted into these three main metabolites (Scripture and Pieper, 2001). Even though 5- ja 6-hydroxy-fluvastatin inhibit HMG-CoA reductase by about 88% and 45% of the effect of parent fluvastatin, their contribution to the cholesterol-lowering efficacy of fluvastatin is insignificant due to their low concentrations (Dain et al., 1993). Approximately 90% of an oral dose of fluvastatin is excreted into the bile and feces, and around 5% into the urine. In addition, all the three metabolites are detected in the feces. Fluvastatin has a very short half-life compared to other statins, only 0.5-1h. (Tse et al., 1992)

1.33 Pitavastatin

Pitavastatin differs from other statins by its structure. It has a synthetic cyclopropyl side group, which gives pitavastatin some unique features (Suzuki et al., 2001). One clear difference of pitavastatin is its ability to decrease cholesterol synthesis with lower doses compared to other statins (Budinski et al., 2009; Ose et al., 2009). It has been estimated to be about 7 times more potent HMG-CoA inhibitor than pravastatin (Aoki et al., 1997). Pitavastatin is administered orally in the active acid from and its absorption occurs mainly in the upper gastrointestinal tract. The absorption of pitavastatin is rapid and extensive, approximately 80%. Like other statins, pitavastatin binds highly to plasma proteins, by more than 96%. (Fujino et al., 1999a) Binding occurs mostly with albumin and alpha 1-acid glycoprotein (Fujino et al., 1999b). The oral bioavailability of pitavastatin is greater

than 60% (Fujino et al., 1999a), which is relatively high compared to other statins. Pitavastatin undergoes only limited metabolism and is mainly metabolized by glucuronidation to form pitavastatinlactone, but some oxidative metabolism also occurs via CYP2C9 and CYP2C8 (Fujino et al., 2003; 2004). Because of the cyclopropyl moiety, CYP3A4 cannot metabolize pitavastatin (Suzuki et al., 2001). As a result of minor metabolism, pitavastatin is mostly excreted into the bile in the unchanged form (Kajinami et al., 2000). Clearance of pitavastatin into the bile is rapid, but the half-life is relatively long due to enterohepatic circulation. Enterohepatic circulation can enhance the oral bioavailability and extend the duration of action of pitavastatin. (Kajinami et al., 2006) The final excretion occurs mainly through feces and less than 5% is excreted in the urine (Catapano, 2010). The half-life of pitavastatin is about 14 hours (Fujino et al., 1999b).

1.34 Pravastatin

Pravastatin was originally derived from fungi and it is one of the most hydrophilic statins (Serizawa et al., 1991). Pravastatin is orally given, and its main absorption site is upper part of small intestine (Triscari et al., 1995). Only 34% of orally give pravastatin is absorbed and this has been suggested to be one of the main reasons why its oral bioavailability is 18% (table 2) (Singhvi et al., 1990). Pravastatin binds to plasma proteins considerably less than other statins, its plasma protein binding is between 43% and 54% (Pan et al., 1993; Singhvi et al., 1990). Between 30% and 50% of pravastatin is metabolized and its metabolites are 3′α-isopravastatin, 3′α,5′β-dihydroxy-pravastatin, desacyl-dehydropravastatin, 3′′-hydroxy-pravastatin and 6′-epipravastatin (Hatanaka, 2000). 3’alpha-isopravastatin isomer is the main pravastatin metabolite (Jacobsen et al., 1999). Both 3′α-isopravastatin and 6′-epipravastatin can be formed in the stomach even before absorption by nonenzymatic acid-catalyzed isomerization and even 25% of pravastatin can be biotransformed in the stomach (Everett et al., 1991; Triscari et al., 1995). 3′α-isopravastatin has a low inhibitory effect towards HMG-CoA reductase and this does not significantly contribute to the overall LDL-cholesterol-lowering efficacy of pravastatin (Everett et al., 1991; Pan et al., 1990). CYP3A4 is the main metabolizing enzyme for converting pravastatin into 3’’-hydroxy pravastatin, but CYP-dependent metabolism is not as significant for pravastatin’s metabolism as it is for some other statins (Everett et al., 1991; Jacobsen et al., 1999). Pravastatin is mostly excreted into the feces, approximately 70%, but it also has a relatively high urinary excretion, 20% of oral dose. The peak plasma concentration of pravastatin is reached in approximately 1 hour and its half-life is about 2 hours. (Singhvi et al., 1990)

1.35 Rosuvastatin

Like atorvastatin and fluvastatin, also rosuvastatin is a synthetic statin. Rosuvastatin is relatively hydrophilic (Rosenson, 2003). The oral bioavailability of rosuvastatin has been estimated to be 20% and its hepatic extraction ratio is 0.63 (Martin et al., 2003a). The peak plasma concentration of rosuvastatin is reached in 3 to 5 hours (Cooper et al., 2002). Rosuvastatin is 88% bound to plasma protein (Tomlinson and Hu, 2013). Rosuvastatin is excreted mainly into bile, 90% of a dose is found in feces, and around 77% of that in unchanged form. The remaining 10 % is excreted into urine. The high proportion of unchanged drug in the excreta indicates that metabolism is only a minor route of elimination for rosuvastatin (Martin et al., 2003b). The metabolic pathways of rosuvastatin include CYP2C9-mediated N-demethylation and uridine diphosphate-glucuronosyltransferase 1A1- and uridine diphosphate-glucuronosyltransferase 1A3-mediated acyl-glucuronidation followed by spontaneous lactonization (Marin et al., 2003b; Prueksaritanont et al., 2002). The elimination half-life of rosuvastatin is about 20 hours (Martin et al., 2003b).

1.36 Simvastatin

Simvastatin is a fungal-derived statin like pravastatin. Unlike other statins, simvastatin is a prodrug administered as the inactive simvastatin lactone. Simvastatin lactone is converted into the active simvastatin acid in the plasma, liver and intestinal mucosa (Tubic-Grozdanis et al., 2008). Conversion from lactone to acid can occur spontaneously or via enzyme-catalyzed hydrolysis mediated by carboxylesterases (Vickers et al., 1990b). Simvastatin lactone and acid are mainly metabolized by CYP3A4, but a minor part is also metabolized by CYP3A5 (Prueksaritanont et al., 2003). The acid form can be converted back to lactone via CoASH-dependent pathway or acyl glucuronide intermediate (Prueksaritanont et al., 2001, 2002). Simvastatin acid has three main oxidative metabolites, which are 6′-hydroxy, 6′-hydroxymethyl, and 6′-exomethylene (Vickers et al., 1990b). Simvastatin is well-absorbed from gastrointestinal track (60%) (Cheng et al., 1994) and undergoes major first-pass metabolism, occurring in the intestinal wall and in the liver (Kato, 2008). The oral bioavailability of simvastatin is low (less than 5%) (Tubic-Grozdanis et al., 2008). Plasma protein binding of simvastatin is high (98%) (Vickers et al., 1990b). Its half-life is around 2 hours (Mousa et al., 2000), and 60% of the oral dose is excreted into feces and 13% into urine (Todd and Goa, 1990). Simvastatin lactone is highly and simvastatin acid relatively lipophilic. Its lipophilicity is suspected to be the reason why it is challenging to demonstrate simvastatin acid transport in vitro, for example for OATP1B1 (Sharma et al., 2012), even though simvastatin acid

is a highly sensitive OATP1B1 substrate *in vivo* (Pasanen et al. 2006, SEARCH Collaborative Group et al.,2008).

Table 2. Summary of the pharmacokinetic parameters of statins. Adapted from "Drug interactions with lipid-lowering drugs: mechanisms and clinical relevance" by Neuvonen, P. J., Niemi, M., and Backman, J.T., 2006, Clin Pharmacol Ther, 80(6):565-81. Copyright (2006) by the American Society for Clinical Pharmacology and Therapeutics

	Atorvastatin	**Fluvastatin**	**Pitavastatin**	**Pravastatin**	**Rosuvastatin**	**Simvastatin**
Absorption (%)	30	98	80	35	50	60-85
Bioavailability (%)	12	29	60	18	20	< 5
Hepatic extraction (%)	70	>70	70	45	63	>80
Protein binding (%)	>98	>98	96	50	90	>95
Elimination half-life (h)	7-20	1-3	10-13	1-3	20	2-5
Main metabolizing CYP	CYP3A4	CYP2C9	-	CYP3A4	CYP2C9	CYP3A4

1.4 Statins as substrates for transporters

Transporters play a major part in transporting statins across biological membranes. It is important to study which drugs are substrates for each transporter, since these have an influence on drugs' ADME and safety features. With this information, unnecessary drug-drug interactions could be avoided and drugs prescribed more individually. Statins' passage through efflux transporters have been studied. BCRP and MRP2 have been shown to transport atorvastatin (Lee et al., 2019; Shin et al., 2017), fluvastatin (Lin et al., 2011), pravastatin (Afrouzian et al., 2018) and rosuvastatin (Lee et al., 2019; Knauer et al., 2010). In addition, BCRP transports pitavastatin (Vildhede et al., 2016). MRP3 is demonstrated to transport pitavastatin (Vildhede et al., 2016) and rosuvastatin (Kanda et al., 2018), while MRP4 and P-gp are shown to transport atorvastatin (Hochman et al., 2004; Knauer et al., 2010;), pitavastatin (Kanda et al., 2011; Vildhede et al., 2016), pravastatin (Afrouzian et al., 2018) and rosuvastatin (Kanda et al., 2018; Knauer et al., 2010). Simvastatin acid is a difficult compound to study *in vitro* and there are only a few

reports considering simvastatin and efflux transporters. Simvastatin lactone is shown not to be a substrate of BCRP, MRP2 nor P-gp *in vitro* (Keskitalo et al., 2009b; Li et al., 2011). In addition, statins' uptake via OATP influx transporters have been investigated. OATP1B1 and OATP1B3 are strongly associated to transport atorvastatin (Koenen et al., 2012; Nakakariya et al., 2016), fluvastatin (Kopplow et al., 2005), pitavastatin (Hirano et al., 2006), pravastatin (Hsiang et al., 1999; Seithel et al., 2007)) and rosuvastatin (Ho et al., 2006). The orientation of transporters and direction of statin transport are presented in figure 1.

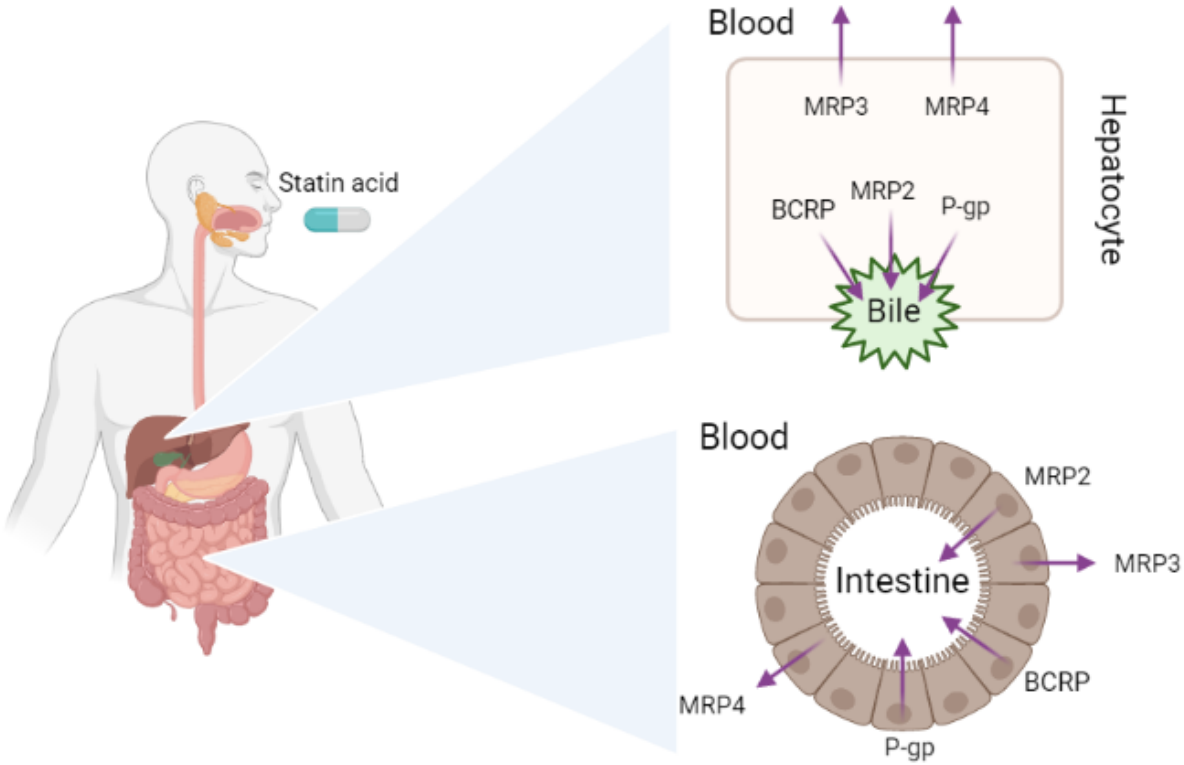

Figure 1. Orientation of selected ABC efflux transporters in the liver and intestine. Figure created with BioRender.com.

1.5 Methods for studying drug transporters

Several cell-based and membrane-based methods are available for investigating the role of transporters in drug pharmacokinetics *in vitro*. Moreover, animal models and human pharmacogenetic and drug-drug interaction studies can be employed to investigate their roles *in vivo*.

Membrane-based assay system is based on vesicles, which includes transporters on their membrane. When studying ABC efflux transporters, commonly used vesicles are inside-out orientated (Keppler et al., 1998). To produce these membrane vesicles, multiple cell

lines expressing the transporter of interest can be used, for example baculovirus-infected insect cells or transfected mammalian cells (Brand et al., 2011; Gu et al., 1996). Vesicles are a good method in mechanic studies, since the interest is in direct interaction of the drug with the transporter protein. The advantage is that the drug of interest has direct access to the transporter and effluxed drug accumulates inside the vesicles (Giacomini, 2010). These properties make membrane vesicle transport assay suitable for transporter kinetic studies and for QSAR analyses (Saito et al., 2006). The weakness is that it might not work for lipophilic compounds, since they are able to pass the membrane by diffusion in or out of the vesicle, or may bind to the membrane (Krumpochova et al., 2012). Vesicle-based assays are also suitable for investigating transporter inhibition (Saito et al., 2006).

