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Intestinal Permeability and Presystemic Extraction of Fexofenadine and R/S-verapamil

BY

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Abstract

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The main objective of this thesis was to investigate the in vivo relevance of membrane transporters and cytochrome P450 (CYP) 3A4-mediated metabolism in the intestine and liver for the bioavailability of drugs in humans after oral administration.

In the first part of the thesis, the main transport mechanisms involved in the intestinal absorption and bioavailability were investigated for fexofenadine, a minimally metabolized drug, which is a substrate for P-glycoprotein (P-gp) and members of organic anion transporting polypeptide (OATP) family. Jejunal perfusion studies revealed that co-perfusion with verapamil increased the bioavailability of fexofenadine by decreasing the first-pass liver extraction as the low intestinal permeability was unchanged by the transport inhibitors studied. The mechanism behind the interaction probably involves inhibition of OATP-mediated sinusoidal uptake and/or P-gp-mediated canalicular secretion of fexofenadine. Results from the Caco-2 model supported that the intestinal absorption of fexofenadine is mainly determined by the low passive permeability of the drug, even though fexofenadine clearly is a P-gp substrate.

In the second part of the thesis, the effect of repeated oral administration of the P-gp and CYP3A4 inducer St. John's wort on the in vivo intestinal permeability and presystemic metabolism of the dual P-gp and CYP3A4 substrate verapamil was investigated in a jejunal perfusion study. St. John's wort decreased the bioavailability of the enantiomers of verapamil by inducing the CYP3A4-mediated presystemic metabolism, probably mainly in the gut. It was also concluded that induction of efflux transporters, such as P-gp, does not affect the intestinal transport or the gut wall extraction of high permeability substrates like verapamil. Data from Caco-2 cells with induced CYP3A4-activity supported these findings. The plasma levels of the enantiomers of norverapamil also decreased despite an increased formation, which was attributed to induction of CYP3A4 and/or other metabolic routes.

Keywords: Bioavailability, Fexofenadine, Verapamil, P-glycoprotein, CYP3A4, OATP, Enantioselective, Permeability, Caco-2, Intestinal perfusion

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Contents

Papers discussed

This thesis is based on the following papers, which will be referred to by the Roman numerals assigned here:

I. Tannergren, C., Knutson, T., Knutson, L., Lennernäs, H. The effect of ketoconazole on the in vivo intestinal permeability of fexofenadine using a regional perfusion technique. Br. J. Clin. Pharmacol. 2003, 55, 182-190.

II. Tannergren, C., Petri, N., Knutson, L., Hedeland, M., Bondesson, U., Lennernäs H. Multiple transport mechanisms involved in the intestinal absorption and first-pass extraction of fexofenadine. Clin. Pharmacol. Ther. 2003, 74, 423-436.

III. Petri, N., Tannergren, C., Rungstad, D., Lennernäs, H. Transport characteristics of fexofenadine in the Caco-2 cell model. *Submitted.*

IV. Tannergren, C., Engman, H., Knutson, L., Hedeland, M., Bondesson, U., Lennernäs H. St John's wort decreases the bioavailability of R- and S-verapamil through induction of the first-pass metabolism. Clin. Pharmacol. Ther. 2004 *In Press.*

V. Engman, H., Tannergren, C., Artursson, P., Lennernäs, H. Enantioselective transport and CYP3A4-mediated metabolism of R/S-verapamil in Caco-2 cell monolayers. Eur. J. Pharm. Sci. 2003, 19, 57-65.

Abbreviations

a-b	apical-to-basolateral
ABC	ATP-binding cassette
Ar	Appearance ratio
AUC	Area under the plasma concentration-time curve
b-a	basolateral-to-apical
BCRP	Breast cancer resistance protein
BCS	Biopharmaceutics classification system
BSP	Sulfobromophthalein
C_{max}	Maximum plasma concentration
CYP	Cytochrome P450
D_3	1α,25-dihydroxy vitamin D_3
E_G	Extraction ratio in the gut wall
E_H	Extraction ratio in the liver
F	Bioavailability
f_a	Fraction absorbed
LOQ	Limit of quantification
MRP	Multidrug resistance associated protein
MDR	Multidrug resistance
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
P-gp	P-glycoprotein
P_{app}	Apparent permeability
P_{eff}	Effective permeability
PXR	Pregnane X receptor
SD	Standard deviation
SLC	Solute carrier
SNP	Single nucleotide polymorphism
TEA	Tetraethylammonium
TEER	Transepithelial electrical resistance
UWL	Unstirred water layer
Å	Ångström

1. Introduction

1.1 Oral drug delivery and bioavailability

Oral administration of solid dosage forms, such as tablets and capsules, is the most common route for administration of drugs (1) because it is convenient and safe, the desired dose can be given with high accuracy and it produces advantages in the manufacturing process (2). However, to exert its effect, an orally administered dose has to reach the systemic circulation, unless a local effect is desired, from where it can be distributed throughout the body to its site of action. To accomplish this, the drug must be dissolved, be stable in the gastrointestinal fluids and be able to pass through the enterocytes lining the intestine during its transit in the intestine as well as pass through the liver. However, both the gastrointestinal tract and the liver constitute potential barriers during this "first passage".

The fraction of an oral dose that reaches the systemic circulation in unchanged form is called the oral bioavailablity, F, which can be described by the following equation:

$$F = fa \cdot (1-E_G) \cdot (1-E_H) \qquad (1)$$

where fa is the fraction of the administered dose that is absorbed across the apical membrane of the enterocytes, $1-E_G$ is the fraction of an absorbed dose that is not metabolized in the gut wall and $1-E_H$ is the fraction that escapes metabolism and biliary excretion in the liver (3-5). All these processes have the potential to affect the bioavailability of a drug (Figure 1).

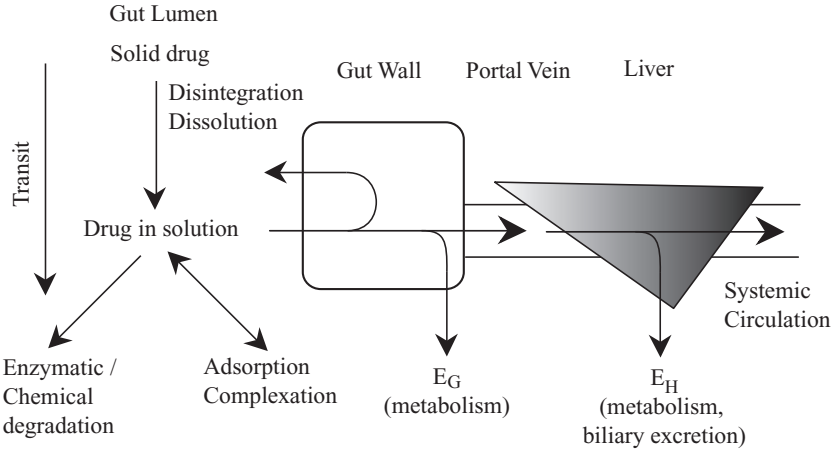


Figure 1. The factors influencing the bioavailability of orally administered drugs.

1.2 Factors influencing intestinal absorption of drugs

A drug given as a solid dosage form must first disintegrate and be dissolved in the gastrointestinal fluids before it can be transported across the intestinal epithelium. The dissolution rate is determined by formulation factors and the physicochemical properties of the drug, such as particle size, solubility, pKa, crystal form, salt and molecular size, as well as by the environment in the gastrointestinal tract including the pH, transit time, blood flow and composition of the gastrointestinal fluid (6-9). The actual absorption step is also influenced by several factors, such as size, lipophilicity, charge, transport mechanism, surface area, blood flow, gastric emptying, intestinal transit time, polar surface area, pH, pKa, dose and disease (3, 10-15).

Intestinal absorption of a drug is thought to mainly be limited by the dissolution rate/solubility or the permeability across the apical membrane of the enterocyte. These factors are also the cornerstones of the Biopharmaceutics Classification System (BCS), which provides a basis for in vitro-in vivo correlations and for prediction of oral drug absorption (16). However, a drug in solution may also be subject to enzymatic and chemical degradation as well as adsorption and complex formation, which would further reduce the amount available for absorption (8-10).

The factors affecting intestinal transport and presystemic extraction of drugs will be described in more depth in the following sections.

1.3 Intestinal transport mechanisms of drugs

Before a drug molecule can be transported across the intestinal epithelium, of which up to 90% is constituted of enterocytes, it first has to pass the unstirred water layer (UWL), which is a more or less stagnant layer of water, mucus and glycocalyx lining the intestinal wall (17). Although this barrier can affect the permeability of rapidly transported compounds in vitro (18), it is unlikely that it limits the intestinal absorption of drugs in humans because the intestinal motility reduces the thickness of the layer (17, 19).

The two transport routes for drugs across the intestinal epithelium, shown in Figure 2, are: transcellular transport, where the molecule is transported across the enterocyte and paracellular transport, which is transport between adjacent enterocytes. The transcellular pathway can be further divided into passive diffusion and carrier-mediated transport. Carrier-mediated transport can either involve uptake or efflux transport.

1.3.1 Passive transcellular diffusion

Passive diffusion across the enterocytes is the most common transport mechanism. The apical membrane is thought to be the rate limiting step in this process as it is thicker than and not as fluid as the basolateral membrane, resulting in a higher resistance to transport (20). The transport rate across the apical membrane can be described by Fick's first law of diffusion:

$$J = P \cdot A \cdot (C_1 - C_2) \quad (2)$$

where J is the mass/time flux, P is the permeability coefficient, A is the surface area available for absorption and (C_1-C_2) is the concentration gradient across the membrane (21). The concentration gradient is maintained by the blood, which acts as a sink, distributing absorbed molecules throughout the body. The permeability (cm/s) is the speed at which a molecule is transported across a membrane and it can be described by the simplified equation:

$$P = \frac{K \cdot D_m}{\lambda} \quad (3)$$

where K is the partition coefficient between the aqueous phase and the membrane, D_m is the membrane diffusion coefficient and λ is the thickness of the membrane (21). It can be anticipated that lipophilic drugs with a rather small molecular size would have a high permeability as these properties would result in high values of K and D_m, respectively. However, if the lipophilicity is too high, the permeability may decrease (22). Molecules with low permeability may still be transported by the transcellular route as they will be exposed to a larger surface area during their diffusion down the length of the villi compared to high permeability drugs, which are absorbed at the tip of the villi (23, 24).

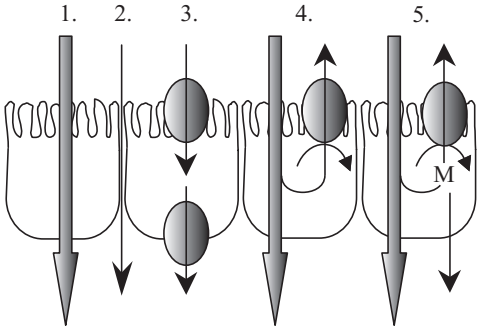


Figure 2. The different transport mechanisms across the intestinal epithelium. 1. Passive transcellular diffusion, 2. paracellular transport, 3. carrier-mediated absorptive transport, 4. carrier-mediated efflux transport. Metabolites formed in the enterocytes may be transported back to the lumen 5.

