Intestinal Barriers to Oral Drug Absorption

Cytochrome P450 3A and ABC-Transport Proteins

BY

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The subject of this thesis was to study two intestinal barriers to oral drug bioavailability, drug efflux proteins of the ABC-transporter family, and in particular ABCB1/P-glycoprotein (Pgp), and the drug metabolizing enzyme cytochrome P450 (CYP) 3A4. At the onset of this thesis, similarities between CYP3A4 and Pgp in terms of their tissue distribution and gene regulation, along with overlapping substrate specificities, had generated the hypothesis that CYP3A4 and Pgp may have a complementary function and thus form a coordinated intestinal barrier to drug absorption and gut wall metabolism.

In the first part of this thesis, a cell culture model of the intestinal epithelium that expressed both functional Pgp and CYP3A4 was developed. This model was then used to investigate the steroselective drug efflux and metabolism of R/S-verapamil. In summary, the results indicated that the two barriers in the cell culture model were in agreement with those in the human intestine.

Both ABC-transporters and CYPs are regulated by drugs that interact with nuclear receptors. However, while the regulation of CYPs is quite well understood, less is known about how repeated drug administration regulates the most abundantly expressed ABC-transporters. Therefore, in the second part of this thesis, the effects of repeated drug administration on the gene regulation of four ABC-transporters and CYP3A4 were studied in intestinal epithelial cell lines in vitro and in the perfused human jejunum in vivo. The in vitro studies revealed that the ABC-transporters are induced by drugs that interact with slightly different sets of nuclear receptors. The in vivo study showed that repeated oral administration of St John’s wort decreased the bioavailability of verapamil, predominantly by induction of intestinal CYP3A4. This part of the thesis provides new information about the regulation of ABC-transporters, shows that the intestinal metabolism is the most significant barrier to oral bioavailability of verapamil and provides evidence for a clinically significant interaction between verapamil and St John’s wort in vivo.
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PAPERS DISCUSSED

This thesis is based on the following papers, which will be referred to by their roman numeral in the text:

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ABBREVIATIONS

a-b  apical-to-basolateral
ABC  adenosine triphosphate (ATP) Binding Cassette
Ar  appearance ratio
b-a  basolateral-to-apical
BCRP  breast cancer resistance protein
CAR  constitutive androstane receptor
CYP  cytochrome P450
D3  1α,25-dihydroxy vitamin D₃, calcitriol
E₉  extraction ratio in the gut wall
E₉  extraction ratio in the liver
F  systemic bioavailability
fₐ  fraction dose absorbed
FCS  fetal calf serum
GR  glucocorticoid receptor
HBSS  Hank’s balanced salt solution
HNF4α  hepatocyte nuclear factor 4α
HPLC  high performance liquid chromatography
i.v.  intravenous
MDR  multidrug resistance
MDR1 Pgp  multidrug resistant gene 1 P-glycoprotein
MRP  multidrug resistance associated protein
Pₐₚₚ  apparent permeability coefficient
Pₐₚₚ  effective permeability coefficient
PXR  pregnane X receptor
RT-PCR  reverse transcription polymerase chain reaction
SD  standard deviation
TER  transepithelial electrical resistance
VDR  vitamin D receptor
Å  Ångström
1. INTRODUCTION

Oral administration of drugs is strongly preferred because of its convenience, the relatively low production cost and the high level of patient safety. However, a considerable proportion of compounds do not display the characteristics required for oral administration. The clinical development of new drugs is frequently hampered by unfavourable pharmacokinetics, such as poor or variable bioavailability of the drug after oral administration.