Whole cell-based assays are useful methods, for example, when investigating the transport of lipophilic compounds, vectorial transport across polarized cells or transporter-enzyme interactions (Desmarais et al., 2009; Wensaas et al., 2007). Cell-based methods are good for perceiving the bigger picture, because they include membranes, original orientation and a number of endogenous of transporters and other cellular mechanisms. The common deficiencies for cell-based methods are that primary cells, e.g., hepatocytes, may lose endogenous expression of transporters over time and, on the other hand, immortalized cells might not sufficiently mimic the endogenous metabolic and transport profiles of primary cells (Giacomini, 2010).

Cell based assays are divided roughly into these four methods:

1) **Polarized cell lines without recombinant transporters** are frequently used methods to investigate transport across important biological membranes, such as the small intestinal wall and the blood-brain barrier (Chabane et al., 2009; Madgula et al., 2007). In this method, the transcellular passage of a substrate can be determined in both directions, apical-to-basolateral and vice versa. Several cell lines can be used, for example Madin-Darby canine kidney (MDCK) cells and Caco-2 cells (Chabane et al., 2009; Madgula et al., 2007; Zhang et al., 2004), but they all share similar limitations. For example these cell lines have low permeability for hydrophilic compounds, transporter composition differs from endogenous pool of transporters (Hilgendorf et al., 2000), and transporter expression can vary between laboratories (Hayeshi et al., 2008).
2) In **single- and double-transfected cell lines**, single or multiple transporters can be expressed at the same time. MDCK and human embryonic kidney 293

(HEK293) cells are often used in this method. These cell lines are oriented as a monolayer and are suitable for influx, efflux and kinetics measurements, especially for influx kinetics (Kameyama et al., 2005; Shin et al., 2017). To determine the quantitative transport of the drug, cell lines can express both, influx and efflux transporters or only one of those categories (Zhang et al., 2009). Even though the cells can be double-transfected, the model seldom sufficiently imitates *in vivo* situation (Bartholomé et al., 2007).

3) **Primary cells** are obtained from intact tissue and due to the origin from living tissue, these cell lines express all the endogenous features of origin. However, primary cells easily adapt to culture conditions and lose their endogenous properties. This phenomenon can be controlled to some extent with suitable culture conditions. For transport experiments, it is important to maintain cell polarization, and the expression and localization of transporters. Primary cells are used for determining drug intrinsic clearance, transport, metabolism, toxicity, disposition, and drug interactions. (Klein et al., 2015; Pichard et al., 1990; Raab et al., 2011; Thomas et al., 2004)
4) **Sandwich-cultured primary hepatocytes** are a suitable method to maintain primary hepatocyte polarity. Sandwich-cultured primary hepatocytes have been demonstrated to imitate endogenous function of hepatic influx transporters (Marion et al., 2007). As mentioned in primary cells, hepatocytes lose their polarity easily. However, when primary hepatocytes are cultured between two gelled collagen layers, cell polarity is possible to regenerate (LeCluyse et al., 1994; Liu et al., 1999). Sandwich-cultured primary hepatocytes also have some limitations. Limitations of this model include decreased activity of cytochrome P450 enzymes and that the method is laborious (Boess et al., 2003).

In vivo transporter studies include studies with transporter-knockout or humanized animal models, inhibition and imaging studies (Feng et al., 2008; Learned-Coughlin et al., 2003; Polli et al., 2009). Knockout models have several limitations, since strain, sex, diet and housing can cause variation in transporter expression (Chu et al., 2006; Giacomini, 2010; Merino et al., 2005; More and Slitt, 2011; Vlaming et al., 2006). Different species have also shown variation in substrate specificities. For example rats express Mrp2 tenfold higher compared to human (Li M et al., 2008) and Bcrp is expressed in higher levels in the liver in males (Merino et al., 2005).

Imaging techniques, such as positron emission tomography and gamma scintigraphy, are non-invasive imaging techniques useful to determine the role of transporters in tissue distribution. Positron emission tomography can be used for example to determine pharmacodynamic endpoints and gamma scintigraphy to study modulation and activity of transporters (Senthilkumari et al., 2009; Young et al., 1999). Imaging techniques are laborious, which limits their usage.

Pharmacogenetic studies in humans have proven highly effective in determining the role of transporters in drug pharmacokinetics, efficacy, and toxicity in humans (Chasman et al. 2012; Keskitalo et al. 2009a; Pasanen et al. 2006; SEARCH Collaborative Group et al., 2008). This is a useful strategy only when sufficiently common functionally significant genetic variability exists in the transporter of interest. Drug-drug interaction studies also provide useful information on the role of transporters in drug pharmacokinetics in humans, but this approach is impeded by the lack of specific transporter inhibitors and substrates (Lehtisalo et al. 2020),

1.6 Aim of the study

In this thesis, the focus is on six ABC-type efflux transporters: BCRP, MRP2, MRP3, MRP4 and MRP8 and P-gp and six statins: atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin acid. These transporters were chose based on their expression in tissues important for pharmacokinetics, possible involvement in drug-drug interactions and genetic variants. Other transporters are associated with statins but MRP8 was included based on its novelty. Lovastatin was not included in the study, since it is relatively old statin and is rarely used in Finland (Kela, 2019). On the other hand, pitavastatin which is not used in Finland was still included, since pitavastatin is relatively new, potent and interesting statin (Budinski et al., 2009; Ose et al., 2009).

The aim is to characterize which of the statins are substrates of these transporters and to determine the respective transport kinetic parameters by using vesicular transport assay. The goal is to establish better understanding of statin pharmacokinetics and statin-transporter interactions. The ultimate goals are to provide reliable data that is useful in systems pharmacology modelling of statins, increase understanding of statin pharmacokinetics and to bring us one step closer to individualized statin treatment.

2 RESULTS

In this thesis, the vesicular transport assay was used to study the accumulation of six statins inside the vesicles with six efflux transporters. The study was divided into three main phases: screening of statin transport, determination of time-dependent transport and determination of concentration-dependent transport. Screening provided first indication which statins are substrate of each transporter. If ratio of transport in the presence and absence of ATP was two or more, statin was considered as substrate. Transporters, which showed statin transport or were associated with the statin in question in the literature, were selected into second phase of the study. The time-dependent transport of statins determined optimal reaction time for the third phase. The third and final phase of the study determined concentration-dependent transport of the statins and pharmacokinetic parameters of the active transport for each statin.

2.1 Optimization:

The vesicular transport assay was first optimized to confirm the quality of the results and reproducibility. Optimization included several experiments where pre-wetting the filters before sample transfer, terminating the reaction with washing buffer, evaporating the samples and dissolving into organic solvent, reduction of the elution volume, elution two times and adding vesicles into external standard were examined.

In the optimization, pre-wetting the filters with MQ-water was evaluated. Results showed that pre-wetting the filter before transferring the samples into filters decreased the apparent statin transport in the presence and absence of ATP compared to dry filters (figure 2 and table 3). Variation between replicates seemed to be lower when filters were pre-wetted. Reaction termination was tested with washing buffer to evaluate possible effect of commercial stopping buffer to mass spectrometric measurements. The results revealed apparent lower statin transport when using washing buffer to reaction termination compared to stopping buffer (figure 2 and table 3). Surprisingly, pravastatin was transported in MRP3-expressing vesicles more in absence than presence of ATP in pre-wetted filters when reaction was terminated with stopping buffer. The impact of pre-washing to external standard was also studied by treating filter plate in two different ways before adding standard solutions: dry filters and filters treated three times with stopping buffer and twice with washing buffer. This filter treatment did not affect the results (figure 3 and table 3).

The effect of samples evaporation was investigated during optimization. Samples were evaporated after experiment and dissolved into acetonitrile aiming to concentrate the samples. Results showed that evaporation did not improve reproducibility. Conversely signals included even larger variation and measured transport values were vague (figure 4 and table 4). Pravastatin's ATP-dependent transport via MRP3 and variation was more consistent without evaporation.

To evaluate appropriate elution volume, the same protocol was used with pitavastatin, pravastatin, rosuvastatin, and 5(6)-Carboxy-2′,7′-dichlorofluorescein (CDCF). Elution volume experiment performed with CDCF showed increased fluorescence with increasing elution volume in the presence of ATP, but in the absence of ATP MRP2 did not transport CDCF (figure 5 and table 5). Experiment performed with statins revealed that decreasing elution volume from 100µl to 50µl increased transported statin concentration to a small extent only, without increasing variety between replicates (figure 6 and table 6). Also, eluting samples two times proved that all the statin is eluted at the first time and second elution is not necessary (figure 7 and table 7). Pravastatin accumulation is higher in absence of ATP in twice eluted 200µl, but variation in the results was notable. The experiment was performed by adding a second eluant after first elution and results were compared to the experiment where pravastatin was eluted only once.

Vesicles were added into external standard to evaluate vesicles' possible statin or internal standard binding. Results showed that vesicles did not affect results, which indicated that vesicles did not bind to internal standard or statin (figure 7 and table 7).

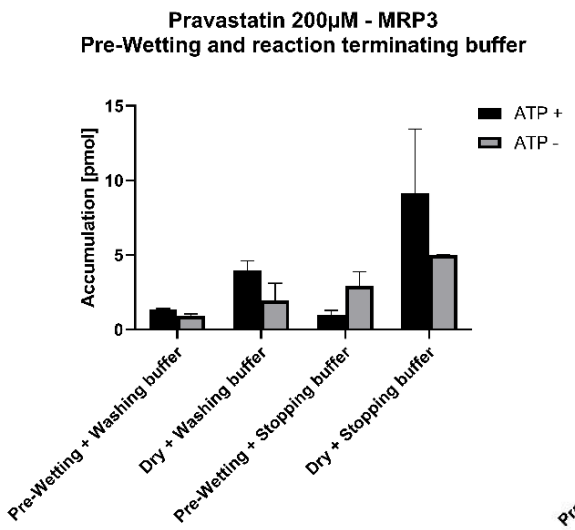

Figure 2. Pre-wetting the filters with MQ-water before transferring samples into filters and terminating reaction with two different buffers. Pravastatin accumulation in MRP3 was studied with pre-wetted and dry filters. Reaction was terminated by using ice cold washing buffer and commercial stopping buffer, to detect possible effect of proteins in stopping buffer on mass spectrometric measurements.

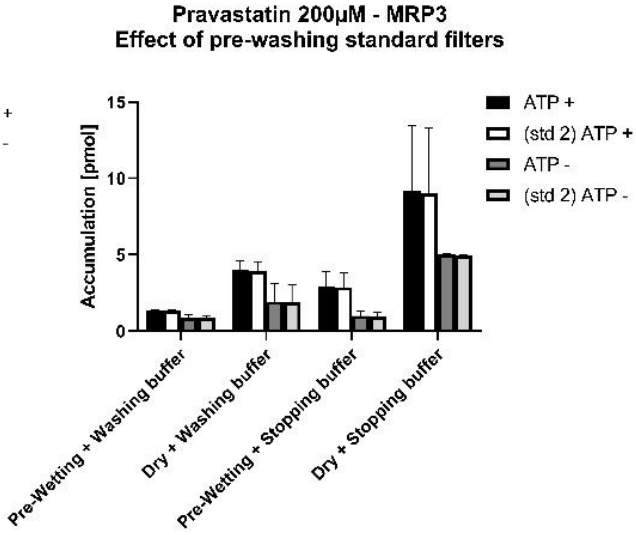

Figure 3. Effect of pre-washing filters before adding external standard. Experiment is the same as in figure 1, but the results were adjusted with two different standards. Second standard filter was pre-washed three times with stopping buffer and twice with washing buffer before adding external standard on the filters.

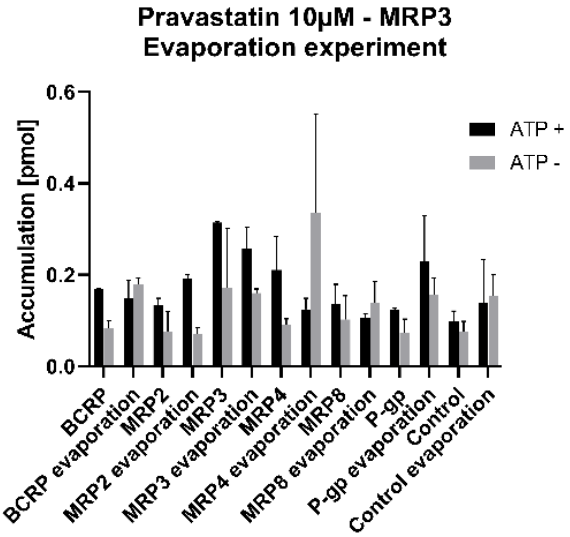

Figure 4. Evaporating samples and dissolving into organic solvent. Effect of evaporating and dissolving accumulated statin in acetonitrile was studied with 10µl pravastatin and MRP3.

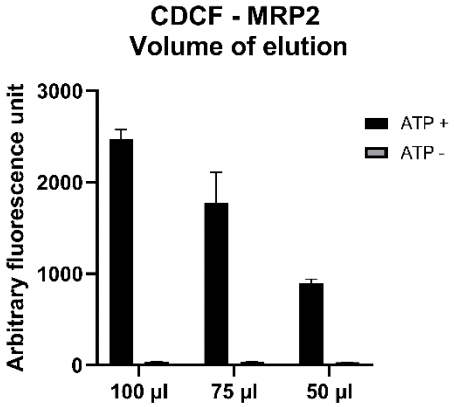

Figure 5. Evaluation of the effect of elution volume on CDCF fluorescence. The elution volume was studied first with fluorescent substrate CDCF and MRP2. CDCF accumulation was studied with three elution volumes 100µl, 75µl and 50µl.