1.3.2 Paracellular transport

The rate-limiting step in paracellular transport is thought to be the tight junctions, which separate the apical and basolateral membranes (25, 26). This transport mechanism is believed to predominantly concern compounds with a low molecular weight that are hydrophilic or charged. The drugs that are thought to be transported by this route include atenolol, furosemide and cimetidine (27-30). Passive diffusion is believed to be the mechanism for the transport of drugs by this route, but it appears that cationic drugs are more readily transported than

anionic and neutral compounds (31, 32). As the paracellular pores only constitute 0.1% of the small intestinal surface area (33), drugs transported by this route are often incompletely absorbed. Furthermore, it has been suggested that paracellular transport is of minor importance in the upper part of the small intestine in humans for drugs with a molecular weight above 200 (34, 35).

1.3.3 Carrier-mediated transport

Some drugs are able to utilize transport proteins present in the apical membrane of the enterocytes, in addition to passive diffusion, to enhance their absorption (36). The transporters, which are mainly located in the small intestine (37), are specialized in transporting nutrients such as amino acids, oligopeptides and monosaccharides, as well as bile acids (36, 38, 39). Drugs transported by this mechanism are usually structurally similar to the nutrient and hydrophilic in nature. Active carrier-mediated transport is energy dependent and is characterized by the ability to transport compounds against a concentration gradient. Also, as there is a limited capacity, this transport mechanism is saturable at high substrate concentrations as well as inhibitable (10, 40). This may result in a decreased fraction absorbed and a lower bioavailability of the transported drug. Carrier-mediated transport down a concentration gradient that is independent of the energy input it is known as facilitated transport (10).

There are also efflux transporters present in the membranes of the enterocytes that actively extrude drugs from the interior of the cells where they are expressed, and may, therefore, restrict the absorption of drugs (see Section 1.5) (41-43).

In Sections 1.4 and 1.5 various intestinal absorptive and efflux transporters are described in greater detail.

1.4 Intestinal absorptive transporters

The human di/tri-peptide transporter (hPepT1) is located in the apical membrane of the enterocytes and in Caco-2 cells (44, 45) and is driven by an inwardly directed H^+-gradient (36, 46). hPepT1 facilitates the intestinal absorption of peptidomimetic drugs such as β-lactam antibiotics and angiotensin-converting enzyme inhibitors (46-48).

Some members of the human organic anion transporting polypeptide (OATP) family (solute carrier family 21A, SLC21A), namely OATP-B (SLC21A9), OATP-D (SLC21A11) and OATP-E (SLC21A12), are expressed in the small intestine and colon (49, 50). It was recently shown that OATP-B is localized to the apical membrane of the human enterocytes (51) (Figure 3) and that it transports pravastatin and fexofenadine in a pH-dependent manner (51, 52). This may confirm that the interaction by which various fruit juices reduce the oral bioavailability of fexofenadine in humans is caused by inhibition of intestinal OATP (53). The results suggest that intestinal OATPs may be involved in the in vivo absorption of drugs in humans.

Of the organic cation transporters (SLC22A family), human OCTN2 (SLC22A5) is expressed in the small intestine and is localized to the apical membrane in Caco-2 cells (54-56). It transports carnitine in a Na^+-dependent

manner and organic cations, such as tetraethylammonium (TEA), quinidine and verapamil, in a Na^+-independent manner (56-58). However, the clinical importance of OCTN2 for the absorption of drugs is unknown. Human OCT1 is located to the basolateral membrane of the enterocytes (59).

Transport systems for nucleosides, amino acids, glucose and bile acids are also present in the intestine (36, 55, 60).

1.5 Intestinal efflux transporters

The relevant efflux transporters for drugs in clinical use belong to the ATP-binding cassette (ABC) family. These transporters extrude drugs, metabolites and conjugates in an ATP-dependent manner from the cells in which they are expressed (41, 61). Examples of efflux transporters localized at the apical membrane of the intestinal epithelium include MDR1 P-glycoprotein (P-gp, ABCB1), the breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance associated protein 2 (MRP2, ABCC2) (Figure 3) (41-43, 61-63). They may restrict the intestinal absorption and hence the bioavailability of drugs (64, 65). Because of the tissue distribution and cellular localization it is believed that their normal function, in addition to limiting intestinal absorption of potentially harmful compounds, is to facilitate their excretion into urine and bile and limit the tissue distribution, for example to the brain (42, 43). They are also involved in the multidrug resistance (MDR) in cancer cells (66).

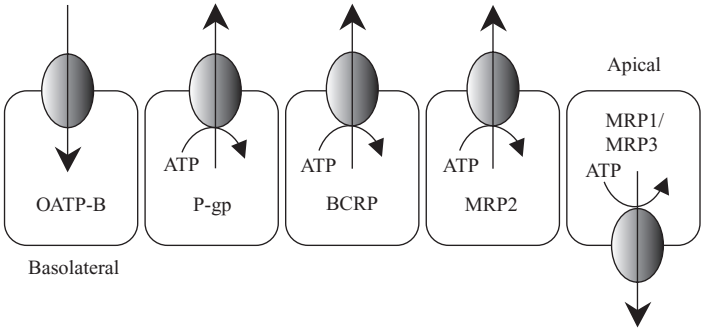


Figure 3. Subcellular localization of OATP-B, P-gp, BCRP and MRP1-3 in the human enterocytes.

1.5.1 P-glycoprotein

Intestinal P-gp is localized at the villus tip of the apical membrane of the enterocytes and is expressed throughout the small intestine and colon, and, although the expression seems to increase from the stomach to the colon (42, 43, 67, 68), conflicting data exist (69). Furthermore, there does not appear to be any P-gp expression in the crypt cells (43). P-gp has a very broad substrate specificity and transports a vast number of structurally diverse compounds such as anticancer drugs, HIV protease inhibitors and antibiotics (61, 70). Other than lipophilicity**,** no clear structural requirement for a P-gp substrate has been established, but the transporter does seem to prefer neutral and weakly basic drugs as substrates (41,

71). The orphan nuclear pregnane X receptor (PXR) regulates the expression of the MDR1 gene encoding for P-gp (72). Drugs that are PXR-ligands, such as rifampicin and St. John's wort, increase the expression of P-gp (65, 72, 73).

Most of the evidence for the involvement of P-gp in the intestinal absorption of drugs has emerged from bidirectional transport and inhibitor studies in cell lines (70, 74, 75). Digoxin (76), celiprolol (77) and cyclosporin A (78) are examples of drugs transported by P-gp in the Caco-2 model. In vivo evidence has been provided from knockout mice models. For example, the AUC of paclitaxel was 2- and 6-fold higher in mdr1a (-/-) mice than in wild-type mice after intravenous and oral administration, respectively, which shows that P-gp affects the bioavailability and clearance of the drug (79). Moreover, the P-gp inhibitor quinidine increased the plasma levels of digoxin in wild-type but not in knockout mice (80).

Even though in vitro and animal data are convincing, surprisingly few clinical examples are known where P-gp alone affects the bioavailability of a drug in humans. The quantitative contribution of P-gp is often difficult to assess because of overlapping substrate specificity with metabolizing enzymes, such as CYP3A4 (81), and commonly used inducers and inhibitors are often unspecific (82). However, the intestinal absorption of the drugs talinolol and digoxin, which are negligibly metabolized, seems to be limited by intestinal P-gp as the AUCs for the drugs correlate with intestinal P-gp expression and, since the P-gp inducer rifampicin decreased the plasma levels to a larger extent after oral administration than it did when administered by the intravenous route (65, 83, 84). In addition, mechanistic perfusion studies have shown that both drugs are secreted into the intestine (85, 86). Talinolol and digoxin are also involved in drug-drug interactions with known P-gp inhibitors and inducers (73, 87-89).

The expression of intestinal P-gp varies 2-8-fold between individuals (90, 91), which may result in variable absorption and differences in the bioavailability of P-gp substrates. Single nucleotide polymorphism (SNP) has been suggested to contribute to the variable expression levels and the variations in the activity of the transporter (92, 93). The C3435T mutation is associated with lower intestinal P-gp expression and higher plasma levels of digoxin (92), but this was not seen in a similar study (94). Similar discrepancies have been reported for fexofenadine (95, 96).

Recently, it was suggested that the importance of P-gp for the intestinal absorption of drugs has been overemphasized as the transporter is easily saturated by the high luminal concentrations achieved after oral administration unless small doses are given or the dissolution and/or the membrane diffusion is slow (97-100).

1.5.2 MRP2 and BCRP

MRP2 is located at the apical membrane in human enterocytes and the expression in rats is highest in the proximal part of the jejunum (62, 101). MRP2 transports anionic drugs and endogenous compounds, including their glutathione-, glucuronide- and sulfate conjugates (102). Examples of unconjugated drugs that are substrates for the transporter include vinblastine, methotrexate, ampicillin and pravastatin (102-104). The substrate specificity is similar to that of MRP1 (ABCC1) and MRP3 (ABCC3), which are believed to be located in the basolateral membrane of the enterocytes (Figure 3) (105). Taking the number of

possible substrates and the high expression in the intestine into account, it is possible that MRP2 could limit the intestinal absorption of drugs in humans (91). As several phase II enzymes are present in the human intestine (106-108), MRP2 may be responsible for the intestinal secretion of the conjugates formed in the intestine.

Human BCRP is expressed in the apical membrane of the enterocytes in the small intestine and colon (63, 91) and the anticancer drugs topotecan and mitoxantrone have been reported to be substrates (109). In mdr1a/1b knockout mice the P-gp and BCRP inhibitor GF120918 increased the bioavailability and decreased the elimination of topotecan after oral and intravenous administration, respectively, owing to inhibition of the transporter in the intestine and liver (109). Evidence that BCRP may limit the bioavailability of drugs in humans was provided recently, where the effect of GF120918 on the bioavailability of topotecan was, at least in part, attributed to BCRP (110).

1.6 Presystemic metabolism

Both the intestine and the liver constitute metabolic barriers where an absorbed drug molecule may be metabolized before reaching the systemic circulation. Metabolism is divided into phase I reactions, where the parent drug is modified by oxidation, for example by Cytochrome P450 (CYP) enzymes, and phase II reactions, where the parent drug or its phase I metabolite is rendered more hydrophilic by conjugation with glutathione, sulfate or glucuronic acid. Drug metabolism could be saturated, with dose-dependent bioavailability being the result (111).