A prerequisite for a drug to be efficient after oral administration is that it, largely, avoids a sequential series of barriers in the gastrointestinal tract and in the liver. Until recently, systemic bioavailability of orally administered drugs has, primarily been considered to be a function of intestinal drug absorption and subsequent phase I metabolism in the liver. However, the human small intestine has increasingly been recognized as an important site for first-pass extraction. Both cytochrome P450 (CYP) 3A isoenzymes (1, 2) and efflux proteins, belonging to the ATP-binding cassette (ABC) transporter family (e.g., ABCB1/P-glycoprotein, Pgp) (3-5), are abundantly expressed in the human small intestine and contribute to a reduced bioavailability of many drugs (6-8). Among the members of the CYP3A subfamily, CYP3A4 is the most important, both quantitatively and qualitatively (9). Recently, similarities between the tissue distribution and gene regulation of CYP3A4 and Pgp, along with their overlapping specificities for substrates, inhibitors, and inducers (10) generated the hypothesis that CYP3A4 and Pgp have a complementary function and thus form a coordinated intestinal barrier to drug absorption and gut wall metabolism (11). The lack of direct evidence supporting the concerted action between CYP3A4 and Pgp is partly due to the lack of inhibitors specific for the enzyme and transporter, respectively, but also to difficulties in separating the relative contribution of the two proteins in vivo. For this purpose, reproducible in vitro models allowing detailed mechanistic studies would be useful. The aim of the first part of this thesis was therefore to establish and characterize a cell culture model of the intestinal epithelium for application in studies of both CYP3A4-mediated drug metabolism and Pgp-mediated efflux activity.

Induction of CYP3A and, potentially also ABC-transporters in the human small intestine is an important source of drug-drug interactions and variations in oral bioavailability (8, 12-14). Many clinically relevant drugs are substrates for both ABC-transporters and CYP3A4. Several nuclear receptors, such as PXR, CAR, and VDR have recently been shown to be important participants in the transcriptional pathways that control the adaptive response of ABC-transporters and CYP450 to both exogenous and endogenous compounds (15-19). These compounds include many clinically relevant drugs. While the induction of CYP3A in the intestine has been thoroughly investigated, much less is known about how drugs regulate intestinal ABC-transporters. Thus, the aim of the second part of this thesis was therefore to investigate the effects of repeated drug administration on the gene regulation and function of ABC-transporters and CYP3A4 in the human intestine. These studies were performed in two human intestinal epithelial cell models in vitro, and in the human intestine in vivo.
1.1 Factors Influencing Oral Drug Absorption and Bioavailability

The bioavailability (F) of a drug is defined as the fraction of the dose that appears intact in the systemic circulation (20). The various factors that influence the systemic bioavailability following oral administration can be described by the following equation:

\[ F = f_a \cdot (1-E_G) \cdot (1-E_H) \]  

where \( f_a \) is the fraction of the dose absorbed over the mucosal membrane of the enterocyte, \( (1-E_G) \) is the fraction of the drug that escapes metabolism in the gut wall, and \( (1-E_H) \) is the fraction of the drug that escapes metabolism and biliary excretion in the liver (21).

As can be seen in Figure 1, the intestinal absorption and bioavailability after oral drug administration is governed by several factors (20, 22, 23).

![Figure 1. Schematic illustration of factors influencing oral drug absorption and bioavailability.](image)

Drugs designed to be systemically active must be absorbed from the site of administration in order to be efficient. Furthermore, to allow passage through biological membranes, the drug must be in solution. Since most drugs are administered as solid dosage forms, disintegration of the formulation must precede dissolution of the drug in the surrounding media. The disintegration rate is influenced by characteristics of the pharmaceutical formulation and by physiological factors such as the gastric emptying rate, the transit time and the pH in the gastrointestinal fluids. The dissolution rate is not only dependent on the physico-chemical properties of the particle and the drug molecule in itself (e.g., pKa, molecular size, lipophilicity and hydrogen bonding), but also on the luminal pH and the composition of the luminal contents. Once in solution, the drug is susceptible to both chemical and enzymatic degradation. The oral bioavailability may be
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further reduced by efflux mechanisms or first-pass metabolism in the intestinal epithelium and/or the liver.

The intestinal solubility of and permeability to a drug are considered to be the two most important determinants of oral absorption e.g., (24, 25). The latter is defined as the transport of the unchanged drug molecule from its dosage form across the apical membrane of the enterocyte (20). In this thesis, the focus is on drugs in solution, and hence the effects of drug solubility and dissolution are not considered. If, however, one wished to consider aspects of solubility and permeability, these parameters have been incorporated into the biopharmaceutical classification system, BCS (26), which allows e.g., predictions of the oral drug absorption to be made in humans. The interplay between permeability and solubility can also be roughly assessed by calculating the maximum absorbable dose, MAD (24). However, valid use of MAD requires the assumption that complicating factors such as luminal degradation are negligible.