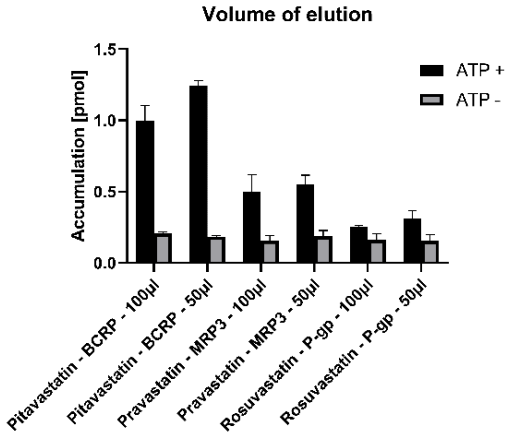

Figure 6. Evaluation of the effect of elution volume on pitavastatin, pravastatin and rosuvastatin transport. Statins were studied with two elution volumes 100µl and 50µl. Pitavastatin was accumulated in BCRP, pravastatin in MRP3 and

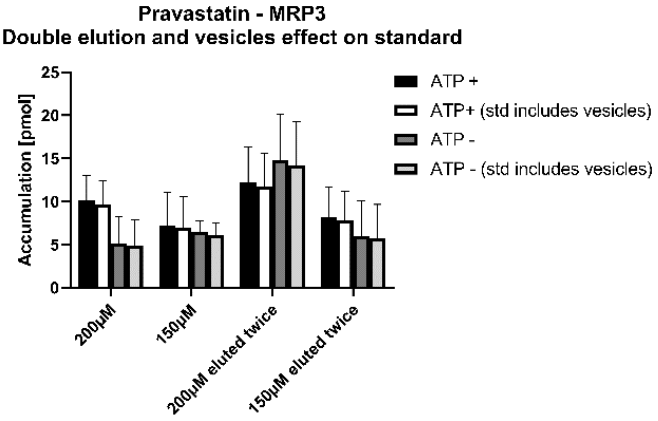

Figure 7. Double elution and vesicles effect on external standard. Samples were eluted twice to confirm all the accumulated statin is eluted. Vesicles were added to external standard to evaluate vesicles possible effect on results. Experiment was performed with two concentrations 200µM and 150µM pravastatin and MRP3.

Table 3. Results of optimizing pre-wetting of filters and reaction termination buffer experiments. The mean and SD of statin accumulation in the presence and absence of ATP, ATP-dependent transport and ratio between ATP + and ATP – for 200µM pravastatin and MRP3 in different experimental conditions. Wetting of filters with MQ-water before transferring samples into filters and terminating reaction with two different buffers. Reaction was terminated by using ice cold washing buffer and commercial stopping buffer, to detect possible effect of proteins in stopping buffer on mass spectrometric measurements. Results were determined by using two external standards. Filters of second external standards (std 2) were washed three times with stopping buffer and twice with washing buffer before adding standard.

	ATP + (pmol/min/mg)	SD	ATP - (pmol/min/mg)	SD	ATP-dependent transport (pmol/min/mg)	SD	Ratio of ATP + / ATP -	SD
Pre-Wetting + Washing buffer	35.56	2.02	23.82	4.10	11.73	4.57	1.49	0.27
Pre-Wetting + Washing buffer (std 2)	35.02	2.02	23.42	4.10	11.60	4.57	1.50	0.28
Dry + Washing buffer	106.22	16.65	51.33	31.33	54.89	35.48	2.07	1.30
Dry + Washing buffer (std 2)	104.84	16.50	50.58	31.03	54.27	35.14	2.07	1.31
Pre-Wetting + Stopping buffer	77.56	25.60	26.49	7.62	-51.07	26.71	0.34	0.15
Pre-Wetting + Stopping buffer (std 2)	76.49	25.25	26.04	7.54	50.44	26.35	2.94	1.29
Dry + Stopping buffer	244.22	114.47	133.33	1.33	110.89	114.48	1.83	0.86
Dry + Stopping buffer (std 2)	241.02	113.18	131.87	1.54	109.16	113.19	1.83	0.86

Table 4. Evaporation studies. The mean and SD of statin accumulation in presence and absence of ATP, ATP-dependent transport and ratio between ATP + and ATP – for 10µM pravastatin and BCRP, MRP2, MRP3, MRP4, MRP8 and P-gp.

	ATP + (pmol/min/mg)	SD	ATP - (pmol/min/mg)	SD	ATP-dependent transport (pmol/min/mg)	SD	Ratio of ATP + / ATP -	SD
BCRP	2.25	0.03	1.10	0.22	1.15	0.23	2.04	0.45
BCRP evaporation	2.00	0.50	2.39	0.19	-0.39	0.54	0.84	0.22
MRP2	1.77	0.22	1.02	0.57	0.75	0.61	1.73	0.99
MRP2 evaporation	2.56	0.11	0.93	0.18	1.63	0.21	2.74	0.54
MRP3	4.19	0.02	2.29	1.73	1.90	1.73	1.83	3.12
MRP3 evaporation	3.43	0.62	2.14	0.12	1.29	0.63	1.60	0.30
MRP4	2.81	0.98	1.21	0.18	1.60	1.00	2.33	1.05
MRP4 evaporation	1.67	0.32	4.49	2.87	-2.82	2.88	0.37	0.25
MRP8	1.80	0.59	1.36	0.69	0.44	0.91	1.32	1.07
MRP8 evaporation	1.41	0.13	1.86	0.62	-0.45	0.64	0.76	0.26
P-gp	1.64	0.07	0.98	0.39	0.66	0.40	1.67	0.65
P-gp evaporation	3.08	1.30	2.08	0.50	1.00	1.39	1.48	0.72
Control	1.31	0.29	1.02	0.30	0.30	0.42	1.29	0.48
Control evaporation	1.84	1.27	2.06	0.61	-0.22	1.41	0.89	0.67

Table 5. The impact of elution volume on CDCF transport. The mean and SD of statin accumulation in the presence and absence of ATP and ratio between ATP + and ATP – for CDCF and MRP2 in three different elution volumes. AFU = Arbitrary fluorescence unit.

Volume	AFU in presence of ATP	SD	AFU in absence of ATP	SD	Ratio of ATP + / ATP -	SD
100µl	2474.33	102.77	36.67	3.21	67.48	6.55
75µl	1773.33	337.92	34.67	3.21	51.15	10.84
50µl	894.33	43.62	30.67	1.53	29.16	2.03

Table 6. Results of elution volume experiments. The mean and SD of 10µM statin accumulation in presence and absence of ATP, ATP-dependent transport and ratio between ATP + and ATP – in two different elution volume.

Statin	Volume	Transporter	ATP + (pmol/min/mg)	SD	ATP - (pmol/min/mg)	SD	ATP-dependent transport (pmol/min/mg)	SD	Ratio of ATP + / ATP -	SD
Pitavastatin	100µl	BCRP	13.31	1.41	2.73	0.17	10.58	1.42	80.01	9.78
Pitavastatin	50µl	BCRP	16.55	0.48	2.44	0.12	14.12	0.50	134.01	7.84
Pravastatin	100µl	MRP3	6.64	1.64	2.06	0.47	4.57	1.70	14.21	4.76
Pravastatin	50µl	MRP3	7.31	0.90	2.48	0.55	4.83	1.05	13.29	3.37
Rosuvastatin	100µl	P-gp	3.40	0.06	2.15	0.61	1.24	0.62	5.54	1.58
Rosuvastatin	50µl	P-gp	4.17	0.70	2.05	0.58	2.11	0.91	7.22	2.37

Table 7. Results of double elution and vesicles effect on standard experiments. The mean and SD of statin accumulation in the presence and absence of ATP, ATP-dependent transport and ratio between ATP + and ATP – for pravastatin and MRP3. Results were determined with two external standards. Second standard included the same vesicles (MRP3) as the actual sample.

	ATP + (pmol/min/mg)	SD	ATP - (pmol/min/mg)	SD	ATP-dependent transport (pmol/min/mg)	SD	Ratio of ATP + / ATP -	SD
200µM	134.67	38.18	68.53	41.74	132.27	113.13	1.96	1.32
150µM	96.51	50.21	85.67	18.10	21.69	106.73	1.13	0.63
200µM eluted twice	162.71	54.79	162.00	49.97	1.42	148.31	1.00	0.46
150µM eluted twice	109.29	46.27	79.78	54.52	59.02	143.00	1.37	1.10
200µM (vesicles in the standard)	258.67	72.82	131.38	79.95	127.29	108.14	1.97	1.32
150µM (vesicles in the standard)	185.29	96.44	164.22	34.67	21.07	102.48	1.13	0.63
200µM eluted twice (vesicles in the standard)	311.82	104.69	378.76	135.87	-66.93	171.52	0.82	0.40
150µM eluted twice (vesicles in the standard)	209.42	88.76	153.07	104.44	56.36	137.06	1.37	1.10

2.2 Screening

The screening experiments were performed with two statin concentrations: 1µM and 10µM. In 10µM experiments, statin accumulation was more detectable and deviation was smaller compared to 1µM experiments. In addition, at 1 µM, the quantities of some statins were below limit of quantification. Based on these reasons, 10µM experiment results were used to evaluate which statins are substrates of which transporters (figure 8 and table 8). The mean and SD of statin transport in presence and absence of ATP, ATP-dependent transport and ratio between ATP + and ATP – for all the statins and transporters are summarized in the table 8.

BCRP, MRP3 and P-gp showed effective transport of atorvastatin (ratios of 1.6, 2.22 and 3.06). A lower transport was observed with MRP2 and MRP4 (ratios of 1.51 and 1.39).

ATP-dependent transport of atorvastatin by MRP8 (1.3 pmol/min/mg) was slightly higher compared to control vesicles (1.2 pmol/min/mg), but the transport ratio (1.2) was lower than in control (1.3). Fluvastatin screening was performed with two isomers: 3R, 5S-fluvastatin and 3S, 5R-fluvastatin. Both isomers were very good substrates of BCRP (ratios of 3.8 and 3.1), MRP3 (ratios of 2.1 and 3.2), MRP4 (ratios of 2.3 and 4.08) and P-gp (ratios of 2.8 and 2.4). MRP8 showed ATP-dependent transport of both fluvastatin isomers, with greater transport of 3S,5R-fluvastatin (4.8 vs. 2.8 pmol/min/mg). However, the uptake ratio of 3R,5S-fluvastatin was at the same level with control (1.3). MRP2 did not transport either of the fluvastatin isomers (ratios of 1.3 and 1.4).

BCRP transported pitavastatin over two-fold compared to control vesicles (ratios of 4.6 and 1.7) and also P-gp showed good transport (ratios of 3.3). MRP8 and MRP3 transported pitavastatin to a minor extent only (ratios of 1.8 and 2.4). MRP2 nor MRP4 did not transport pitavastatin (ratios of 1.4 and 1.5).

Large variability existed in the transport experiments with pravastatin. BCRP was the only transporter which clearly transported pravastatin (ratio of 2.0). MRP3 and MRP4 appeared to transport pravastatin, even though there was large variability between replicates (ratios of 1.8 and 2.33). Transport of pravastatin by MRP2, MRP8 and P-gp was very weak and only slightly higher than control (0.8, 0.4, 0.7 and 0.3 pmol/min/mg).

Rosuvastatin was highly transported by BCRP (ratio of 8.4). MRP4 and MRP8 showed also good rosuvastatin transport (ratios of 2.6 and 5.0), but rosuvastatin transport by MRP8 showed large variation. Rosuvastatin did not accumulate in MRP2 or MRP3 vesicles as compared with control (ratios of 1.0, 1.3 and 1.5). P-gp showed only modest transport of rosuvastatin (ratio of 2.0). Large variation was observed in the transport experiments with simvastatin acid and significant accumulation of simvastatin acid was observed in the absence of ATP. P-gp showed ATP-dependent transport of simvastatin acid, but the difference compared to the control was not notable.

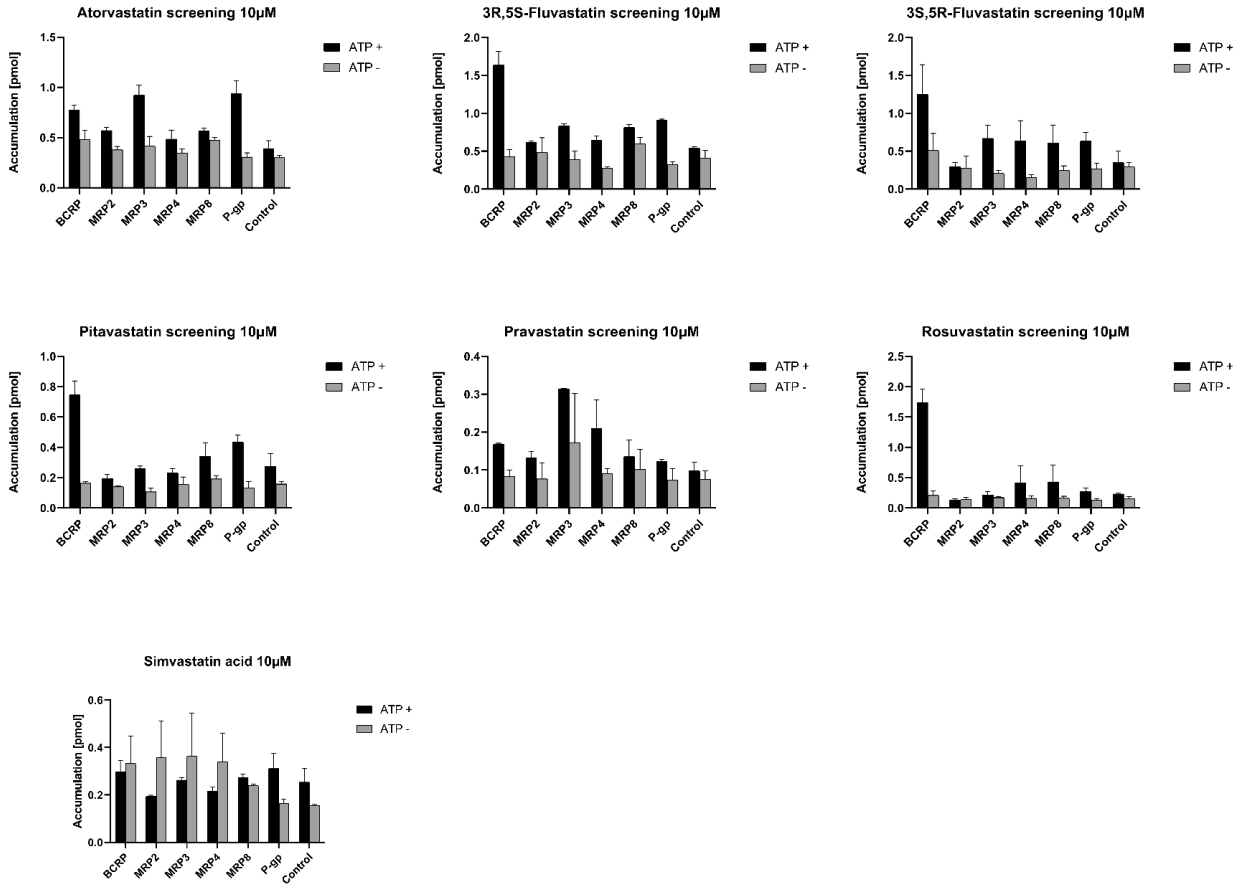

Figure 8. Screening of 10µM atorvastatin, 3R,5S-fluvastatin, 3R,5S-fluvastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin in BCRP, MRP2, MRP3, MRP4, MRP8, P-gp and control vesicles.