It has been reported that CYP enzymes were responsible for 55% of the total elimination of the drugs in a survey (112). The liver is the main metabolizing organ and enzymes in the CYP1-3 families account for about 70% of the total liver CYP content (113), with the CYP3A isoform constituting about 30% of this total (113). A study reported that mRNA of CYP1A1, 1B1, 2C, 2D6, 2E1, 3A4 and 3A5 were expressed in the human small intestinal enterocytes, while only CYP3A4, 2C and 1A1 proteins were detected (114). CYP3A is also the dominant subfamily in the small intestine, where it constitutes about 70% of total CYP protein, which represents 50% of the liver content (115-117).

1.6.1 Cytochrome P450 3A

CYP3A has a great influence on drug bioavailability and elimination in humans, 40-50% of all drugs are metabolized by this family (118). CYP3A contributes significantly to the presystemic metabolism and bioavailability of numerous drugs such as midazolam (119), terfenadine (120), felodipine (121), verapamil (122) and cyclosporin A (123).

Although CYP3A4 is the main isoenzyme (118), significant levels of CYP3A5 are expressed in about 30% of human livers (124, 125). However, it has been suggested that CYP3A5 only plays a minor role in drug metabolism (126, 127). The interindividual variability in protein and mRNA as well as in catalytic activity of CYP3A4 is extensive (128, 129).

Intestinal and hepatic CYP3A4 is inducible by drugs, such as rifampicin and St. John's wort that activate PXR (73, 130-133). For example, treatment with the herbal drug St. John's wort (300 mg 3 times a day) for 14 days decreased the bioavailability of the CYP3A probe drug midazolam in healthy volunteers (82, 134). Similarly, treatment with rifampicin (600 mg daily) for 11 days decreased the bioavailability of S-verapamil 25-fold, while it only increased the systemic clearance 1.3-fold, indicating a preferential induction of prehepatic CYP3A4-mediated metabolism (122).

Inhibition of intestinal and/or hepatic CYP3A also affects the bioavailability and disposition of CYP3A substrates in vivo in humans. For example, it has been reported that grapefruit juice increases the bioavailability of felodipine by inhibition of the gut wall metabolism (135, 136). Also, the extensive presystemic CYP3A-metabolism of terfenadine is inhibited by ketoconazole, resulting in markedly increased plasma levels of the drug and serious cardiac arrhythmias (120).

1.6.2 Coordinated function of CYP3A and P-gp

P-gp and CYP3A have broad substrate specificities and they share many substrates and inhibitors (81, 137). They are also both present in the small intestinal enterocytes and both are activated by PXR (72, 81, 130). On the basis of this, it has been suggested that intestinal CYP3A and P-gp work cooperatively to limit the bioavailability of joint substrates (81, 138, 139) and that P-gp recycles the substrates across the apical membrane, thereby increasing the exposure of drug molecules to CYP3A, with enhanced metabolism being the result (139, 140).

Evidence for this has been collected from Caco-2 cells with induced CYP3A4 activity (139, 141). For example, formation of the main metabolite of the dual P-gp and CYP3A substrate indinavir, was higher for the apical to basolateral transport than for the opposite direction, when normalized to the amount of drug transported (141). However, it should be noted that data from human in vivo studies, for example, drug interaction studies using specific P-gp inhibitors, are lacking. Also, the validity in vivo is not clear as drugs such as midazolam and felodipine undergo extensive gut wall metabolism without being P-gp substrates (119, 121).

Cyclosporin A is an example of a drug where it is considered that drug-drug interactions involving both P-gp and CYP3A are of importance. The potent P-gp and CYP3A inhibitor ketoconazole increases the bioavailability of cyclosporin A (142). Moreover, St. John's wort decreases the plasma levels of cyclosporin A (82). Interestingly, although St. John's wort induces both CYP3A and P-gp, it affected the pharmacokinetics of cyclosporin A to a lesser degree than it affected midazolam, which is not a P-gp substrate (82). In a human jejunal perfusion study ketoconazole inhibited the metabolism of the enantiomers of the dual P-gp and CYP3A4 substrate verapamil but did not affect the intestinal permeability of the drug (143).

1.7 Hepatic uptake and biliary secretion

In addition to passive diffusion, transporters in the sinusoidal membrane of the hepatocytes play an important role in the uptake of drugs from the blood into the hepatocytes where metabolism and/or transport across the canalicular membrane into the bile may occur (Figure 4). Thus, the liver plays an important role, not only for disposition, but also for the bioavailability of drugs.

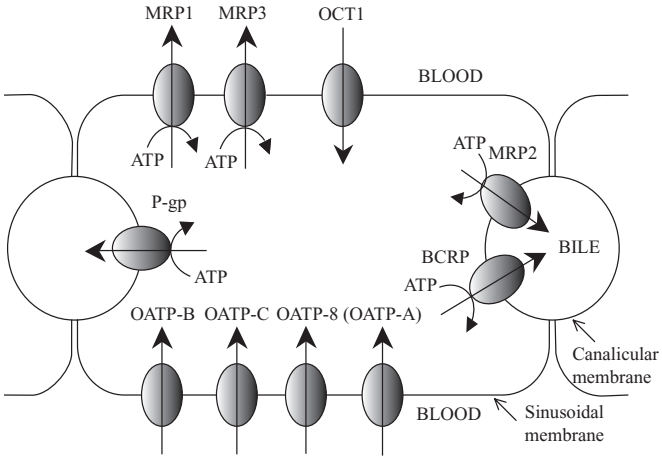


Figure 4. Examples of transport systems involved in the sinusoidal uptake and canalicular secretion of drugs in human hepatocytes.

1.7.1 Sinusoidal transporters

Members of the OATP family play an important role in the sinusoidal transport of drugs (144). The substrates are mainly amphipathic anions of high molecular weight (> 450) that are bound to plasma proteins, i.e., they have similar attributes as drugs secreted into bile; cations, zwitterions and neutral compounds are substrates as well (144). OATPs transport a wide range of compounds in a Na^+-independent manner (144).

OATP-A (SLC21A3) was originally found in the liver, but is predominantly expressed in the brain (145). The substrates include bile acids, hormones and their conjugates, peptides, bulky type II organic cations and the drug fexofenadine (144-147).

OATP-B (SLC21A9) is located to the sinusoidal membrane of the hepatocytes, but is also expressed in other tissues such as the small intestine (49,50). It has a restricted substrate specificity and does not transport bile salts (49, 144), but fexofenadine and pravastatin are substrates (51, 52).

OATP-C (SLC21A6) expression is restricted to the sinusoidal membrane of the hepatocytes (148) where it transports a wide range of compounds such as taurocholate and bilirubin as well as the drugs pravastatin and rifampicin (144). It seems to prefer anionic substrates (49).

The tissue distribution and substrate specificity of OATP-8 (SLC21A8) is similar to that of OATP-C (144, 149). However, OATP8 is the only OATP that transport the neutral drug digoxin (49).

Inhibition of OATP-mediated sinusoidal uptake may affect the disposition and, perhaps, also the bioavailability of drugs. Rifamycin SV and rifampicin, which inhibit several basolateral OATPs, reduce the hepatic elimination of the anion BSP and induce hyperbilirubinemia in humans (150). In addition, cyclosporin A, which inhibits the OATP-C mediated uptake of cerivastatin, increases the AUC of cerivastatin without affecting the metabolism (151, 152). Moreover, it has been suggested that polymorphism in the OATP-C gene affects the pharmacokinetics of pravastatin (153).

Human OCT1 (SLC22A1) is also expressed in the sinusoidal membrane where it mediates Na^+-independent liver uptake of hydrophilic type I organic cations, such as TEA (154, 155). Low levels of MRP1 and MRP3 are located to the sinusoidal membrane where they are thought to mediate secretion into the blood when canalicular transport is blocked (155).

1.7.2 Canalicular transporters

P-gp, MRP2 and BCRP are all expressed in the canalicular membrane of human hepatocytes and mediate transport into the bile (43, 63, 156).

P-gp is responsible for the biliary excretion of many neutral and cationic compounds. For example, the biliary clearance of digoxin was higher in wild-type mice compared to mdr1a knockout mice (157). Other studies in knockout mice have shown that P-gp is also involved in the biliary excretion of various cationic drugs, for example vecuronium (158). In vivo in humans, the steady state plasma concentrations of digoxin increased by 44% after coadministration with verapamil, which was explained by a 43% decrease in digoxin biliary clearance as determined by a perfusion technique (159). The P-gp inhibitor quinidine also reduces the biliary secretion of digoxin (86).

MRP2 plays an important role in biliary excretion of anionic drugs and conjugated metabolites formed via phase II enzymes in the hepatocytes (102-104). The importance of BCRP in canalicular secretion remains to be determined, but it has been suggested that GF120918 increases the bioavailability of topotecan in humans partly by inhibition of BCRP (110). GF120918 also decreased the biliary excretion of topotecan in mdr1a/1b (-/-) mice (109).

1.8 Mechanistic intestinal absorption models

The available models for the mechanistic study of intestinal absorption could, roughly, be divided into in vitro, in situ and in vivo models, in accordance with their increasing complexity (for reviews, see (35, 160-162)).

Examples of in vitro models are brush border membrane vesicles, cell cultures and isolated intestinal segments (160). Caco-2 and transfected MDCK cells are widely used for studies of both passive and active transport mechanisms (163). Excised segments from various intestinal regions from humans or rats can be used for the study of region-dependent transport and metabolism (162, 164, 165). Rat

in situ perfusions in isolated intestinal segments, where the blood supply is intact, can also be used in investigations of region-dependent transport and metabolism (160, 166). Permeability values obtained for passively transported drugs correlate well with those from the human jejunum and the in vivo fraction absorbed (167). In vivo intestinal perfusion techniques in humans have also been developed (35, 162).

The two models used in this thesis are described below.

1.8.1 The Caco-2 cell model

Monolayers of Caco-2 cells grown on permeable supports (Figure 5) are widely used to study in vitro passive (28, 168) and active transport of drugs (169, 170). Several transporters and some enzymes are expressed in the Caco-2 cells (55, 91), which can be advantageous since it more closely mimics the in vivo situation, but it might also represent a limitation as the contribution of a specific transporter is difficult to assess. The Caco-2 permeability values for passively transported drugs have shown a good correlation with the fraction absorbed in humans (171). Values for high permeability drugs are comparable to those of the human jejunum, while low permeability and actively transported drugs have many-fold lower values compared to the human jejunum (24).

Modified Caco-2 cells have been used to study CYP3A-mediated metabolism and transport using the CYP3A5 expressing clone TC7 (172), cells with heterologously expressed CYP3A4 (173) or cells with 1α,25-dihydroxy vitamin D_3 induced CYP3A4 (174, 175).

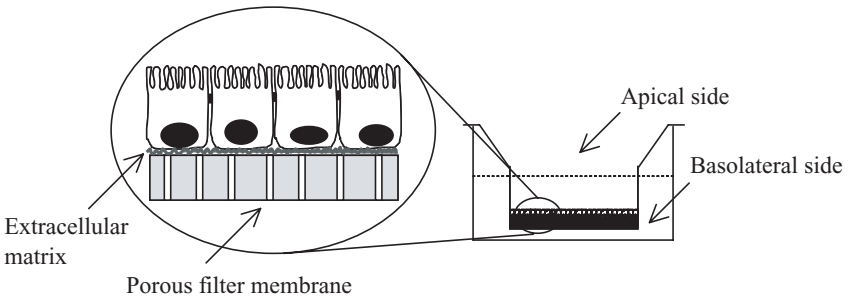


Figure 5. Illustration of the monolayer of Caco-2 cells grown on a permeable support. The apical and basolateral chambers are indicated.

1.8.2 In vivo intestinal perfusion techniques in humans

Human intestinal perfusion techniques are divided into open, semiopen and double balloon methodologies and the calculation of the absorption parameters is based on the disappearance of the drug under investigation from the perfused segment (35, 176, 177). These techniques have been used in mechanistic in vivo studies of intestinal absorption, biliary secretion, and presystemic metabolism (4, 85, 86, 100, 143, 178). A simultaneous assessment of plasma pharmacokinetics of drugs is also possible. In addition, the Loc-I-Gut® technique (Figure 6) enables a

determination of the effective permeability (176), which has been correlated to the extent of absorption in man and used to establish the Biopharmaceutics Classification System (BCS) (16, 162, 176, 179). Enterocytes shed from the intestinal segment have also been collected with these techniques and used to study the expression of transporters and enzymes in the intestine (180, 181). Similar systems have been developed for rectal perfusions (182, 183).

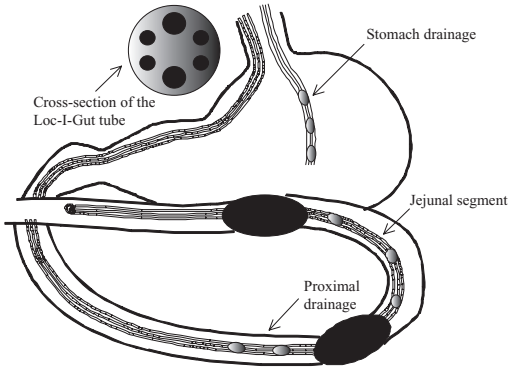


Figure 6. The Loc-I-Gut® perfusion technique makes it possible to obtain a direct measure of intestinal transport (the effective jejunal permeability) and local metabolism simultaneously with an assessment of plasma pharmacokinetics of drugs. Absorption of the drugs occurs from the 10 cm jejunal segment between two inflated balloons.

1.9 Model drugs

1.9.1 Fexofenadine

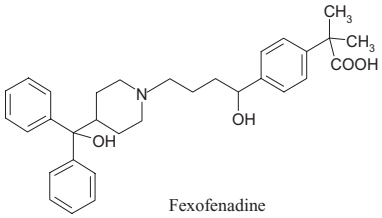
Fexofenadine (Figure 7) is a selective non-sedating histamine H_1 receptor antagonist with approximately dose linear systemic exposure in the 10 to 800 mg dose range (184). Fexofenadine is 60-70% bound to plasma proteins, mainly albumin and α_1-acid glycoprotein (185). After oral administration of 60 mg ^{14}C-fexofenadine, the total dose recovered was 92%, 12% in the urine and 80% in the feces; more than 85% was recovered as parent drug in urine (185, 186).

Ketoconazole has been reported to increase C_{max} and the AUC for fexofenadine in healthy volunteers by 135 and 164%, respectively (185). Since fexofenadine is proposed to not be metabolized to any major extent (186, 187), it has been suggested that this effect is caused by inhibition of intestinal or biliary secretion (185). Fexofenadine is indeed transported by P-gp in various in vitro models (147, 188, 189). The effects of inhibitors and inducers of P-gp on the plasma levels of fexofenadine suggest that this efflux pump is important for the intestinal absorption of the drug in humans (82, 185, 187, 190). Fexofenadine is also a substrate for OATPs, such as OATP-A and OATP-B, which may affect the bioavailability and disposition of the drug (52, 147). It has been suggested that inhibition of intestinal OATP-mediated transport of fexofenadine is the mechanism whereby various fruit juices affect the pharmacokinetics of fexofenadine (53).

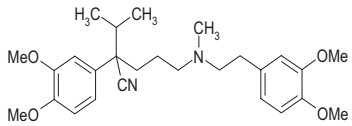
1.9.2 Verapamil

The calcium channel antagonist verapamil (Figure 7) is almost completely absorbed after oral administration (191). Verapamil is a P-gp substrate and inhibitor (192, 193), and the enantiomers have a high but concentration dependent human intestinal permeability, probably owing to saturation of P-gp, predicting a complete fraction absorbed (100).

Verapamil undergoes extensive presystemic (hepatic and intestinal) and systemic metabolism (122, 191), with only 3-4% of an oral dose being excreted unchanged in urine (194). This results in a low and dose-dependent bioavailability of between 10 and 35% (122, 143, 191). The first-pass metabolism is enantioselective and R- and S-verapamil are intermediate and high extraction drugs, respectively (122, 143, 195). The bioavailability for R- and S-verapamil has been reported to be 50 and 20%, respectively (196). The metabolism of verapamil is catalyzed mainly by CYP3A4, the main metabolites norverapamil and D-617 are formed by N-dealkylation, although other CYP enzymes also contribute to some extent (197-200). 84-91% of racemic verapamil is bound to plasma proteins, mainly albumin and α_1-acid glycoprotein. The plasma protein binding for R- and S-verapamil is 94 and 89%, respectively (195).



Fexofenadine



Verapamil

Figure 7. Chemical structures of fexofenadine and verapamil.

2. Aims of the thesis

The overall objective of this thesis was to investigate the in vivo relevance of transporters and cytochrome P450 3A-mediated metabolism in the intestine and liver for the bioavailability of drugs in humans after oral administration. This was examined using fexofenadine and verapamil as model drugs as different mechanisms are involved in their absorption and presystemic extraction processes.

The work included in this thesis may be divided into two parts in accordance with the transport and metabolism processes involved:

(I) Intestinal and liver transport of the P-gp and OATP substrate fexofenadine where the more specific aims were:

- to investigate the main transport mechanisms involved in the intestinal absorption and the bioavailability of fexofenadine in humans (Paper I and II).
- to determine the human effective jejunal permeability and investigate whether the in vivo drug-drug interaction between ketoconazole and fexofenadine could be attributed to inhibition of intestinal P-gp (Paper I).
- to further study the transporters involved in the transport of fexofenadine and to determine the relative contribution of active and passive transport using the Caco-2 model (Paper III).

(II) Intestinal transport and presystemic metabolism of the enantiomers of the dual P-gp and CYP3A substrate verapamil where the more specific aims were:

- to investigate the inducing effect of repeated oral administration of St. John's wort on the in vivo jejunal permeability and presystemic metabolism of R- and S-verapamil and the formation of R- and S-norverapamil in humans (Paper IV).
- to further study the transport and metabolism of the enantiomers of verapamil in CYP3A4-expressing Caco-2 cells (Paper V).

3. Materials and methods

3.1 Drugs and radiolabeled markers

The drugs used in the jejunal perfusion experiments (Paper I, II and IV) were racemic fexofenadine HCl (Hoechst Marion Roussel, Kansas City, MO, USA), ketoconazole, racemic verapamil HCl (Knoll AG, Darnstadt, Germany) and St. John's wort tablets. A low perfusate concentration (10 mg/l, 53 μM) of antipyrine was used as a marker for passive transcellular diffusion in all of the perfusion experiments and ^{14}C labeled polyethylene glycol 4000 (^{14}C-PEG 4000, 2.5 μCi/l) was used as a non-absorbable volume marker.

In the Caco-2 cell studies (Paper III and V), the following drugs were used: fexofenadine HCl, GF120918, racemic verapamil HCl, racemic norverapamil, erythromycin, probenecid, indomethacin, ketoconazole, rifamycin SV, tetrahexylammonium and 1α,25-dihydroxy vitamin D_3 (D3). ^{14}C-mannitol (52 mCi/mmol) was used as a paracellular marker.

3.2 In vivo jejunal single-pass perfusion experiments (Papers I, II and IV)

The jejunal perfusion experiments were performed in 8-10 healthy volunteers using the Loc-I-Gut® perfusion tube, which is a 175 cm long (5.3 mm external diameter) multichannel polyvinyl tube with two inflatable balloons and a tungsten weight at the tip (Figure 6) (176, 177). The subjects had fasted overnight before the experiment. The tube was introduced through the subjects' mouths after applying local anesthesia to the throat with a lidocaine spray. Once the perfusion tube had been positioned in the proximal part of the jejunum, which was checked by fluoroscopy, the two balloons were inflated with approximately 26-30 ml of air, creating a 10 cm long jejunal segment. To avoid nausea, another tube was positioned in the stomach to drain gastric juice during the experiment. The intestinal fluid above the perfused segment was drained as well. After rinsing the jejunal segment with isotonic saline (37°C), the perfusion solution (pH 6.5, 37°C) containing the relevant drugs was pumped into the jejunal segment at a flow rate of 2.0 ml/min. The perfusate leaving the jejunal segment during the perfusion was collected on ice at 10 minute intervals and frozen at -20°C. Immediately after the perfusion experiment, the jejunal segment was rinsed with 120 ml of isotonic saline to terminate the drug absorption process. Venous blood samples were collected immediately before and at intervals during and after completion of the perfusion experiment.

The study in Paper I was divided into two parts, referred to as Treatment 1 and 2. In the control period (0-100 min), of Treatment 1, the jejunal segment was perfused with 50 mg/l (93 μM) fexofenadine. Then in a subsequent period of equal length (100-200 min), fexofenadine was co-perfused with 50 mg/l (94 μM) of the transport inhibitor ketoconazole to study the short-term inhibition. To study any additional effects of longer treatment with the inhibitor, the same experiments were repeated after 5 days of oral treatment with ketoconazole (200 mg once daily) (Treatment 2). The total dose of fexofenadine given in each perfusion experiment was 20 mg.

In Paper II each experiment lasted for 200 min, divided into two equal periods. During the control period (0-100 min), the jejunal segment was perfused with 100 mg/l (186 μM) fexofenadine. In the treatment period (100-200 min), fexofenadine was co-perfused with 500 mg/l (1018 μM) of the transport inhibitor verapamil. The total dose of fexofenadine given in each perfusion experiment was 40 mg.

In Paper IV jejunal single-pass perfusion experiments with 120 mg/l (244 μM) racemic verapamil were performed for 100 min before and after 14 days of oral treatment with St. John's wort (300 mg 3 times a day). The total dose of verapamil given in each perfusion experiment was 24 mg. The last dose of St. John's wort was scheduled for the day before the second jejunal perfusion experiment to avoid any acute inhibitory effects from the extract.