Table 8. Summary of screening results. The mean and SD of statin accumulation in the presence and absence of ATP, ATP-dependent transport and ratio between ATP + and ATP – for all the statins and transporters.

Statin	Transporter	ATP + (pmol/min/mg)	SD	ATP - (pmol/min/mg)	SD	ATP-dependent transport (pmol/min/mg)	SD	Ratio of ATP + / ATP -	SD
10µM Atorvastatin	BCRP	10.32	0.66	6.42	1.24	3.90	1.41	1.61	0.33
	MRP2	7.61	0.45	5.04	0.46	2.57	0.64	1.51	0.16
	MRP3	12.28	1.35	5.53	1.30	6.75	1.87	2.22	0.58
	MRP4	6.45	1.23	4.64	0.53	1.81	1.34	1.39	0.31
	MRP8	7.56	0.39	6.30	0.41	1.26	0.57	1.20	0.10
	P-gp	12.48	1.75	4.07	0.57	8.41	1.84	3.06	0.60
	Control	5.22	1.04	4.03	0.24	1.19	1.07	1.30	0.27
10µM 3R,5S-Fluvastatin	BCRP	21.84	2.41	5.75	1.21	16.09	2.70	3.80	0.91
	MRP2	8.21	0.27	6.46	2.58	1.75	2.59	1.27	0.51
	MRP3	11.08	0.44	5.24	1.43	5.84	1.49	2.12	0.58
	MRP4	8.58	0.75	3.68	0.25	4.90	0.79	2.33	0.26
	MRP8	10.83	0.50	8.01	1.05	2.82	1.16	1.35	0.19
	P-gp	12.16	0.13	4.28	0.50	7.89	0.52	2.84	0.34
	Control	7.16	0.26	5.47	1.34	1.70	1.36	1.31	0.32
10µM 3S,5R-Fluvastatin	BCRP	16.68	5.21	5.40	2.13	11.28	5.63	3.09	2.16
	MRP2	3.99	0.93	2.81	2.08	1.18	2.28	1.42	0.79
	MRP3	8.91	2.31	2.81	0.51	6.10	2.37	3.17	0.72
	MRP4	8.50	1.05	2.08	0.43	6.42	1.14	4.08	0.53
	MRP8	8.14	3.14	3.34	0.75	4.80	3.22	2.44	0.93
	P-gp	8.45	1.52	3.58	0.99	4.87	1.82	2.36	0.72
	Control	4.67	2.06	3.89	0.80	0.78	2.21	1.20	0.58
10µM Pitavastatin	BCRP	9.93	1.24	2.17	0.14	7.76	1.25	4.57	0.64
	MRP2	2.61	0.34	1.86	0.06	0.75	0.35	1.40	0.19
	MRP3	3.44	0.24	1.42	0.31	2.02	0.40	2.43	0.56
	MRP4	3.06	0.39	2.08	0.65	0.98	0.76	1.47	0.50
	MRP8	4.54	1.17	2.54	0.31	2.00	1.21	1.79	0.51
	P-gp	5.79	0.61	1.78	0.52	4.01	0.80	3.25	1.01
	Control	3.67	1.10	2.11	0.22	1.56	1.12	1.74	0.55
10µM Pravastatin	BCRP	2.25	0.03	1.10	0.22	1.15	0.23	2.04	0.45
	MRP2	1.77	0.22	1.02	0.57	0.75	0.61	1.73	0.99
	MRP3	4.19	0.02	2.29	1.73	1.90	1.73	1.83	3.12
	MRP4	2.81	0.98	1.21	0.18	1.60	1.00	2.33	1.05
	MRP8	1.80	0.59	1.36	0.69	0.44	0.91	1.32	1.07
	P-gp	1.64	0.07	0.98	0.39	0.66	0.40	1.67	0.65
	Control	1.31	0.29	1.02	0.30	0.30	0.42	1.29	0.48
10µM Rosuvastatin	BCRP	23.19	2.93	2.78	0.95	20.42	3.08	8.35	3.05
	MRP2	1.80	0.23	1.84	0.49	-0.05	0.55	0.97	0.29
	MRP3	2.88	0.66	2.29	0.24	0.59	0.71	1.26	0.32
	MRP4	5.53	3.72	2.14	0.49	3.39	3.75	2.59	1.84
	MRP8	10.71	9.00	2.15	0.42	8.56	9.01	4.99	4.31
	P-gp	3.65	0.78	1.79	0.29	1.87	0.83	2.04	0.55
	Control	3.07	0.24	2.09	0.41	0.98	0.48	1.47	0.31
10µM Simvastatin	BCRP	3.96	0.65	4.45	1.54	-0.49	1.67	0.89	0.34
	MRP2	2.59	0.06	4.78	2.04	-2.19	2.04	0.54	0.23
	MRP3	3.50	0.14	4.85	2.41	-1.35	2.42	0.72	0.36
	MRP4	2.88	0.25	4.54	1.59	-1.66	1.61	0.63	0.23
	MRP8	3.65	0.19	3.19	0.09	0.47	0.21	1.15	0.07
	P-gp	4.15	0.87	2.19	0.23	1.96	0.91	1.89	0.45
	Control	3.38	0.78	2.07	0.06	1.31	0.78	1.64	0.38

2.3 Time-dependent transport

Transporter-statin pairs showing transport in the screening experiments were selected into time-dependent transport experiments (table 9). In addition, transporters that did not show any signs of ATP-dependent transport or were not differed from the control vesicles, but transport statins according to the literature, were selected. Since simvastatin acid did not show significant transport, it was not included in the further experiments.

Time-dependent transport of each statin was studied with three time points: 5, 10 and 15 min. The overall trend was (with some exceptions), that when the incubation time was increased, statin transport increased also in the control vesicles. In this case, longer incubation time did not increase ATP-dependent transport. Transport may not be linear anymore at the time point of 5 min, which could not be seen since 5 min was the first time point. The 5 min incubation time was selected for the concentration-dependent transport experiments, because good statin accumulation was observed in this time and 5 min was practical. Mean and SD of ATP-dependent transport and ratio in three time points are summarized in the table 10.

Atorvastatin time-dependent transport was studied with BCRP, MRP2, MRP3, MRP4 and P-gp (figure 9). These results confirmed earlier findings indicating that atorvastatin is a substrate of BCRP, MRP3 and P-gp (ratios over 2.0 in all three time points). MRP2 and MRP4 weak transport in both the screening and time-dependent transport, and were not included in the concentration-dependent transport experiments. Atorvastatin accumulation in the control vesicles was low and very similar in all the time points (ratios of 1.2, 1.3 and 1.4).

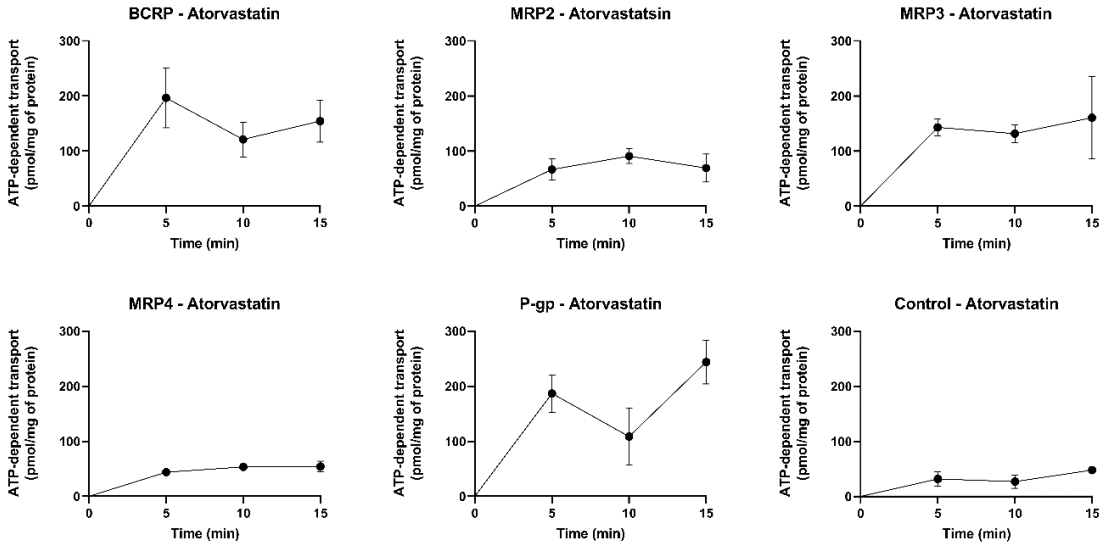

Figure 9. Time-dependent transport of atorvastatin in BCRP, MRP2, MRP3, MRP4 and P-gp vesicles.

Time-dependent transport of 3R, 5S-fluvastatin was studied with BCRP, MRP2, MRP3, MRP4, MRP8 and P-gp (figure 10). Control vesicles showed low ATP-dependent transport of 3R,5S-fluvastatin, and increased slightly along with an increasing incubation time (ratios of 1.1, 1.2 and 1.8). BCRP showed highest 3R,5S-fluvastatin transport of the transporters, confirming screening results (ratios around 4 in all time points). MRP3 and P-gp indicated also good transport (ratio of 3.1 and 3.0 at 5 min). MRP2, MRP4 and MRP8 also transported 3R,5S-fluvastatin, but at a lower rate. 3R,5S-fluvastatin accumulation in MRP8 vesicles was similar to control in the two last time points (ratios of 1.4 and 1.7).

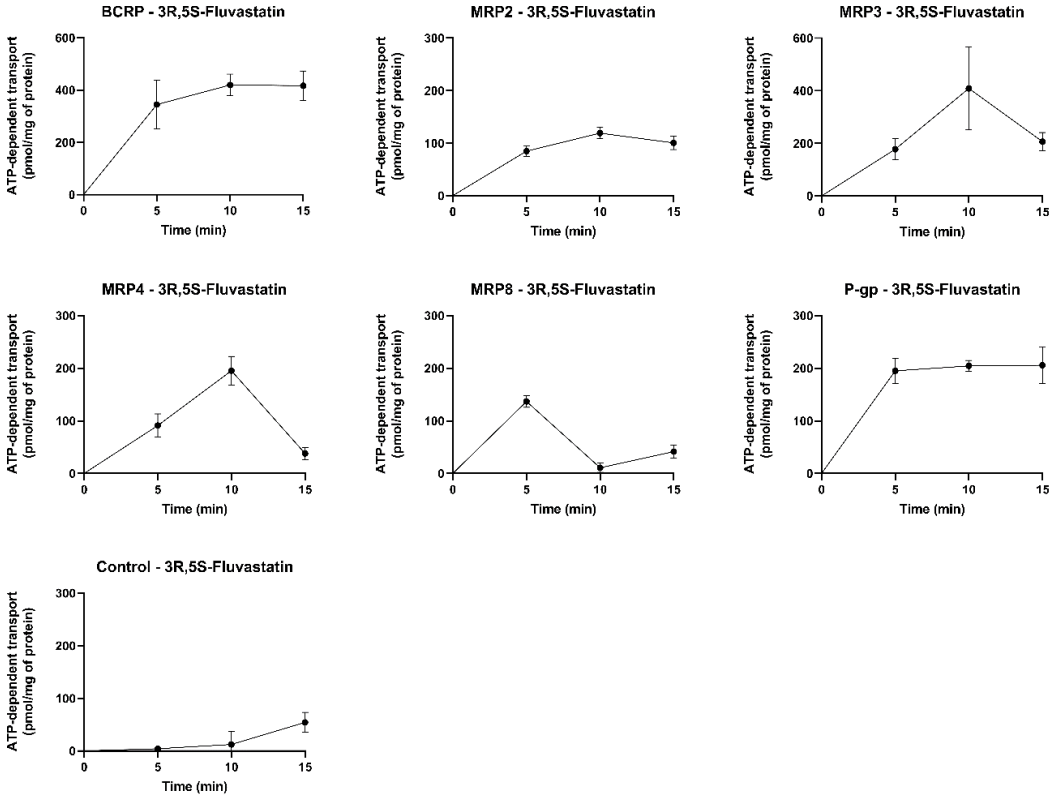

Figure 10. Time-dependent transport of 3R, 5S-fluvastatin in BCRP, MRP2, MRP3, MRP4, MRP8 and P-gp vesicles.

3S,5R-fluvastatin time-dependent transport was investigated with BCRP, MRP2, MRP3, MRP4, MRP8 and P-gp vesicles (figure 11). 3S,5R-fluvastatin seemed to be a good substrate of BCRP, MRP3 and P-gp, as shown in screening experiments results (ratios of 4.4, 3.1 and 2.7 at 5 min). Transport of 3S,5R-fluvastatin in MRP2 vesicles was lower than in the control vesicles (ratios of 1.0 and 1.3 at 5 min), indicating that 3S,5R-fluvastatin is not substrate of MRP2. MRP4 and MRP8 showed some transport of 3S,5R-fluvastatin (ratios of 2.2 and 1.7 at 5 min, 3.6 and 2.0 at 10 min, and 1.9 and 1.9 at 15 min).

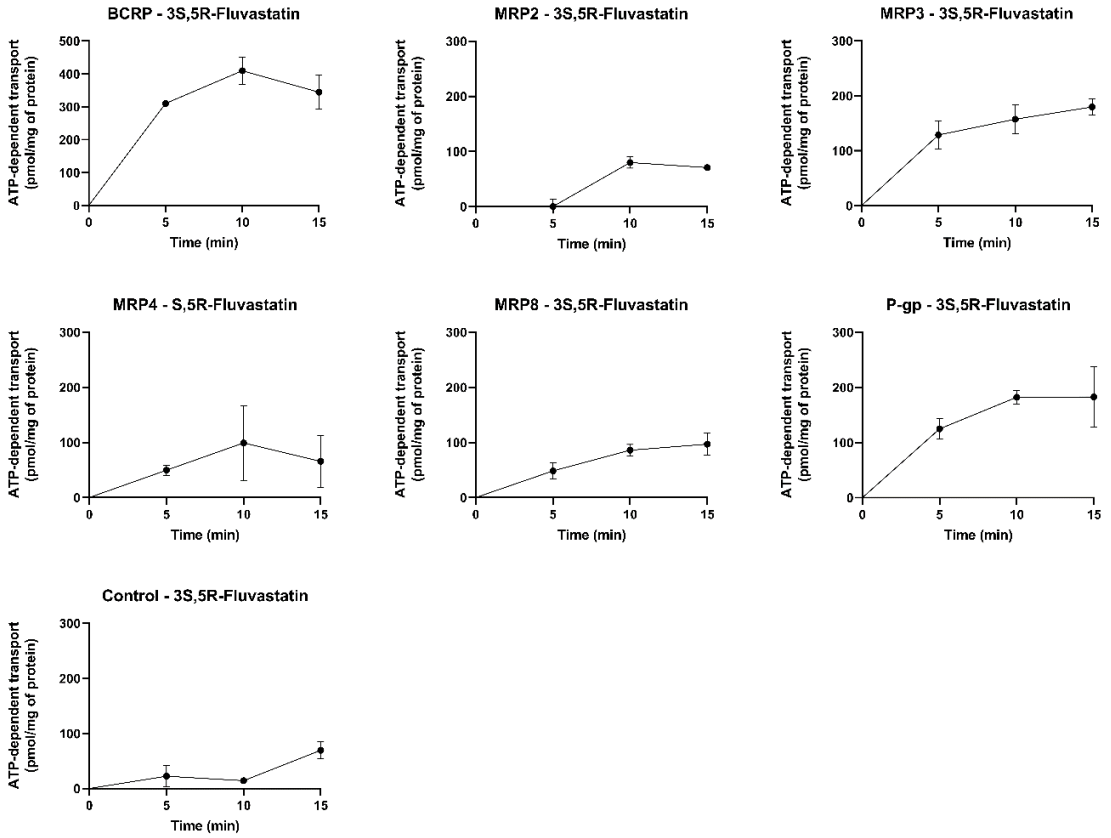

Figure 11. Time-dependent transport of 3S, 5R-fluvastatin in BCRP, MRP2, MRP3, MRP4, MRP8 and P-gp vesicles.

Time-dependent transport of pitavastatin was studied with BCRP, MRP2, MRP3, MRP4 and P-gp vesicles (figure 12). Results showed that pitavastatin transport into the vesicles was highest by BCRP (ratios of 5.2, 5.7 and 5.1). This confirmed the screening results. MRP3 showed only minor transport of pitavastatin (ratios of 1.9, 1.9 and 1.7). MRP2 and MRP8 did not transport pitavastatin more than the control vesicles, which also confirmed the screening results (ratios of 1.6, 1.2 and 1.2 at 5 min). In P-gp vesicles, pitavastatin transport in time points 5 and 10 min was almost two-fold compared to control vesicles (ratios of 3.6 and 1.2 at 5 min), which indicated pitavastatin possibly being a substrate for P-gp.

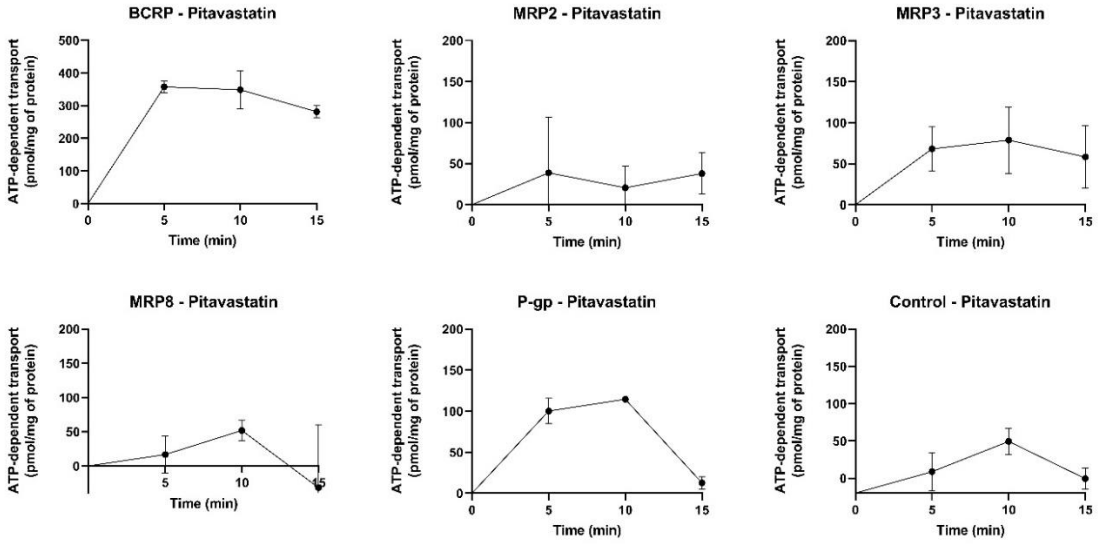

Figure 12. Time-dependent transport of pitavastatin in BCRP, MRP2, MRP3, MRP4 and P-gp vesicles.

Time-dependent transport of pravastatin was studied with BCRP, MRP2, MRP3 and MRP4 vesicles (figure 13). Significant variation was observed in the pravastatin experiments. MRP3 showed accumulation of pravastatin in all three time points, even though some variation was observed (ratios of 2.2, 1.5 and 1.7). MRP3 transported pravastatin to the highest extent of the investigated transporters. Experiments with BCRP vesicles showed high variation, but indicated some transport of pravastatin (ratio of 1.6 at 5 min). MRP2 and MRP4 did not show signs of pravastatin transport (ratios of 1.0 and 1.0 at 5 min). The transport of pravastatin in control vesicles was very low (ratios of 0.8, 1.0 and 0.9).

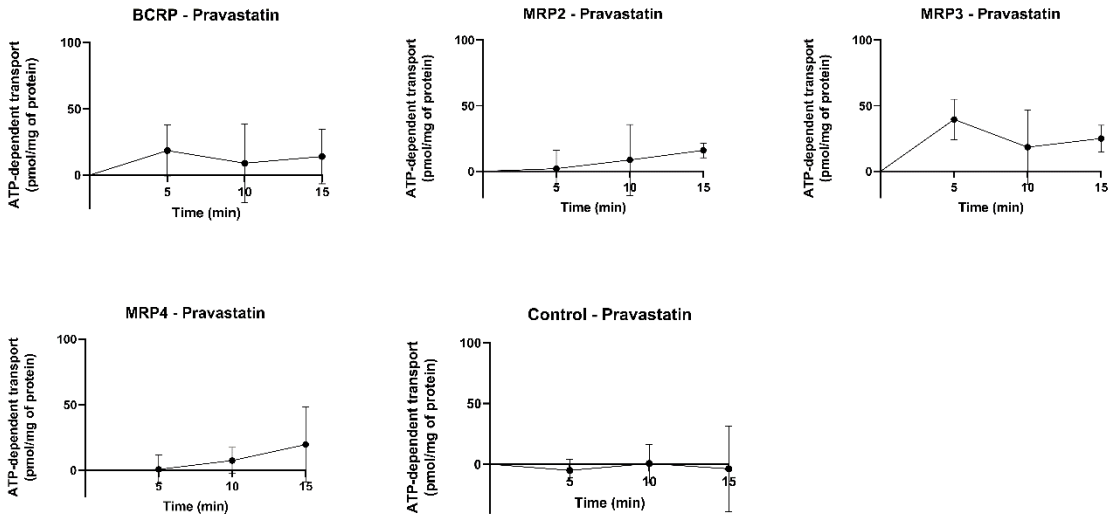

Figure 13. Time-dependent transport of pravastatin in BCRP, MRP2, MRP3 and MRP4 vesicles.

Time-dependent transport of rosuvastatin was investigated with BCRP, MRP2, MRP4 and P-gp vesicles (figure 14). As expected based on the screening experiments, rosuvastatin was a good substrate of BCRP (ratio of 18.5, 15.5 and 11.0). MRP4 transported rosuvastatin two-fold compared to the control (ratios of 2.0 and 1.0). Transport of rosuvastatin was poor at the 5 min time point (ratio of 1.1) in P-gp vesicles but indicated accumulation in 10 and 15 min time points (ratios of 1.9 and 2.0). MRP2 did not show signs of transport (ratios of 1.1, 1.3 and 1.3).

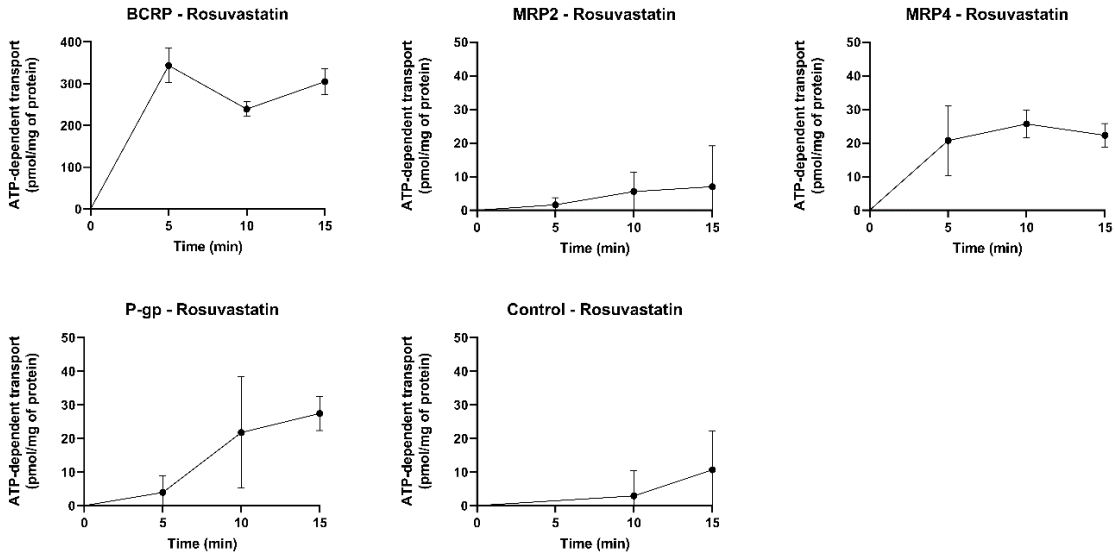

Figure 14. Time-dependent transport of rosuvastatin in BCRP, MRP2, MRP4 and P-gp vesicles.

Table 9. Transporters selected from the screening experiments to the time-dependet transport experiments. MRP2 did not show transport of fluvastatin (both isomers), pitavastatin, pravastatin or rosuvastatin in the screening, but was selected to be investigated based on the literature.

	BCRP	MRP2	MRP3	MRP4	MRP8	P-gp
Atorvastatin	X	X	X	X		X
Fluvastatin (both isomers)	X	X	X	X	X	X
Pitavastatin	X	X	X		X	X
Pravastatin	X	X	X	X		
Rosuvastatin	X	X		X	X	X
Simvastatin						

Table 10. Summary of time-dependent transport results. Mean and SD of ATP-dependent transport and ratio between ATP + and ATP – in the three time points.

Statin	Transporter	ATP-dependent transport at 5 min (pmol/min/mg)	SD	Ratio of ATP +/ ATP - at 5min	SD	ATP-dependent transport at 10 min (pmol/min/mg)	SD	Ratio of ATP +/ ATP - at 10 min	SD	ATP-dependent transport at 15 min (pmol/min/mg)	SD	Ratio of ATP +/ ATP - at 15 min	SD
Atorvastatin	BCRP	196.59	54.50	2.53	0.43	121.09	31.70	2.70	0.58	154.30	38.42	2.31	0.57
	MRP2	66.67	19.07	1.61	0.21	90.70	13.26	2.09	0.27	69.30	25.41	1.75	0.41
	MRP3	143.20	15.75	2.16	0.23	131.83	16.10	2.57	0.21	160.63	74.25	2.22	0.74
	MRP4	43.76	5.83	1.49	0.09	53.38	3.82	1.75	0.08	54.18	9.83	1.71	0.15
	P-gp	186.72	33.98	2.46	0.36	108.68	51.98	3.12	1.03	243.93	40.00	3.41	0.66
	Control	31.94	13.17	1.22	0.11	27.05	12.22	1.27	0.13	47.81	4.97	1.42	0.05
3R,5S-Fluvastatin	BCRP	344.89	92.83	4.05	0.85	419.53	41.64	4.48	0.78	416.61	56.74	4.05	0.84
	MRP2	84.79	9.50	2.33	0.17	118.92	10.78	2.60	0.25	100.56	12.72	2.52	0.29
	MRP3	177.58	39.73	3.06	0.63	408.84	158.13	4.59	1.49	206.42	34.85	3.72	0.46
	MRP4	91.16	22.13	2.50	0.47	195.18	27.30	2.76	0.30	37.86	11.63	2.23	0.68
	MRP8	136.75	11.31	2.03	0.12	10.81	8.55	1.36	0.30	41.59	12.11	1.73	0.26
	P-gp	195.07	23.71	2.96	0.32	204.26	10.54	2.78	0.12	205.66	35.41	3.54	0.89
	Control	4.27	4.81	1.14	0.17	12.42	25.00	1.23	0.46	54.12	19.29	1.83	0.44
3S,5R-Fluvastatin	BCRP	310.16	8.85	4.42	0.29	409.59	41.49	4.49	0.48	344.35	51.60	4.25	0.65
	MRP2	0.11	13.22	1.00	0.25	79.82	10.24	2.79	0.31	70.75	4.14	2.50	0.12
	MRP3	128.54	25.62	3.07	0.75	157.27	26.81	3.47	0.50	179.84	14.52	4.19	0.43
	MRP4	49.58	9.54	2.16	0.45	99.10	67.90	3.56	6.18	65.57	47.26	1.89	1.18
	MRP8	48.28	14.63	1.71	0.25	86.03	10.38	2.04	0.13	96.89	19.86	1.93	0.27
	P-gp	124.87	18.58	2.74	0.33	182.22	12.80	3.08	0.32	182.87	55.35	2.52	0.72
	Control	22.36	19.56	1.27	0.27	14.39	3.09	1.62	0.15	69.45	15.89	1.98	0.44
Pitavastatin	BCRP	357.56	17.96	5.24	0.58	348.81	58.47	5.71	0.80	281.17	18.38	5.14	0.39
	MRP2	38.81	67.72	1.63	1.34	20.66	26.56	1.74	1.00	37.96	25.16	1.71	0.48
	MRP3	68.22	26.79	1.92	0.46	78.87	40.53	1.91	0.50	58.31	38.11	1.68	0.75
	MRP8	16.66	26.97	1.16	0.28	51.77	14.93	1.68	0.23	-31.74	92.08	0.79	0.48
	P-gp	100.11	15.45	3.63	1.12	114.52	3.80	376.65	61.38	12.81	7.30	1.64	0.53
	Control	8.84	25.63	1.20	0.59	49.34	17.85	2.41	0.94	-0.48	13.79	0.99	0.41
Pravastatin	BCRP	18.43	19.19	1.56	0.78	8.92	29.59	1.22	0.89	13.98	20.61	1.38	0.72
	MRP2	1.99	13.86	1.04	0.26	8.58	27.04	1.18	0.58	15.91	5.64	1.68	0.34
	MRP3	39.46	15.45	2.24	0.53	18.31	28.36	1.51	0.81	24.99	10.13	1.72	0.47
	MRP4	0.64	10.99	1.02	0.30	7.39	9.96	1.25	0.42	19.58	28.65	1.75	1.31
	Control	-5.11	8.90	0.84	0.28	0.64	15.63	1.02	0.55	-3.78	35.25	0.91	0.82
Rosuvastatin	BCRP	343.11	41.39	18.49	2.76	238.86	17.26	15.46	4.08	304.67	30.74	11.03	1.81
	MRP2	1.62	2.11	1.06	0.07	5.58	5.70	1.33	0.36	7.02	12.14	1.28	0.50
	MRP4	20.74	10.33	1.98	0.66	25.66	4.16	2.84	0.55	22.28	3.53	2.08	0.18
	MRP8	4.03	13.18	1.13	0.48	3.88	6.27	1.22	0.40	13.68	11.26	1.65	0.57
	P-gp	3.92	4.97	1.14	0.18	21.73	16.56	1.85	1.14	27.43	5.08	2.03	0.19
	Control	-0.60	6.60	0.97	0.34	2.88	7.50	1.18	0.49	10.65	11.54	1.51	0.58