3.3 In vitro transport and metabolism studies (Papers III and V)

3.3.1 Caco-2 cells (Paper III)

Caco-2 cells, obtained from American Type Culture Collection (ATCC; Rockville, MD, USA), were used at passage intervals 28-45. The cells were cultured at 37ºC, 90% relative humidity and 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine containing 10% fetal bovine serum (FBS), 1% non-essential amino acids (MEM), 100 U/ml penicillin and 100 μg/ml streptomycin. After reaching confluency, the cells were seeded at a density of 88 500 cells/cm^2 on 12 mm (internal diameter) Transwell polycarbonate membrane inserts (0.4 μm pore size).

The transport experiments in Paper III were conducted 21-25 days post seeding at 37°C in Hank's Balanced Salt Solution (HBSS) pH 7.4 containing 1% dimethylsulfoxide at a constant stirring rate (450 rpm). Prior to each experiment the cells were preincubated with the relevant HBSS solution (37°C) for 20 min. Samples were drawn from the receiver chamber after 90-180 min and were replaced with HBSS. Donor samples were taken before and after each experiment. The permeability of fexofenadine was studied in the concentration range 10-1000 μM in both apical-to-basolateral (a-b) and basolateral-to-apical (b-a) directions. The effect of the inhibitors GF120918, verapamil, erythromycin,

ketoconazole, indomethacin, probenecid and rifamycin SV on the bidirectional transport of 150 μM fexofenadine were also examined.

3.3.2 Caco-2 cells with D3-induced CYP3A4 activity (Paper V)

Parental Caco-2 (ATCC) cells were used at passage intervals 92-105 and cultured as described previously (168, 175). The cells were seeded at a density of 400 000 cells/cm^2 onto Transwell cell culture inserts (filter diameter 24 mm mean pore size 0.45 μm) coated with a mixture of extracellular matrix proteins (Matrigel, 15 μg/cm^2). At confluency, the culture medium was supplemented with 0.5 μM of D3 (174).

Bidirectional transport experiments with verapamil (2.5-80 μM) were performed in HBSS pH 7.4 at 37°C in a humidified atmosphere whilst stirring at 500 rpm. Samples were withdrawn from the receiver chamber at 2-3 regular intervals until 16 min had passed. The permeability was determined in both untreated and D3-treated monolayers. The effect of the inhibitors GF120918 (0.2 μM), indomethacin (20 μM) and tetrahexylammonium (50 μM) on the transport of the enantiomers of verapamil was also investigated. Prior to the experiments, both compartments were incubated with each inhibitor for 30 min in HBSS.

The enantioselective metabolism of verapamil was determined in CYP3A4-expressing (D3-treated) Caco-2 cell monolayers, using untreated cells as controls. The cell monolayers were washed and equilibrated for 15 min in pre-warmed HBSS, at pH 7.4. Thereafter, racemic verapamil (5-150 μM) was added to the apical side and the monolayers were incubated at 37ºC for 120 min. After the incubation, samples from the apical and basolateral sides were collected as was the cells for determination of intracellular content.

In Papers III and V, the integrity of the monolayers was checked with ^{14}C-mannitol and by measuring the TEER. Also, in both studies, the inhibitors were added to both the donor and receiver sides to maintain a constant inhibiting concentration throughout the experiment. In addition, the solution for volume compensation contained the relevant inhibitor.

3.4 Analytical methods

The concentrations of fexofenadine in the intestinal perfusate and in HBSS were measured by HPLC with mass, UV and fluorescence detection in Papers I, II and III, respectively. The concentrations of fexofenadine in plasma were measured by HPLC with mass detection. The LOQ was 1 ng/ml and 0.17 ng/ml in Papers I and II, respectively. The enantiomers of verapamil and norverapamil in intestinal perfusate or HBSS were quantified by chiral-HPLC with fluorescence detection (Papers II, IV and V) (201). To quantify the enantiomers of verapamil and norverapamil in plasma a sensitive chiral-HPLC method with MS/MS detection was developed (Paper IV) (202).

The concentrations of antipyrine were analyzed using an HPLC method with UV detection. The total radioactivity of ^{14}C-PEG 4000 in the perfusion solution and the perfusate samples was determined by liquid scintillation counting.

3.5 Data analysis

3.5.1 Assessment of in vivo intestinal permeability and local metabolism (Papers I, II and IV)

All calculations from the single-pass perfusion experiments were made from steady-state concentrations in the outlet jejunal perfusate, which were attained after 50-60 min. Since the drugs under study did not bind to the perfusion tube and were stable in the perfusion solution, the amount that disappeared during the single passage through the jejunal segment was considered to be absorbed.

The fraction of the drug absorbed in the segment during a perfusion (f_{abs}) was calculated using equation 4:

$$f_{abs} = 1 - \left(\frac{C_{out} \cdot PEG_{in}}{C_{in} \cdot PEG_{out}} \right) \quad (4)$$

where C_{in} and C_{out} are the concentrations entering and leaving the jejunal segment, respectively, and PEG_{in} and PEG_{out} are the concentrations of ^{14}C-PEG 4000 (dpm/ml) entering and leaving the segment, respectively.

The effective jejunal permeability (P_{eff}) of each drug was calculated according to a well-mixed tank model, as shown in equation 5 (203):

$$P_{eff} = \frac{C_{in} - C_{out}}{C_{out}} \cdot \frac{Q_{in}}{2\pi rL} \quad (5)$$

where the cylindrical area representing the jejunal segment ($2\pi rL$) was calculated using the intestinal radius (r = 1.75 cm) and the length (L = 10 cm) of the segment. To correct for water flux across the jejunal segment, C_{out} was multiplied by the ratio of the inlet to outlet concentrations of the non-absorbable ^{14}C-PEG 4000.

In Paper IV the appearance ratio (Ar), defined as the ratio between the concentration of formed norverapamil appearing in the perfusate and the concentration of verapamil that is absorbed in the segment (on a molar basis), was calculated for each enantiomer as shown in equation 6:

$$Ar = \frac{C_{out}(\text{norverapamil})}{C_{in}(\text{verapamil}) \cdot f_{abs}} \quad (6)$$

where C_{out} (norverapamil) is the concentration of R- or S-norverapamil in the perfusate leaving the segment and C_{in} (verapamil) is the concentration of the corresponding enantiomer of verapamil entering the segment (100, 143).

3.5.2 Pharmacokinetic data analysis (Papers I, II and IV)

The area under the plasma concentration time curve from time zero to infinity ($AUC_{0-\infty}$) was calculated using the linear and the logarithmic trapezoidal rules for ascending and descending plasma concentrations, respectively, up to the last time point. The AUC beyond the last time point to infinity was estimated by dividing the predicted plasma concentration at the last time point by the calculated terminal elimination rate constant, k. The linear trapezoidal method was used to calculate the AUC up to 100 min (AUC_{0-100}) and for the 100–200 min interval ($AUC_{100-200}$). To compensate for the fexofenadine concentration remaining from the previous period, when calculating $AUC_{100-200}$ in Paper II, a calculated concentration, obtained using the first-order elimination equation, was subtracted for each time point from the corresponding measured concentration for the 100–200 min interval. It was assumed that the terminal half-life ($t_{1/2}$), obtained from k, was not changed by verapamil. An increase in $t_{1/2}$ would result in a more pronounced difference in AUC and the apparent absorption rate constant between the control and treatment period.

In Paper II, the apparent absorption rate constant for the transport of fexofenadine into the systemic circulation (k_a) was calculated using the Wagner-Nelson method (204). In Paper IV, the maximum plasma concentration (C_{max}) of the enantiomers of verapamil and norverapamil were obtained by visual inspection.

3.5.3 Assessment of in vitro permeability and metabolism (Papers III and V)

In Paper III the apparent permeability coefficients (P_{app}, cm/s) of fexofenadine were calculated using Equation 7,

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{(A \times C_0)} \qquad (7)$$

where dQ/dt is the linear appearance rate of the mass in the receiver solution transported during sink conditions. A is the surface area of the membrane (1.13 cm^2) and C_0 is the initial donor concentration.

Since sink conditions could not be maintained for the enantiomers of verapamil (Paper V), P_{app} was calculated from (205),

$$C_{R(t)} = (M/V_D+V_R) + (C_{R,0} - (M/V_D+V_R)) \cdot e^{-PappA(1/V_D + 1/V_R)t} \qquad (8)$$

where $C_{R(t)}$ is the time-dependent drug concentration in the receiver compartment, M is the total amount of drug in the system, V_D and V_R are the volumes of the donor and receiver compartments, respectively, $C_{R,0}$ is the drug concentration in the receiver compartment at the beginning of the interval, and t is the time from the start of the interval. P_{app} was obtained from nonlinear regression, minimizing the sum of squared residuals ($\Sigma(C_{R,i,obsd} - C_{R,i,calcd})^2$), where $C_{R,i,obsd}$ and $C_{R,i,calcd}$

are, respectively, the observed and calculated receiver concentrations at the end of the interval.

The efflux ratio (ER) was calculated as the ratio P_{app}(b-a)/P_{app}(a-b). Apparent maximum velocities (V_{max}) and the Michaelis-Menten constants (K_m) were determined using nonlinear regression.

3.6 Statistical analysis

When comparing the mean between two groups the student's t-test for paired or unpaired data was used where appropriate. For multiple comparisons, one-way or repeated measures analysis of variance was performed followed by the Bonferroni, Dunnet's, Tukey's or Newmann-Keuls post test. After a variance stabilizing logarithmic transformation, the plasma pharmacokinetic data in Paper IV was analyzed by analysis of variance using the SAS statistical package. Differences between the control and treatment phases and the enantiomers of norverapamil and verapamil were estimated and then transformed back to the original scale. The differences between mean values were considered significant at $P < 0.05$. All data are expressed as mean values ± one SD unless stated otherwise.

4. Results and discussion

4.1 Intestinal permeability of fexofenadine in humans and in vitro (Papers I-III)

The human in vivo jejunal P_{eff} of fexofenadine was low ($\leq 0.3 \cdot 10^{-4}$ cm/s). The measured P_{eff} values were 0.11 ± 0.11 (93 μM) and $0.06 \pm 0.07 \cdot 10^{-4}$ cm/s (186 μM) and f_{abs} was 3 ± 3 and 2 ± 2% in the control periods in Papers I and II, respectively (Table 1 and 2). This classified fexofenadine as a low permeability drug according to the BCS (16) and predicts an incomplete fraction absorbed in humans (206). Indeed, the bioavailability has been estimated to 30% (147). The low permeability could also, at least in part, explain the high recovery of unchanged fexofenadine in feces after oral dosing (186). High intersubject variability is commonly seen for incompletely absorbed drugs (207).