2.4 Concentration-dependent transport experiments

In the concentration-dependent transport experiments each statin was investigated with several concentration points, in order to determine kinetic parameters for the statins by fitting results into of the Michaelis-Menten equation. All the statins did not reach plateau in ATP-dependent transport experiments. Concentration-dependent transport of fluvastatin was investigated with racemic fluvastatin. All the pharmacokinetic parameters are summarized in the table 11.

Concentration-dependent transport experiments of atorvastatin confirmed earlier results, which indicated that atorvastatin is substrate for BCRP, MRP3 and P-gp (figure 15). Increasing transport of atorvastatin with concentration occurred, but a clear plateau of ATP-dependent transport was not observed in the BCRP vesicles and only nearly in the MRP3 vesicles. In P-gp vesicles, concentration-dependent accumulation reached a plateau already after the 25µM concentration point. The K_m value of BCRP, MRP3 and P-gp seemed to be below 100µM. The K_m value of BCRP for atorvastatin was 82µM, that of MRP3 was 33µM and that of P-gp was 11µM. V_{max} was estimated to set around 135 pmol/min/mg in the BCRP, 79 pmol/min/mg in MRP3 and 33 pmol/min/mg in P-gp vesicles.

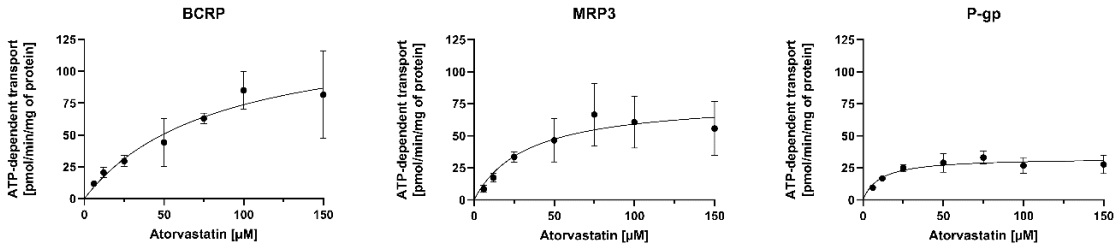

Figure 15. ATP-dependent transport of atorvastatin in BCRP, MRP3 and P-gp vesicles.

Concentration-dependent transport of fluvastatin was investigated with racemic fluvastatin for all the transporters (figure 16). BCRP was clearly the best transporter for racemic fluvastatin and the concentration-dependent transport curve fitted well with the Michaelis-Menten equation. The calculated V_{max} and K_m values were 340 pmol/min/mg and 23 µM for BCRP. MRP2, MRP3, MRP4, MRP8 transported racemic fluvastatin in concentration-dependent manner, but plateau of ATP-dependent transport was not observed. The calculated V_{max} and K_m values were 181 pmol/min/mg and 133 µM for MRP2. MRP3 was a good transporter for racemic fluvastatin and steady plateau was

observed after 100µM. The calculated V_{max} and K_m for MRP3 were 156 pmol/min/mg and 43 µM. Transport by MRP4, MRP8 and P-gp occurred in concentration-dependent manner, but clear plateaus were not observed, especially so for P-gp. The estimated V_{max} and K_m values were 54 pmol/min/mg and 33 µM for MRP4, and 85 pmol/min/mg and 62 µM for MRP8. The estimated V_{max} and K_m were high for P-gp.

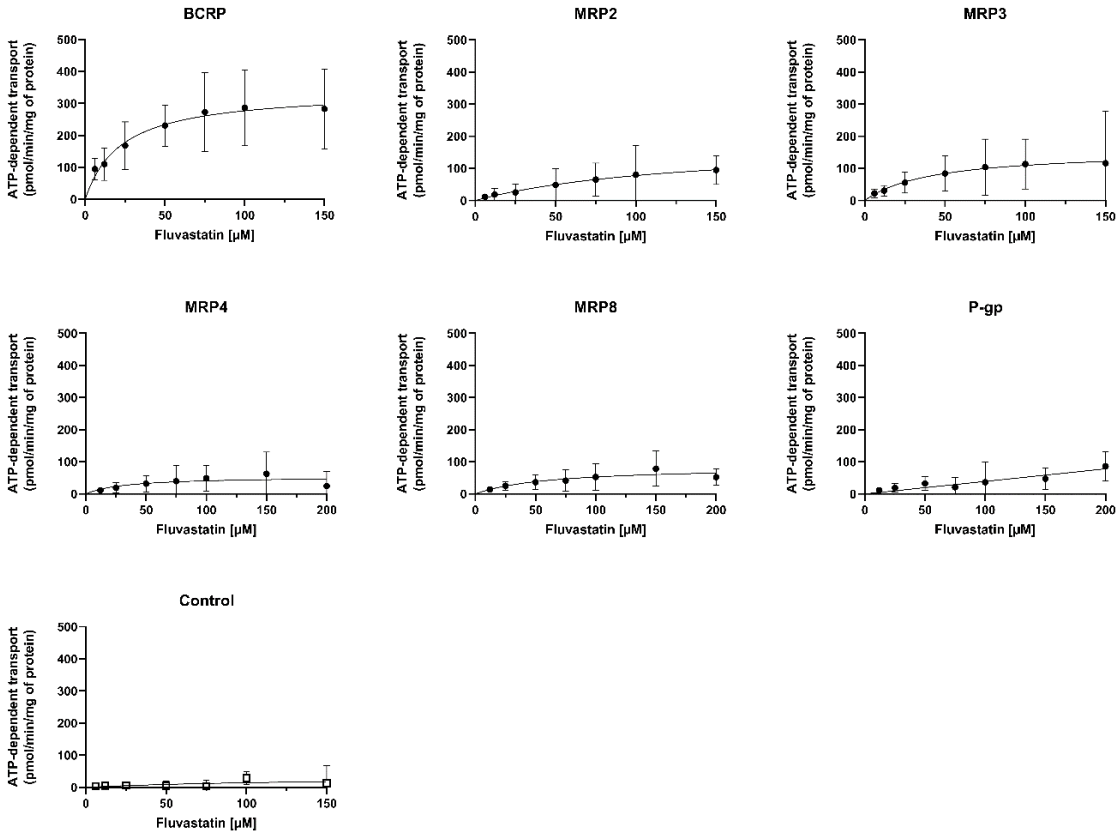

Figure 16. ATP-dependent transport of racemic fluvastatin in MRP2, MRP3, MRP4 and P-gp vesicles.

Concentration-dependent transport of pitavastatin was studied with BCRP, MRP3, P-gp vesicles (figure 17). BCRP transported pitavastatin at the highest level, but with significant variation. MRP3 and P-gp showed minor transport of pitavastatin. Full concentration-depend transport and saturation of pitavastatin accumulation was not observed for MRP3. Transport by P-gp decrease after 100µM and curve-fitting was not complete. The estimated V_{max} for BCRP was 94 pmol/min/mg, for MRP3 it was 20 pmol/min/mg and for P-gp it was 22 pmol/min/mg. Approximated K_m values were 15 µM for BCRP, 15 µM for MRP3 and 15 µM for P-gp.

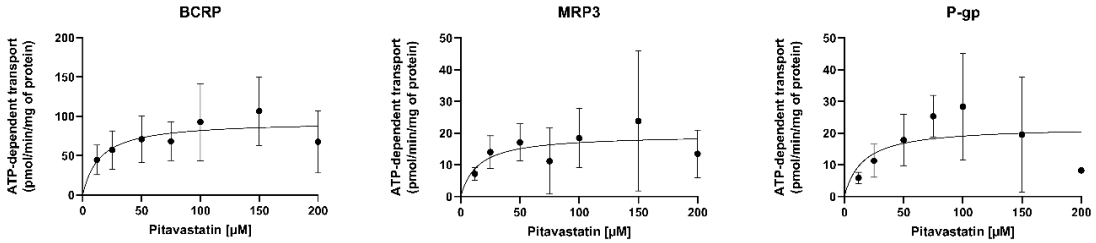

Figure 17. Concentration-dependent transport of pitavastatin in BCRP, MRP3, and P-gp vesicles.

Concentration-dependent transport of pravastatin was studied with MRP3 vesicles (figure 18). MRP3 was the only transporter that showed clear transport of pravastatin in the time-dependent transport experiment and hence it was the only transporter studied in the concentration-dependent experiment. ATP-dependent transport of pravastatin increased with concentration, but plateau was not achieved. The V_{max} of MRP3-mediated pravastatin transport was estimated as 26 pmol/min/mg of protein and K_m as 67 µM.

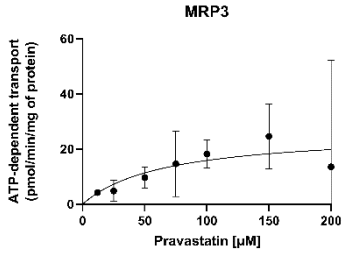

Figure 18. Concentration-dependent transport of pravastatin in MRP3 vesicles.

Concentration-dependent transport of rosuvastatin was studied with three transporters: BCRP, MRP4 and P-gp (figure 19). Concentration-dependent transport of BCRP was investigated with lower concentration points, which revealed rosuvastatin's concentration-dependent accumulation before saturation. Transport by MRP4 or P-gp was much lower compared to BCRP. Transport in MRP4 vesicles at first three concentration points (12 µM, 25 µM and 50 µM) increased, but after that variation increased and the calculated ATP-dependent transport decreased almost to zero. Transport of rosuvastatin by P-gp increased with the concentration, but with significant variation in the last three concentration points (100 µM, 150 µM and 200 µM). Concentration-dependent transport by P-gp did not fit the Michaels-Menten equation and plateau of ATP-dependent transport was not clearly seen. The estimated V_{max} for BCRP was 87 pmol/min/mg, for MRP4 4 pmol/min/mg and for P-gp 20 pmol/min/mg. The K_m values were 5 µM, 1 µM and 60 µM for BCRP, MRP4 and P-gp.

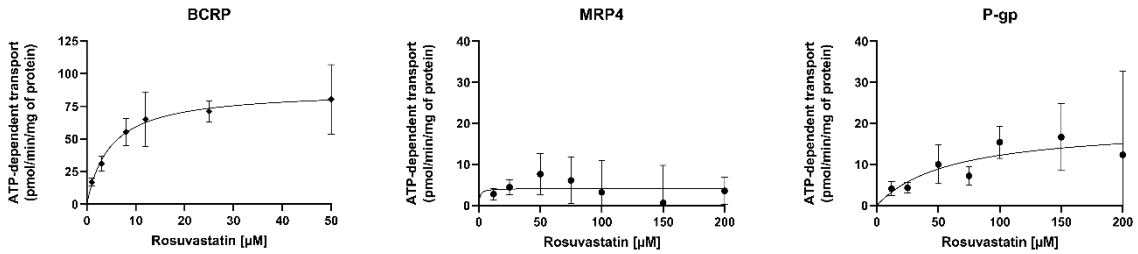

Figure 19. Concentration-dependent transport of rosuvastatin in BCRP, MRP4 and P-gp vesicles.

Table 11. The summary of kinetic parameters of atorvastatin, racemic fluvastatin, pitavastatin, pravastatin and rosuvastatin transport in BCRP, MRP2, MRP3, MRP4, MRP8 and P-gp vesicles.