Using the Caco-2 model, it was found in Paper III that the P_{app}(a-b) of fexofenadine was low and independent of concentration in the range 50-1000 μM (Table 3). The P_{app}(a-b) of 150 μM fexofenadine, an in vivo relevant concentration, was $0.31 \pm 0.03 \cdot 10^{-6}$ cm/s, predicting a low fraction absorbed after an oral dose. The results are in line with the human P_{eff} values and previous in vitro data (147) as well as with results from pharmacokinetic studies showing that fexofenadine displays approximately dose-linear systemic exposure in the 10-800 mg dose range (184). However, the P_{app}(b-a), $8.24 \pm 0.31 \cdot 10^{-6}$ cm/s for 150 μM, was both concentration dependent and several-fold higher than in the absorptive direction (Table 3).

Several explanations for the low intestinal permeability and low bioavailability of fexofenadine after oral dosing exist. First, a low membrane penetrating ability and hence a low fraction absorbed may be a consequence of the physicochemical properties of a drug (12, 13, 15). Second, fexofenadine is suggested to be minimally metabolized (186), but may be actively secreted back into the intestinal lumen directly from the enterocytes and/or via biliary secretion as it is a P-gp substrate (147, 188, 189). The main transport mechanisms of fexofenadine are discussed in the following section.

Table 1. Absorption parameters and AUC of fexofenadine (mean ± SD) administered alone (0–100 min) or with ketoconazole (100–200 min) before (Treatment 1) and after 5 days pretreatment (Treatment 2) with ketoconazole.

		Treatment 1		*Treatment 2*	
		0-100 min	*100-200 min*	*0-100 min*	*100-200 min*
P_{eff}	(10^{-4} cm/s)	0.11 ± 0.11	0.04 ± 0.13	0.29 ± 0.47	0.22 ± 0.31
f_{abs}	(%)	3 ± 3	1 ± 3	7 ± 10	6 ± 8
AUC_{100}	(ng/ml ·min)	55 ± 101		51 ± 33	
AUC_{720}	(ng/ml ·min)	318 ± 426		426 ± 232	

No significant changes were observed

Table 2. Absorption and plasma pharmacokinetic parameters of fexofenadine (mean ± SD) administrated alone (Control) or with 500 mg/L verapamil (+ Verapamil).

		Control	*+ Verapamil*	
P_{eff}	(10^{-4} cm/s)	0.06 ± 0.07	0.04 ± 0.07	
f_{abs}	(%)	2 ± 2	1 ± 2	
ka	(min^{-1})	0.0030 ± 0.0012	0.0255 ± 0.0103	***
AUC	(ng/ml·min)	161 ± 181	664 ± 537	**

** Significantly different from the control (0–100 minutes) (P < .01). *** P < .001.

Table 3. Concentration dependent permeability of fexofenadine in the Caco-2 model.

	$P_{app} \cdot 10^{-6}$ cm/s							
Donor (μM)	*a-b*		*P*	*b-a*			*P*	*ER*
10				14.22	±	0.53		-
50	0.17	± 0.08		14.02	±	1.71	ns	85
100	0.23	± 0.03	ns	14.82	±	0.95	ns	66
200	0.27	± 0.04	*	10.19	±	0.23	***	37
500	0.22	± 0.03	ns	8.78	±	1.04	***	40
1000	0.19	± 0.01	ns	5.43	±	0.42	***	28

* Significantly different from 50 or 10 μM (P < .05). *** P < .001. ns non significant

4.2 Main transport mechanisms affecting the oral bioavailability of fexofenadine (Papers I-III)

4.2.1 Human in vivo transport mechanisms of fexofenadine

The reported drug-drug interaction between fexofenadine and ketoconazole (185), inspired a study to determine whether ketoconazole increases the intestinal absorption of fexofenadine by inhibition of an apical efflux pump, such as P-gp in vivo in humans. In the first part of the study the short-term effect of ketoconazole on the P_{eff} of fexofenadine was investigated. To mimic the reported drug-drug interaction, the effect of oral pretreatment with ketoconazole was investigated in the second part. Ketoconazole produced no significant change in P_{eff} or f_{abs} for fexofenadine either before or after pretreatment with ketoconazole, despite the fact that the maximum soluble concentration of the inhibitor was used (Table 1). This concentration is known to inhibit P-gp in vitro (208, 209) and to inhibit the CYP3A-mediated metabolism of verapamil in a previous perfusion study (143). No significant effect of ketoconazole was seen on the plasma levels either (Table 1).

In Paper II fexofenadine was co-perfused with the transport inhibitor verapamil to further investigate the main in vivo transport mechanisms involved in the intestinal absorption and bioavailability of fexofenadine. In line with the results from Paper I, verapamil did not significantly affect the P_{eff} or f_{abs} of fexofenadine compared with control (Table 2, Figure 8). On the other hand, verapamil increased k_a and AUC for fexofenadine 12- and 4-fold, respectively (Table 2, Figure 9 and 10).

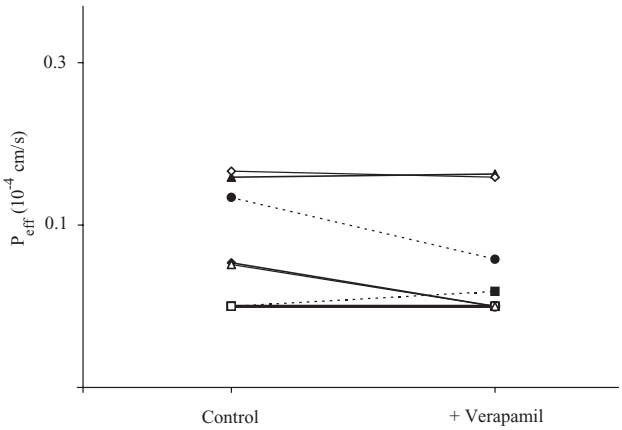


Figure 8. Individual P_{eff} values of fexofenadine administered alone (Control) or with verapamil (+Verapamil).

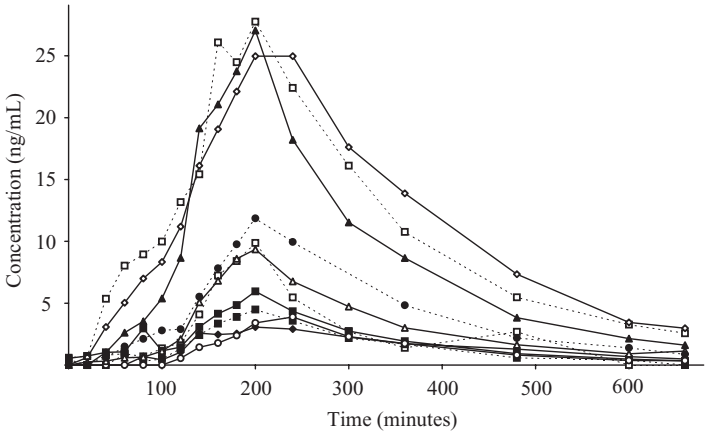


Figure 9. Individual plasma fexofenadine concentration-time profiles after jejunal perfusion of fexofenadine alone (0-100 min) and with verapamil (100-200 min).

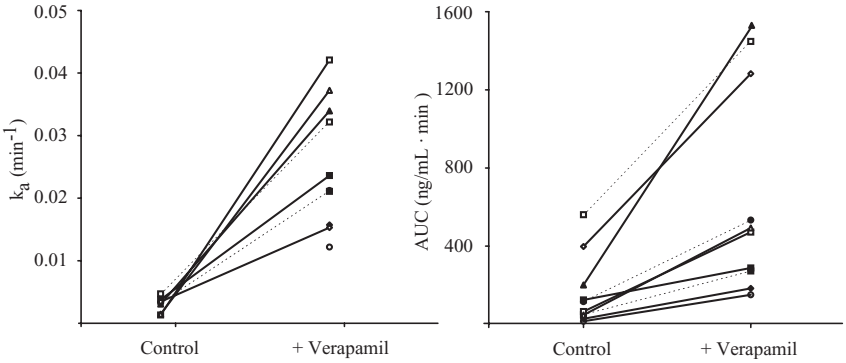


Figure 10. Individual apparent k_a based on plasma concentrations (left) and plasma AUC values (right) after jejunal perfusion of fexofenadine alone (Control) and with verapamil (+ Verapamil).

When just taking plasma data into account, the effect of verapamil on the bioavailability of fexofenadine is in line with previous pharmacokinetic studies, which have suggested that P-gp is important for the intestinal absorption of the drug (187, 190). However, the effect of verapamil and the previously reported effect of ketoconazole (185) on the plasma levels of fexofenadine, along with the observed unchanged intestinal absorption after both ketoconazole and verapamil coadministration, suggest that, instead, the liver transport of fexofenadine is affected. The most probable mechanism for the increased bioavailability is that the carrier-mediated membrane transport of fexofenadine in the liver is significantly affected by verapamil, by inhibition of OATP-mediated uptake of fexofenadine across the sinusoidal membrane and/or P-gp mediated secretion across the canalicular membrane during the first-pass through the liver. This is

supported by in vitro data showing that P-gp and members of the OATP-family are involved in the transport of fexofenadine (52, 53, 147, 187, 190) and that both ketoconazole and verapamil are P-gp and OATP inhibitors (147). Also, verapamil is known to increase the steady state plasma concentrations of the P-gp and OATP8 substrate digoxin by 44%, which is explained by a 43% decrease in digoxin biliary clearance (159). Assuming a high extraction ratio, a dual inhibition of the liver OATPs and P-gp would be effective as these transporters act in the same direction.

In conclusion, verapamil increased the bioavailability of fexofenadine. As the intestinal permeability was unchanged by both ketoconazole and verapamil, it was suggested that the major reason for this effect was a decreased first-pass liver extraction of fexofenadine. The most plausible mechanism for this would be a decreased OATP-mediated sinusoidal uptake and/or P-gp-mediated canalicular secretion of fexofenadine.

4.2.2 Transport characteristics of fexofenadine in the Caco-2 model

In Paper III, the Caco-2 model was used to study the bidirectional concentration-dependent permeability and the effect of various transport inhibitors on the permeability of fexofenadine.

The P_{app} of fexofenadine was highly polarized (Table 3). While P_{app}(a-b) remained unchanged, P_{app}(b-a) decreased at higher donor concentrations, suggesting saturation of an apical efflux transporter (Table 3). The saturated transport was characterized by a V_{max} and K_m of 5.21 nmol/s/cm^2 and 150 μM, respectively (Figure 11).

The P-gp inhibitors GF120918, ketoconazole, verapamil and erythromycin, affected both the absorptive and secretory P_{app} of 150 μM fexofenadine (Figure 12 and 13). For example, 2 μM of the specific P-gp inhibitor GF120918 increased the P_{app}(a-b) 3-fold and decreased the efflux ratio of fexofenadine to one (Figure 13). GF120918 is also known to inhibit BCRP at concentrations of 10 μM and above (210) but, since no additional effect was observed at that concentration, it may be speculated that BCRP is not involved in the transport of fexofenadine. However, this may also be the result of a low BCRP expression in the Caco-2 model (91).