		V_{max} (pmol/min/mg of protein)	**95% CI of V_{max}**	**K_m (µM)**	**95% CI of K_m**	**CL (µl/min/mg)**
Atorvastatin	BCRP	134.5	90.7-273.4	82.4	32.4 - 270.5	1.4
	MRP3	78.7	58.4 -118.1	33.0	12.7 - 89.8	2.2
	P-gp	32.9	28.4 - 38.3	10.7	5.4 - 19.7	1.4
Fluvastatin (racemic)	BCRP	339.8	246.2 - 535.0	22.7	5.9 - 80.1	15.8
	MRP2	180.6	61.0 - INF	132.7	8.0 - INF	1.9
	MRP3	156.3	68.3 - INF	42.9	0.6 - INF	3.5
	MRP4	54.1	21.9 - INF	32.8	N/A	1.7
	MRP8	85.3	44.7 - 818.7	62.0	5.3 – 1820	1
	P-gp	~3.8e+015	N/A	~9.9e+015	N/A	0.6
Pitavastatin	BCRP	93.8	67.1 - 142.1	14.5	0.1 - 66.5	8.8
	MRP3	19.5	11.9 - 40.23	14.7	-INF - 141.6	1.2
	P-gp	22.1	13.6 - 37.4	15.8	-INF - 91.1	1.8
Pravastatin	BCRP	26.4	9.3 - INF	66.6	N/A	0.2
Rosuvastatin	BCRP	87.5	80.6 - 95.1	4.78	3.5 - 6.4	20.4
	MRP4	4.2	1.7 - 10.3	0.9	-INF - 165.3	0.3
	P-gp	19.6	9.4 - INF	60.5	2.0 - INF	0.4

Vmax = maximal velocity, CI = confidence interval, Km = Michaels constant, CL = clearance, INF = infinity and N/A = not available.

3 DISCUSSION:

This thesis evaluated the transport of six clinically used statins by six efflux transporters important for drug pharmacokinetics. The results demonstrated significant differences in the efflux transport profiles of statins.

The vesicular transport assay protocol was first optimized with several experiments. The aim of the optimization was to ensure the quality and reproducibility of the results. Vesicular membrane assay has disadvantages that are mostly related to the lipophilicity of the substrate of interest (Gilibili et al., 2017). Lipophilic compounds may diffuse in and out of the vesicles, which is why determining their ATP-dependent transport is difficult in membrane vesicles. The hydrophilic pravastatin (Log D -0.75) proved to be a challenging substrate in the vesicle experiments. Therefore, optimization experiments were mostly carried out with pravastatin.

According to the manufacturer's instructions, pre-wetting of the filters on the filter plates is not necessary (MultiScreen HTS plate protocol). The impact of pre-wetting was studied by adding samples to dry and pre-wetted filters (figure 2). When using dry filters, the results showed higher overall transport, but also higher apparent transport in the absence of ATP, and more variation. Based on these results, pre-wetted filters were used in the subsequent experiments. The impact of stopping buffer on mass spectrometric measurements was also investigated. The commercial stopping buffer was delivered with the vesicles. To avoid a possible matrix effect on mass spectrometric analysis, the vesicles were always washed well with washing buffer to get all the stopping buffer away. The stopping buffer was foamy and bubbly, which covered the whole well and formed a film over the filter. Bubbles were broken manually with pipet tips so that the washing buffer had better access. Even though the stopping buffer is washed away by using washing buffer, it may still affect the subsequent measurement. Therefore, the stopping and washing of the reaction was tested with washing buffer only. The results showed lower apparent statin transport and indicated that the stopping buffer was a better buffer for terminating the reaction than washing buffer (figure 2). The experiment was not completely successful since in the pre-wetted filters, when reaction was terminated with stopping buffer, pravastatin accumulated in MRP3 vesicles more in the absence than in the presence of ATP. Results were not completely unambiguous, and it was decided to stick with vesicle manufacturer's recommendation and use the commercial stopping buffer.

The level of pravastatin transport was very low and its concentration in the vesicles was near the mass spectrometric quantification limit. Therefore, the samples were concentrated by evaporating the samples after and diluting them into a smaller volume of acetonitrile (figure 4). However, this procedure did not improve statin quantification and also increased variation in the measurements. Evaporation was therefore not implemented in the assay protocol.

The elution volume was optimized by investigating statin concentration in the eluant at different elution volumes. The first experiment was performed by using the fluorescent substrate CDCF and MRP2 (figure 5). The aim was to improve statin concentration in eluant by decreasing elution volume. However, the measured fluorescence increased with elution volume. In fluorescence measurements it is possible that a higher liquid column gives a higher signal (the Beer-Lambert law), which probably happened in this experiment. For this reason, elution volume was studied also with three statins (pitavastatin, pravastatin and rosuvastatin) each with different transporter (BCRP, MRP3 and P-gp) (figure 6). Lower elution volume of 50µl did not decrease statin yield or increase variation, and it was implemented to the protocol.

In the optimizing, it was also studied if elution is complete after one elution. The filter plate was eluted twice, by adding a second fresh eluant after the first elution, which confirmed that all the statin is collected (figure 7). A minor difference between once and twice eluted statin concentration was observed, which can be explained by normal variation between replicates.

In the assay filters, the external standard and samples were treated differently. Sample filters were pre-wetted, washed with stopping and washing buffer, but external standard was added to dry filters without washes. External standard also did not contain any vesicles. To rule out bias due to the differing treatment of the samples and external standard, the possible effects of vesicles and washes on the external standard were investigated. To investigate the effects of different treatment of external standard filters on the results, results of double elution were determined by using normal external standard and external standard which included vesicles (figure 7), and results of pre-wetting the filters were determined with external standard added to dry and pre-washed filters (washed three times with stopping buffer and twice with washing buffer) (figure 3). In both of these experiments, different treatment of external standard did not affect the results. Therefore, the external standard was decided to be added to dry filters without vesicles in the subsequent experiments.

The experiments of this thesis consist of three main phases: screening, time-dependent transport and concentration-dependent transport experiments. In the screening, BCRP, MRP2, MRP3, MRP4, MRP8 and P-gp were investigated as potential transporters for all the statins of interest (atorvastatin, fluvastatin, pravastatin, pitavastatin, rosuvastatin and simvastatin). Screening was performed with two concentrations (1 µM and 10 µM), but statin transport was more detectable in 10 µM experiments and deviation was smaller compared to 1 µM experiments. In addition, at 1 µM, the quantities of some statins were below the limit of quantitation. Therefore, 10 µM experiments results were used to evaluate which statins are substrate of each transporter. In the time-dependent studies, the optimal reaction incubation time was examined more specifically by using one statin concentration and three incubation times of 5, 10 and 15 minutes. The trend was with few exceptions that when increasing the incubation time, the statin transport increased also in absence of ATP and in the control vesicles. This means that ATP-dependent transport did not increase, and time-dependent saturation had already occurred before the 5 min time point. The 5 min incubation time was selected for the concentration-dependent experiments, because statin transport was clearly observed in this time and 5 min was practical. In the concentration-dependent experiments, statin transport was examined with several concentration points and with the incubation time selected based on time-linearity studies. The aim was to detect concentration-dependent transport of statins and to determine the transport kinetic parameters for the statins by fitting the results into a Michaelis-Menten equation. All the statins did not reach plateau in ATP-dependent transport experiments and did not fit well the Michaelis-Menten equation.

In the screening and time-dependent transport experiments, control vesicles were an important tool to confirm statin transport. Control vesicles were not included in the concentration-dependent transport experiments, because difference from control vesicles was already demonstrated in the time-dependent transport experiments. Control vesicles are prepared form HEK293 cells like other vesicles, but they do not contain any transfected transporters. It is possible that control vesicles express endogenous transporters which may play a role in statin transport (Ahlin et al., 2009; Reid et al., 2003). Most statins were transported slightly better in the presence than absence of ATP in control vesicles, which supports the presence of endogenous statin transporters in HEK293-cell-derived membrane vesicles.

Previous studies have shown with different methods that atorvastatin is a substrate of BCRP (Lee et al., 2019), MRP2 (Shin et al., 2017), MRP4 (Knauer et al., 2010) and P-gp

(Hochman et al., 2004; Shin et al., 2017). Transport of atorvastatin by P-gp has also been studied in Caco-2 cells and an apparent K_m was calculated as 110µM (Boyd et al., 2000). The present results indicate a low K_m (11µM) of atorvastatin for P-gp, indicating high affinity. The present results cannot be directly compared to the results of Boyd et al (2000) since the method is different. BCRP and P-gp are both good transporters for atorvastatin, but an interesting note in the results is that P-gp shows better transport than BCRP, which is often a superior transporter for many statins. Another interesting finding is MRP3, which also transports atorvastatin with relatively good affinity. MRP2 transported atorvastatin in the screening and time-dependent transport, but to a small extent only and was therefore not included in the concentration dependent experiments.

The screening and time-dependent transport of fluvastatin was conducted with both isomers of fluvastatin (3R,3S-fluvastatin and 3S,5R-fluvastatin). However, concentration-dependent transport experiments were performed with racemic fluvastatin, because isomers were not at that time any more commercially available to purchase, and previous stocks were too diluted. BCRP, MRP3, MRP4 and P-gp were the most promising transporters for both isomers in the screening and time-dependent transport studies but all the transporters were included in concentration-dependent transport of racemic fluvastatin. Fluvastatin has been reported to be a substrate of BCRP, MRP2 (Lin et al., 2011) and P-gp (Li et al., 2011). In the concentration-dependent experiments, BCRP was a very good transporter for racemic fluvastatin and MRP3 also showed clear transport of fluvastatin. Other transporters might also transport racemic fluvastatin, but that could not be confirmed in these experiments. This is slightly surprising, because in the screening and in time-dependent transport experiments, both isomers were found to be potential substrates of MRP3, MRP4, MRP8 and P-gp in addition to BCRP. Based on this study, further studies are required to determine the enantiospecific transport of fluvastatin isomers by efflux transporters.

BCRP, MRP3, P-gp (Vildhede et al., 2016) and MRP4 (Kanda et al., 2011) have previously been shown to transport pitavastatin. Vildhede et al (2016) determined the K_m and V_{max} of pitavastatin by using membrane vesicles. K_m was 1 µM, 448 µM and 84 µM for BCRP, MRP3, P-gp, respectively, and V_{max} was 95, 433 and 2380 pmol/min/mg protein. MRP2 or MRP4 were not found to transport pitavastatin. Hirano et al (2005) reported values for K_m and V_{max} of 6 µM and 1106 pmol/min/mg protein with BCRP, also using membrane vesicles. In the present study, pitavastatin was found to be a substrate of BCRP, MRP3 and P-gp. These results are similar to the results of Vildhede et al (2016).

The estimated K_m values for BCRP, MRP3 and P-gp were 13, 20 and 20 µM, respectively, and V_{max} values 118, 24 and 35 pmol/min/mg. Both the K_m and V_{max} values were much lower than those reported by Vildhede et al (2016) results. The difference in parameter values could be a consequence of variation between measurements or difference in vesicle quality and protein expression levels.

Afrouzian et al (2018) have shown with membrane vesicles that pravastatin is substrate of BCRP, MRP2, MRP4, and P-gp. K_m and V_{max} were estimated as 3 µM and 3 pmol/min/mg for pravastatin transport by BCRP. In the present study, only MRP3 transported pravastatin. BCRP and MRP4 looked also promising transporters in the screening, but time-dependent experiments showed only weak if any transport. The V_{max} and K_m were estimated for MRP3, but the ATP-dependent transport did not reach plateau. Therefore, the results should be interpreted with caution. Pravastatin was a very challenging compound in the vesicle experiments from the start. Pravastatin is a relatively hydrophilic compound, indicating that diffusion in or out from vesicles cannot explain this. Variation between replicates was significant. This made the results unreliable and difficult to choose the transporters to the next phase from the screening and time-dependent transport experiments. MRP3 was the only transporter, which indicated frequently signs of accumulation of pravastatin and was therefore the only transporter chosen into pravastatin concentration-dependent transport experiments.

Previous reports have shown rosuvastatin to be substrate of BCRP (by using membrane vesicles) (Lee et al., 2019), MRP2 (Knauer et al., 2010), MRP3, MRP4 (by using membrane vesicles) (Kanda et el., 2018) and P-gp (Knauer et al., 2010). Transport studies of rosuvastatin by BCRP in membrane vesicles have resulted in K_m and V_{max} values of 2 µM and 304 pmol/min/mg (Kitamura et al., 2008). MRP4-expressing membrane vesicles have yielded K_m and V_{max} values of 21 µM and 1140 pmol/min/mg (Pfeifer et al., 2013). Rosuvastatin is known to be a good substrate of BCRP, which was confirmed in the present study. This verifies the functionality of the used method and gives confidence also for the other findings. Concentration-depend transport of BCRP was investigated at lower concentration than other transporters, because of the known high affinity of rosuvastatin to BCRP. The calculated K_m was 5 µM and V_{max} was 88 pmol/min/mg for BCR. Km value is similar compared to study conducted by Kitamura et al (2008), but the Vmax value is lower. In the present study, P-gp also clearly transported rosuvastatin. MRP4 seemed to transport rosuvastatin in the screening and time-dependent transport experiment, but in the concentration-dependent transport experiments at higher

concentration rations, the accumulation dropped and kinetic parameters could therefore not be determined.

Simvastatin is a difficult compound to study transport *in vitro* and there are only a few reports investigating the efflux transport of simvastatin. The parent simvastatin lactone are demonstrated not to be a substrate of BCRP, MRP2 nor P-gp *in vitro* (Li et al., 2011). However, *in vivo* studies have demonstrated simvastatin lactone to be substrate of BCRP and P-gp (Hsyu et al., 2001; Polli et al., 2013). In the present study, the transport of active simvastatin acid was investigated. In the screening experiments, no accumulation of simvastatin acid could be determined, but the results showed considerable variation. Simvastatin acid is a relatively lipophilic compound (Log D 1.5-1.75), which indicates that there may be significant diffusion of simvastatin acid into and out of the vesicles. More studies, potentially with different experimental systems are required to confirm whether simvastatin acid is a substrate of the studied efflux transporters.

All of the statin and transporter combinations did not reach the point where ATP-dependent transport plateaus. In cases of atorvastatin transport by BCRP and racemic fluvastatin transport by MRP2, reaching the plateau could have needed higher concentrations, or cannot be achieved with this method. On the contrary, atorvastatin transport by P-gp, racemic fluvastatin transport by MRP4, MRP8 and P-gp, pitavastatin transport by MRP3 and P-gp and rosuvastatin transport by MRP4 might have needed lower concentration points to achieve a better view of concentration-dependent transport and plateau of ATP-dependent transport. As a consequence, the calculated transport kinetic parameters for these statins and transporters should be interpreted with caution.