It has also been reported that verapamil, ketoconazole and erythromycin inhibit the OATP-mediated uptake of fexofenadine (147). However, in Paper III, no OATP-mediated uptake could be distinguished from the efflux transport. This might be explained by low expression of functional OATPs in the Caco-2 model and/or by a low OATP activity at pH 7.4. (51, 52). The absent OATP activity was confirmed by rifamycin SV, an inhibitor of several members of OATP (Figure 12) (150).

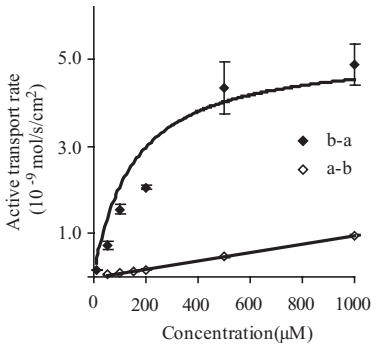


Figure 11. Concentration dependent transport of fexofenadine in the Caco-2 model.

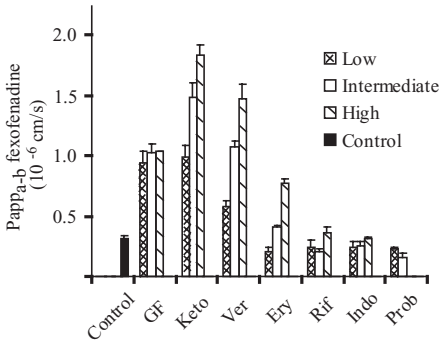


Figure 12. The concentration-dependent effect of inhibitors of P-gp, BCRP, OATP and MRP on the P_{app}(a-b) for fexofenadine.

As probenecid and indomethacin, which are MRP, but not P-gp inhibitors (211, 212), produced little or no effect on the P_{app}(a-b) and as GF120918, which does not inhibit MRP1 or MRP2 (213), completely inhibited the efflux transport (Figure 12), it was concluded that MRP is probably not involved in the membrane transport of fexofenadine. The results agree with previously reported in vitro studies (188).

As the molecular weight of fexofenadine is 538 and as the P-gp inhibitors increased the P_{app}(a-b), it may be concluded that fexofenadine is transported transcellularly rather than by the paracellular route. Also, the higher in vivo permeability compared to the Caco-2 permeability for fexofenadine may be explained by a larger absorptive surface area being available in vivo for low permeability drugs, as they may diffuse down the length of the villi, compared to the Caco-2 model, whose area represents the tip of the villi only (23, 24).

In conclusion, the results from Paper III clearly show that P-gp is the major active membrane transporter of fexofenadine in the Caco-2 model

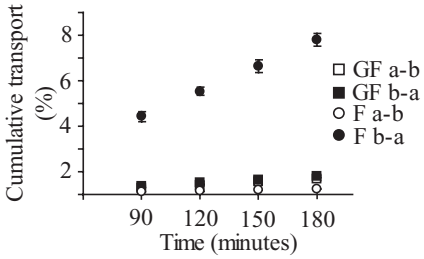


Figure 13. The cumulative transport of fexofenadine (150 μM) alone or in the presence of the specific P-gp inhibitor GF 120918 (2 μM), in the absorptive (a-b) and secretory (b-a) direction across Caco-2 monolayers.

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4.2.3 Possible reasons for the unchanged P_{eff} of fexofenadine: Comparative importance of the passive permeability and the active transport

Why do ketoconazole and verapamil not affect the human in vivo intestinal permeability of fexofenadine in Papers I and II, respectively, when fexofenadine clearly is a substrate for P-gp, as shown in Paper III and as previously reported (147, 188, 189)? There are several possible explanations for this:

First, the intestinal absorption of fexofenadine might involve both uptake and efflux transporters. If intestinal P-gp alone was the main mechanism for the low bioavailability, a non-linear increase in the intestinal absorption of fexofenadine would have been anticipated at higher oral doses instead of the reported dose-independent systemic exposure (97, 184). However, fexofenadine is also a substrate for OATP-B located in the apical membrane of the human enterocytes (51, 52). P-gp and OATPs transport drugs in opposite directions across the apical membrane of the enterocytes and both ketoconazole and verapamil are known to inhibit both transporters (53, 147), from which it is concluded that a plausible reason for the unchanged permeability for fexofenadine observed in Papers I and II might, therefore, be a simultaneous short-term inhibition of both uptake and efflux by OATPs and P-gp, respectively.

Second, the intestinal absorption of fexofenadine might not be limited by P-gp, but could instead be determined by the passive permeability of the drug. Even if the P-gp inhibitors increased the P_{app}(a-b) of fexofenadine in the Caco-2 model, where OATP-activity was absent, the permeability remained low. For instance, when the efflux was completely inhibited by 2 μM GF120918, the P_{app}(a-b) was $1.03 \pm 0.07 \cdot 10^{-6}$ cm/s, still classifying fexofenadine as a low permeability compound with incomplete intestinal absorption. The results show that the absorptive transport of fexofenadine is mainly determined by passive diffusion rather than P-gp-mediated efflux. The apparent effect of the P-gp inhibitors on the efflux ratio was somewhat misleading as the most pronounced effect was seen on the secretory transport (Figure 13). The high polar surface area (124 $Å^2$) of fexofenadine, predicting a low passive permeability and a low fraction dose absorbed (12, 15), as well as the zwitterionic character of fexofenadine, further support the idea that passive diffusion is rate-limiting process in the intestinal absorption of the drug.

According to the classification recently proposed by Troutman and Thakker, the absorptive quotient (AQ) of fexofenadine was calculated to be 0.7 (class I), meaning that P-gp attenuates the passive permeability during absorption by 70% in the Caco-2 model (214). However, inhibition of P-gp would still not dramatically increase the intestinal absorption as the passive permeability is so low.

In conclusion, the results suggest that either low passive permeability or oppositely directed transporters limit the intestinal absorption of fexofenadine.

4.3 The effect of St. John's wort on the in vivo P_{eff} and presystemic metabolism of R- and S-verapamil

The herbal drug St. John's wort (SJW) is known to induce expression of intestinal and hepatic CYP3A4 and intestinal P-gp, through activation of PXR (73, 131, 132). PXR ligands are also known to regulate the genes for members of the CYP2C family (215), phase II enzymes (216) and MRP2 (62, 217). In Paper IV, the P_{eff} and presystemic metabolism of the CYP3A4 and P-gp substrate R- and S-verapamil was studied before and after 14 days of oral treatment with SJW.

It was found that SJW decreased the AUC for both R- and S-verapamil by 78 and 80%, respectively (Figure 14 and 15). The corresponding decreases in C_{max} were 76 and 78%, respectively, while the terminal half-life was unchanged (Figure 14). Also, the AUC of R-verapamil was six times higher than for the S-enantiomer both before and after treatment with SJW. Such an absence of any preferential effect of SJW on any of the enantiomers is in contrast to a previous report with the inducer rifampicin (122), but expected as the AUC should decrease after induction, irrespective of the liver extraction ratio (218).

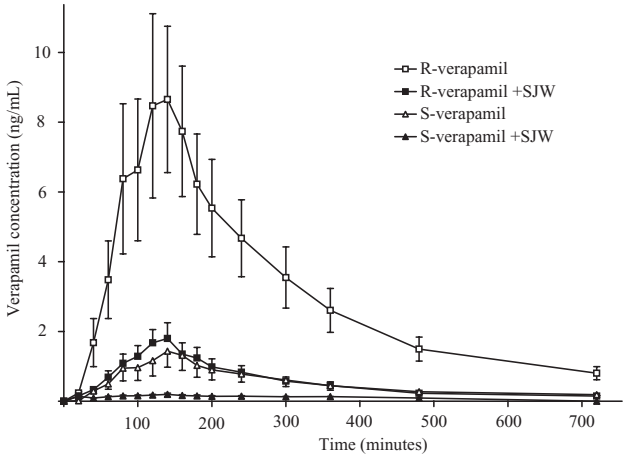

Figure 14. Plasma concentration-time profiles (mean ± standard error) for R- (squares) and S-verapamil (triangles) after 100 minute jejunal perfusion with racemic verapamil before (Control) (open symbols) and after 14 days of treatment with St John's wort (+ SJW) (filled symbols) (300 mg 3 times a day).

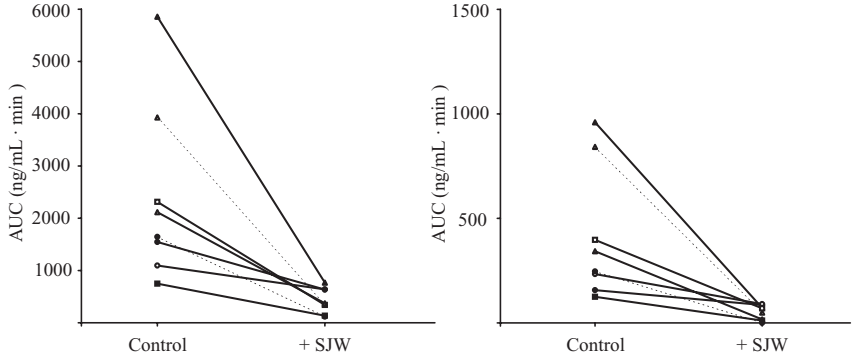

Figure 15. Individual $AUC_{0-\infty}$ values for (left) R- and (right) S-verapamil after a 100 minute jejunal perfusion with racemic verapamil before (Control) and after 14 days of treatment with St John's wort (+ SJW) (300 mg 3 times a day).

The decrease in the bioavailability of verapamil was not attributed to a decreased intestinal absorption caused by an increased P-gp-mediated intestinal secretion of the drug, as the P_{eff} remained high and was unaffected by the SJW treatment. P_{eff} for R- and S-verapamil was 7.37 ± 6.13 and 6.05 ± 4.13 before and 7.06 ± 4.65 and 5.43 ± 2.84 · 10^{-4} cm/s, respectively after treatment with SJW. f_{abs} was approximately 60% for each verapamil enantiomer. The results are in line with previous results, which have shown that the P-gp and CYP3A4 inhibitor ketoconazole does not change the human in vivo permeability of verapamil either (143).

Verapamil is mainly metabolized by CYP3A4 (197-200). Hyperforin, the active constituent of SJW induces the CYP3A4 expression through activation of PXR (73, 131, 132). It was thus concluded that SJW mediates its effects on the verapamil bioavailability through induction of CYP3A4 metabolism. Even though SJW most likely induces both intestinal and liver CYP3A4, it could be argued that the strongest effect was probably achieved in the intestine as the enterocytes were exposed to higher concentrations than the hepatocytes. Indeed, it has previously been reported that rifampicin, another PXR-ligand, increases the presystemic metabolism of R- and S-verapamil mainly by increasing the gut wall extraction ratio (122).