Regarding individual transporters, BCRP transported rosuvastatin best of the studied statins in all the experiments. In addition, both fluvastatin enantiomers and racemic fluvastatin seemed to be substrates of BCRP. Atorvastatin and pitavastatin were well transported by BCRP, but to a smaller extent than rosuvastatin. The transport rates of MRP2 were very low for all the statins. MRP2 transported atorvastatin to the highest extent and signs of fluvastatin enantiomer transport were also observed, whereas pitavastatin, pravastatin and rosuvastatin did not differ from the control. In the previous literature, atorvastatin, fluvastatin, pravastatin and rosuvastatin have been shown to be substrates of MRP2 (Afrouzian et al., 2018; Knauer et al., 2010; Lin et al., 2001; Shin et al., 2017). In contrast, in the present study, none of these statins were well-transported by MRP2. This raises the question of whether the vesicles expressing MRP2 were somehow defective. It is possible that vesicles contain less protein than expected or contain wrongly

orientated transporters, which could alter the results. MRP2 protein quantification, determination of the membrane orientation of MRP2 in the vesicles and investigation of the transport of a prototype substrate would be required to verify the functionality of the used MRP2 vesicles.

MRP3 was shown to transport atorvastatin, both fluvastatin enantiomers, racemic fluvastatin and pravastatin, but pitavastatin and rosuvastatin did not differ from control in the screening and time-dependent transport experiments., pravastatin was only transported by MRP3. Both fluvastatin enantiomers and rosuvastatin were well transported by MRP4 in the screening and time-dependent transport experiments, but transport was weak in the concentration-dependent transport experiments. Also variation in rosuvastatin transport was relatively high. Atorvastatin and pravastatin transport by MRP4 differed only slightly from the control and pitavastatin uptake did not ascend above the control levels. MRP8 showed transport of 3S,5R-fluvastatin and racemic fluvastatin. Pitavastatin also appeared to be transported by MRP8 in the screening, but in the time-dependent transport experiment no pitavastatin transport by MRP8 could be observed. In the screening experiment, only pravastatin and rosuvastatin seemed not to be substrates of P-gp. However, in the time-dependent and concentration-dependent transport experiments, rosuvastatin was found to be transported by P-gp. Other experiments also revealed that racemic fluvastatin and pravastatin were not transported by P-gp.

In conclusion, these data demonstrate that atorvastatin is a substrate of BCRP, MRP3 and P-gp, racemic fluvastatin is a substrate of BCRP, MRP2 and MRP3, pitavastatin is a substrate of BCRP, MRP3 and P-gp, pravastatin is a substrate of MRP3 and rosuvastatin is a substrate of BCRP and P-gp *in vitro*. The transport kinetic parameters K_m and V_{max} were successfully calculated for BCRP, MRP3- and P-gp-mediated transport of atorvastatin, BCRP-mediated transport of racemic fluvastatin, BCRP- and MRP3-mediated transport of pitavastatin, and BCRP- and P-gp-mediated transport of rosuvastatin.

This thesis improves general understanding of pharmacokinetic properties of atorvastatin, fluvastatin, pitavastatin, pravastatin and rosuvastatin. Efflux transporters play an important role in limiting the intestinal absorption and facilitating the excretion of statins and other drugs from the body. These data are useful in predicting the effects of transporter-mediated drug-drug interactions and genetic variability in transporter function on the pharmacokinetics, efficacy and safety of statin therapy.

4 MATERIALS AND METHODS

4.1 Materials

Vesicles were purchased as commercially available from Pharmtox, Radboud UMC, Nijmegen, the Netherlands. Assay buffer and stopping buffer were delivered included with the vesicles. All commercially purchased reagents and materials are shown in the table 12. For incubating plates in warm with mixing shake, ThermoMixer C (Eppendorf-AG, Hamburg, Germany) and Thermo-Shaker PST-60HL (BioSan, Riga, Latvia) were used. Manifold 96-well Plate (Waters, Milford, Massachusetts, USA) were used to filtrate samples through the filtrate plates and in the end, samples were eluted by using Centrifuge 5430 (Eppendorf, Hamburg, Germany). Storage plate containing samples were sealed with heat sealer model of 5390 000.034 (Eppendorf, Hamburg, Germany) and substrate concentrations were measured with liquid chromatography-tandem mass spectrometry. In the optimizing, the evaporation was conducted by using miVac DUO conctartor (Genevac, Ipsxich, UK) and CDCF fluorescence was measured with Cytation 5 (Biotek, Winooski, Vermont, U.S.A). pH balance of the solutions (washing buffer) was determined by using model 3510 pH meter (Jenway, Staffordshire, UK).

Table12. Table of materials including catalogue number and supplier.

Material	Catalogue number of the supplier	Supplier
3R,5S-Fluvastatin	SC-206732	Santa Cruz Biotechnology (Dallas, Texas, USA)
3S,5R-Fluvastatin	SC-206738	Santa Cruz Biotechnology (Dallas, Texas, USA)
96-well Clear Flat Bottom TC-treated Microplate	3585	Corning (Corning, New York, USA)
96-well Clear Round Bottom Polypropylene Not Treated Microplate	3365	Corning (Corning, New York, USA)

Atorvastatin Calcium salt	A791750	Toronto research chemicals (Ontario, Canada)
Atorvastatin-D5 sodium salt	A791753	Toronto research chemicals (Ontario, Canada)
CDCF) (5(6)-Carboxy-2′,7′-dichlorofluorescein)	21882	Sigma-Aldrich (Saint Louis, Missouri, USA)
DMSO	D4540	Sigma-Aldrich (Saint Louis, Missouri, USA)
Fluvastatin racemic	SC-279169	Santa Cruz Biotechnology (Dallas, Texas, USA)
Fluvastatin-D8		Toronto research chemicals (Ontario, Canada)
FrameStar 96 Non-Skirted PCR plate	4ti-0710/R	4titude (Wotton, UK)
KCl (potassium chloride)	1.04936.0500	Merck (Darmstadt, Germany
MeOH	34966	Honeywell (Charlotte, North-Carolina, USA)
$MgCl_2$ (magnesium chloride hexahydrate)	A450933	Merck (Darmstadt, Germany)
MOPS (3-(N-morpholino) propanesulfonic acid)	M3183-100G	Sigma-Aldrich (Saint Louis, Missouri, USA)
MultiScreenHTS FB Filter Plate 1.0 µm / 0.65 µm	MSFBN6B50	Merck (Darmstadt, Germany
Na_2ATP	A2383-5G	Sigma-Aldrich (Saint Louis, Missouri, USA)
NaOH	1.06498.1000	Merck (Darmstadt, Germany

Pitavastatin Calcium	P531000	Toronto research chemicals (Ontario, Canada)
Pitavastatin-D5 sodium salt	P531007	Toronto research chemicals (Ontario, Canada)
Pravastatin Sodium	F702000	Toronto research chemicals (Ontario, Canada)
Pravastatin-D9 sodium salt	P702004	Toronto research chemicals (Ontario, Canada)
Rosuvastatin Calcium salt	R700500	Toronto research chemicals (Ontario, Canada)
Rosuvastatin-D6	R700502	Toronto research chemicals (Ontario, Canada)
Simvastatin acid		SynFine Research (Ontario, Canada)
Simvastatin-D6	S485002	Toronto research chemicals (Ontario, Canada)
Tris (tris(hydroxymethyl)aminomethane)	T-1378	Sigma-Aldrich (Saint Louis, Missouri, USA)

4.2 Methods

The vesicular transport assay was used to study the transport of statins by different efflux transporters. Vesicles were made from HEK293 cells transfected with efflux-transporter encoding baculovirus. Cells were single transfected, which enables overexpression and study of the specific transporter of interest.

Vesicles were thawed on ice and the assays were performed on a 96-well round bottom plate. Vesicles were mixed with substrate solution (concentration of reagents: statins with

varying concentration, 100mM $MgCl_2$, 1.5% DMSO and assay buffer provided with the vesicles). Substrate solution with vesicles, ATP and MQ-water were preincubated separately at 37°C with shaking for 10 min before the reaction. The active transport was initiated by adding ATP (final concentration of 4 mM) to the reaction mixture. In blank samples MQ-water was added instead of ATP. The reaction was performed at 37°C with shaking. Reaction time depended on the phase of the experiment (see below). Reaction was terminated by adding ice-cold stopping solution and samples were immediately transferred and filtered through 96-well filter plate. Vesicles, which were retained on the filter, were instantly washed again once with cold stopping solution and after terminating all the samples, again twice with stopping solution and twice with washing buffer (0.04M MOPS-Tris and 0.07M KCl). Filters were dried with dry filtration and lysis buffer was added to vesicles (25ng/ml internal standard and 50% MeOH). Lysis buffer was incubated for 10 min on ice to break the vesicles. Meanwhile, external standard of the statin was added to the filter plate during incubation. Lysis buffer and standards were eluted by centrifuging at 2204 g (+4500 rpm) for 10 minutes. Similarly, statin accumulation was investigated in control vesicles, which were control-transfected. Statin accumulation inside the vesicles were measured by liquid chromatography-tandem-mass spectrometry.

Study was divided into optimizing and into three larger main phases:

0) Optimizing vesicular transport assay:

Before vesicular transport studies, the assay protocol was further optimized. Optimizing included 5 parts:

i. Pre-wet of filter before sample transfer: To evaluate the effect of pre-wetting filter plate before transferring the samples into filters, experiments were performed by transferring samples to dry filter plate after terminating the reaction and by transferring samples into the filter plate, which had been pre-wetted with MQ-water.
ii. Evaporating of samples and dissolving into organic solvent: To improve signals, after performing the experiment, samples were evaporated at 37°C for 30 min and 25% acetonitrile was added.
iii. Reduction of elution volume and eluting twice: To evaluate appropriate elution volume, same protocol was used with three statins (pitavastatin, pravastatin and rosuvastatin) and fluorescein substrate, CDCF. Several statins were used to study whether the effect varies between different statins. By using fluorescein substrate, it was possible to measure fluorescein right after experiment and pace up optimizing.

Elution was tested with volumes of 100µl and 50µl with statins, and volumes of 100 µl, 75µl and 50µl with CDCF. CDCF was lysed with NaOH. To make sure all the statin is collected from the filters, the experiment was conducted with pravastatin and MRP3, and the filter plate was eluted twice by adding a second fresh eluant to filters and centrifuging.

iv. Addition of vesicles into standards and pre-washing of filters before adding standard: To examine whether statins bind into vesicle matrix and affect measured signals, external standard was prepared both with and without vesicles. Impact of pre-washing to standard was studied by treating filter plate in two different ways before adding standard solutions: dry filters and filters treated three times with stopping buffer and twice with washing buffer.

v. Termination of reaction with ice cold washing buffer: To evaluate effect of possible proteins in stopping buffer to mass spectrometric measurements, reaction was terminated with ice cold washing buffer and only washed with washing buffer.

1) Screening of statin transport:

Which transporters transport each statin was studied in the screening. BCRP, P-gp, MRP2, MRP3, MRP4 and MRP8 transporters were studied as potential transporters for all the statins of interest (atorvastatin, fluvastatin, pravastatin, pitavastatin, rosuvastatin and simvastatin acid). Screening was performed with two concentrations (1 µM and 10 µM), which gave a general insight of transport for further studies and reaction incubation time was 10 minutes. Based on these results, it was evaluated which transporters show active transport for each statin. Transporters and statin concentration for the further parts of the study were selected based on the screening results.

2) Determination of time-dependent transport:

In the transport-time linearity studies, the optimum reaction incubation time was examined more specifically. The experiment was performed by using one statin concentration and incubation times were 5, 10 and 15 minutes, aiming to see the time linearity in the statin transport. Based on these results, the incubation time for the transport kinetic experiments was selected.

3) Determination of concentration-dependent transport:

In the final kinetic studies, several statin concentrations were examined, with the incubation time selected based on time-linearity studies. The aim was to determine concentration-dependent accumulation of statins. The results were used to determine kinetic parameters of the active transport for each statin.

4.3 Data analysis

ATP-dependent transport was determined by calculating the difference between the statin accumulation inside the vesicles in the presence and absence of ATP (Equation 1). By dividing ATP-dependent transport with incubating time and transporter protein abundance, ATP-dependent transport obtains the form pmol/min/mg. Standard deviation (SD) for ATP-dependent transport was determined by summing second power of statin accumulation SD in presence and absence of ATP and dividing with two (Equation 2). The ratio for statin transport was calculated by dividing accumulated statin (pmol) in presence of ATP with the amount accumulated in absence of ATP (Equation 3). The SD for ratio is calculated with the equation [(SD1/T1)^2+(SD2/T2)^2] x 0.5 x Ratio (Equation 4). In equations 2, 3 and 4, T1 is statin transport in the presence of ATP and T2 in the absence on ATP, SD1 is the deviation for accumulation in presence of ATP and SD2 in the absence of ATP. The kinetic parameters of substrate transport were calculated by curve fitting using the Michaelis-Menten equation, by GraphPad Prism version 8.1.2 (GraphPad Software Inc., La Jolla, CA). Michaelis-Menten equation is $V=V_{max}[S]/([S]+K_m)$, where V is the velocity of ATP-dependent transport, V_{max} is maximal velocity, [S] is substrate concentration (μM) (statin concentration in the study), and K_m is the Michaelis-Menten constant (Equation 5). In addition, GraphPad Prism was used to draw all the figures.

$$ATP\text{-}dependent\ transport = Transport_{+ATP} - Transport_{-ATP} \quad \text{(Eq.1)}$$

$$SD\ of\ ATP-dependent\ transport = \frac{(SD1)^2+(SD2)^2}{2} \quad \text{(Eq.2)}$$

$$Ratio = \frac{T1}{T2} \quad \text{(Eq.3)}$$

$$SD\ of\ ratio = \frac{(SD1/T1)^2+(SD2/T2)^2}{2} * Ratio \quad \text{(Eq.4)}$$

$$v = \frac{Vmax\,[S]}{Km+[S]} \quad \text{(Eq.5)}$$

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6 ABBREVIATIONS

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
CDCF	5(6)-carboxy-2,'7'-dichlorofluorescein
CYP	Cytochrome P450
HMG-CoA	3-hydroxy-3-methylglutary-coenzyme A
LDL	Low-density lipoprotein
MRP	Multidrug-resistance associated protein
NBD	Nucleotide-binding domain
OATP	Organic anion transporting polypeptides
P-gp	P-glycoprotein
SD	Standard deviation
SLC	Solute carrier
TMD	Transmembrane domain

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