SJW also affected the plasma levels of norverapamil, one of the main metabolites of verapamil. The AUC decreased by 51 and 63% for R- and S-norverapamil, respectively (Figure 16). The further metabolism of R/S-norverapamil is not well characterized, but the results in Paper IV suggest involvement of CYP3A4, which is in line with previous in vitro reports (198, 200). However, other elimination routes cannot be ruled out. For example, SJW slightly increased the appearance ratio of S-norverapamil. A potential explanation for this effect would be an induced transporter-mediated intestinal secretion of the enantiomers of norverapamil, but the increased appearance in the jejunum could just reflect increased formation of norverapamil in the enterocyte.

In conclusion, SJW decreased the oral bioavailability of the enantiomers of verapamil by inducing the CYP3A4-mediated presystemic metabolism, most

likely mainly in the gut. The unchanged intestinal permeability suggests that intestinal efflux transporters like P-gp do not limit the absorption of highly permeable drugs like verapamil and that, instead, the bioavailability is limited by presystemic metabolism.

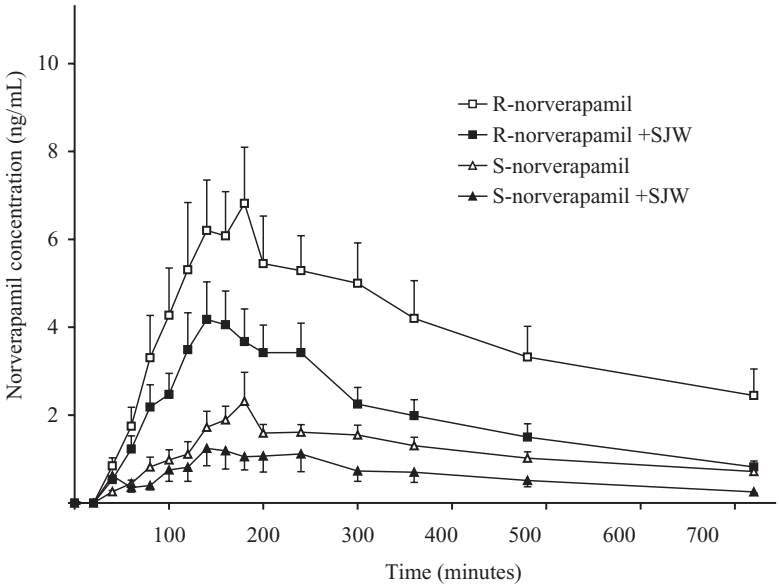

Figure 16. Plasma concentration-time profiles (mean ± standard error) for R- (squares) and S-norverapamil (triangles) after 100 minute jejunal perfusion with racemic verapamil before (Control) (open symbols) and after 14 days of treatment with St John's wort (+ SJW) (filled symbols) (300 mg 3 times a day).

4.4 In vitro transport and CYP3A4 metabolism of the enantiomers of verapamil

In Paper V the intestinal permeability and CYP3A4-mediated metabolism of the enantiomers of verapamil were further studied in Caco-2 cells with D3-induced CYP3A4 activity at clinically relevant concentrations using untreated Caco-2 cells as the control.

The P_{app}(a-b) of the enantiomers of verapamil was high and only weakly concentration dependent in the 2.5-80 μM range in both untreated and D3-treated cells and lacked enantioselectivity (Table 4). For 8 μM racemic verapamil the efflux ratio was 1.9 ± 0.2 for both enantiomers, which was brought close to unity by GF120918, indomethacin and tetrahexylammonium, an OCT inhibitor. On the basis of this, it is clear that even if verapamil is a substrate for transporters such as P-gp, as previously reported (100, 192, 193), the absorption of verapamil will still be determined by the high passive permeability. The P_{app}(a-b) for R- and S-

norverapamil was high and no net secretion was seen. Moreover, the human in vivo P_{eff} from previous perfusion studies (100, 143) was about 3-fold higher than the P_{app} in Paper V. This may be attributed to the intact blood flow in vivo, which makes a significant contribution to the sink conditions, or to a more efficient reduction of the unstirred water layer in vivo (17). As the effective absorptive surface area is thought to be the same in both models for high permeability drugs, it is unlikely that this could explain the difference (23, 24).

As seen in Paper IV and previous reports (122, 143, 195, 196), enantioselective CYP3A4-mediated metabolism of R/S-verapamil was observed (Figure 17). The respective apparent V_{max} and K_m values for the formation of R-norverapamil were 3.2 pmol/min/insert and 0.7 μM, and 5.4 pmol/min/insert and 0.6 μM for S-norverapamil. The levels of R- and S-norverapamil formed constituted 2.8% and 3.4% of the apically applied dose, respectively. Lower levels of norverapamil were formed in comparison to the human jejunum (100, 143), which is in accordance with a lower CYP3A4 expression and activity than in vivo (55, 91). The distribution of verapamil and norverapamil were comparable for the apical and basolateral sides, which is in line with the high passive permeability and low polarized efflux of the two drugs (193). Studies of CYP3A4-mediated or other metabolism of norverapamil were not included in this investigation.

In conclusion, verapamil and norverapamil are mainly transported by passive diffusion and are not limited by transporter-mediated efflux. Like in humans, CYP3A4-mediated metabolism was enantioselective in this model.

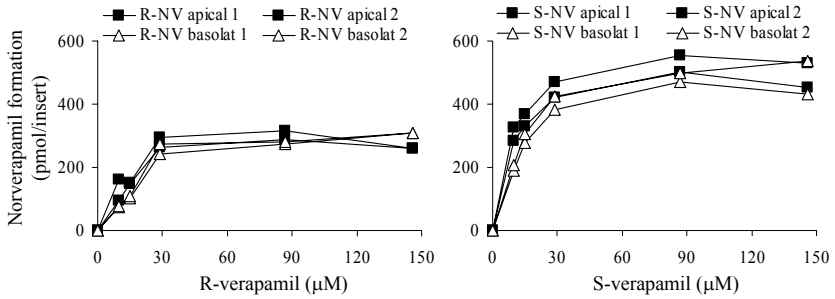

Figure 17. Concentration-dependent demethylation of R/S-verapamil to (left) R-and (right) S-norverapamil (NV) in 1α,25-dihydroxy vitamin D_3 (D3)-treated Caco-2 cells. Samples were drawn separately from the apical (apical) and the basolateral (basolat) compartments. Data from duplicate determinations are shown for each substrate concentration.

Table 4. P_{app}(a-b) and efflux ratios determined for R-and S-verapamil in untreated and 1α,25-dihydroxy vitamin D_3 (D3)-treated Caco-2 cell monolayers.

Racemic verapamil (μM)	*Untreated cells*			*D3-treated cells*		
	R-verapamil	*S-verapamil*		*R-verapamil*	*S-verapamil*	
	P_{app} ($\cdot 10^{-6}$ cm/s)		R/S	P_{app} ($\cdot 10^{-6}$ cm/s)		R/S
2.5	68.9 ± 13.0[b,c]	84.6 ± 13.1[c]	0.81	66.8 ± 1.7[a,b,c]	70.8 ± 3.6[c]	0.94
8	96.3 ± 23.7[c]	124.4 ± 24.1	0.77	80.1 ± 5.9[b,c]	85.7 ± 5.6[c]	0.94
25	123.5 ± 12.9	129.0 ± 12.2	0.96	97.1 ± 6.0[c]	87.6 ± 24.9[c]	1.05
80	156.6 ± 19.1	142.6 ± 16.7	1.10	129.1 ± 3.8	127.0 ± 2.5	1.02
	Efflux ratios			Efflux ratios		
8	2.0 ± 0.6	1.8 ± 0.4		1.9 ± 0.2[d]	1.9 ± 0.2[d]	
80	1.0 ± 0.1	1.0 ± 0.1		1.1 ± 0.0	1.0 ± 0.0	

[a] Significantly different from P_{app} at 8 μM; [b] 25 μM; [c] 80 μM
[d] Significantly different from unity, $p < 0.05$

5. Concluding remarks

The main objective of this thesis was to increase the mechanistic understanding of the importance of membrane transporters and CYP3A-mediated metabolism for the oral bioavailability of drugs in humans, with the main focus being on the in vivo situation. In vitro methods were used to further clarify in vivo findings. From the investigations presented in this thesis, it can be concluded that:

- Fexofenadine has a low effective jejunal permeability in vivo in humans ($\leq 0.3 \cdot 10^{-4}$ cm/s), which predicts an incomplete fraction absorbed after an oral dose. The absorptive permeability in the Caco-2 model was also low ($0.31 \pm 0.03 \cdot 10^{-6}$ cm/s at 150 μM) and independent of the donor concentration of the drug.

- The low human jejunal permeability of fexofenadine was not changed by the transport inhibitors ketoconazole and verapamil. This finding could be explained by a complex absorption mechanism, involving both OATP-mediated uptake and P-gp-mediated efflux. Alternatively, the intestinal absorption of fexofenadine may not be limited by intestinal efflux, suggesting that the low passive permeability limits the small intestinal absorption of the drug in humans, which is predicted from its physicochemical properties.

- In contrast, in the Caco-2 model the absorptive and the concentration-dependent secretory permeability was increased and decreased, respectively, by various P-gp inhibitors and it was concluded that P-gp is the major active membrane transporter of fexofenadine in the Caco-2 model. However, as the absorptive permeability remained low even when P-gp was totally inhibited, it was also concluded that the absorption is mainly determined by the low passive permeability of the drug. Neither BCRP, nor any MRPs or OATPs contributed significantly to the absorptive permeability of fexofenadine in this in vitro cell model.

- Co-perfusion with verapamil increased the bioavailability of fexofenadine, most likely by decreasing the first-pass liver extraction as the intestinal permeability did not change. The mechanism probably involves inhibition of OATP-mediated sinusoidal uptake and/or P-gp-mediated canalicular secretion as fexofenadine is suggested to be minimally metabolized.

- Repeated oral administration of St. John's wort decreased the bioavailability of the enantiomers of verapamil. As no change was seen in the intestinal permeability, it was concluded that it was most likely that the effect was caused by induction of the presystemic CYP3A4-mediated metabolism, probably mainly in the gut. This also shows that induction of efflux

transporters, such as P-gp, does not affect the intestinal transport of high permeability compounds like verapamil. Moreover, because of the high passive diffusion of verapamil, apical recycling was probably not important for the high extraction in the gut. St. John's wort also decreased the plasma levels of the enantiomers of norverapamil despite an increased formation, which was attributed to the induction of CYP3A4-mediated elimination and/or another PXR-dependent metabolic route.

- In Caco-2 cells with 1α,25-dihydroxy vitamin D_3 induced CYP3A4 activity, the enantiomers of verapamil and norverapamil have a high permeability and passive diffusion is the dominant transport mechanism. As in humans, the metabolism of verapamil is enantioselective, but less norverapamil is formed.

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