1.2 THE INTESTINAL EPITHELIUM

Once in solution, a drug molecule encounters a system of sequential barriers during its transport from the intestinal lumen into the blood, figure 2. The rate and extent of intestinal absorption is dependent on the epithelial permeability to the drug (27, 28) and the epithelial surface area. The epithelial surface available for contact with the luminal contents is greatly amplified by the folds, villi, and microvilli structures (29).

**Figure 2.** The crypt-villus functional unit of the human small intestine. The mucosal epithelium is rapidly renewing, and the proliferative cells, which arise from the stem cells located at the bottom of the crypts, lose their ability to proliferate. They start to differentiate in the upper third of the crypt and then migrate toward the tip of the villus, before being exfoliated into the intestinal lumen. Caco-2 cells are commonly used as a model of the human intestinal epithelium. Their loss of proliferating and acquisition of differentiating characteristics appears gradually along the crypt-villus axis. Redrawn from (30).
The functional unit in the intestinal epithelium is the crypt-villus axis, figure 2. Within the axis, the epithelium is spatially separated into proliferating and differentiating cells (in the lower and upper crypt regions, respectively) cells, with functional, absorptive cells (called enterocytes) situated on the villus tip (30). The intestinal epithelium contains three types of cells with distinct functions: endocrine, exocrine and absorptive cells. The endocrine cells secrete digestive hormonal peptides, while the exocrine ones secrete mucus (goblet cells) or antimicrobial peptides (Paneth cells). The enterocytes are the most abundant cells accounting for 80-90% of the total number of epithelial cells. The three groups of differentiated cells originate from the same multipotential stem cell that proliferate near the bottom of the crypt (29).

The properties that are relevant for drug absorption differ between the cells in one region and another along the crypt-villus axis. For instance, in several species, the paracellular space between the cells seems to have a lower permeability at the villus tips than further down the crypt-villus axis (31, 32). High permeability drugs are believed to be absorbed mainly at the tips of the villi (33), while low-permeability drugs may diffuse down the length of the crypt-villus axis to be absorbed over a larger absorptive surface area (34).

1.3 MECHANISMS OF INTESTINAL DRUG TRANSPORT

Drug transport across absorptive epithelia is mediated by one or several of the following mechanisms: passive transcellular or paracellular processes, carrier-mediated absorptive or secretory flux and transcytosis, figure 3.

1.3.1 Passive transcellular transport

A drug molecule must penetrate the membrane surrounding the epithelial cell in order to transverse the cell. Thus, the passive transport is largely determined by the biophysical properties of the membrane. Cell membrane consist of a double layer of phospholipids and cholesterol, with proteins embedded in the layer (35). The type and relative quantities of lipids and proteins differ between different cell types, thus creating a basis for unique permeability properties. Like other epithelial cells, the enterocytes are polarised with marked differences in membrane composition of the apical and basolateral membranes (36).

The first step of passive transcellular transport is the penetration of the apical membrane by the drug, which is generally assumed to be followed by diffusion through the cytoplasm of the cell interior, and subsequent permeation of the basolateral membrane. Very lipophilic drugs may become trapped in the apical membrane, or their transport may involve lateral diffusion in the cell interior. However, diffusion of small molecules in the cytoplasm is normally a rapid process, and therefore the apical membrane is usually considered to be the rate-limiting barrier to passive transcellular transport (37).

For the distribution to the apical membrane and the subsequent transcellular diffusion to be effective, the drug must be sufficiently lipophilic and moderate in size. Nevertheless, several studies suggest that the majority of completely absorbed drugs are transcellularly transported, some of these studies are reviewed in (38). Thus, the rather complex process of intestinal drug absorption can often be satisfactorily described by
just considering the passive transport across the apical membrane only. Thereby explaining why experimental and theoretical models describing transport by this mechanism have received particular attention (39, 40). Different theoretical models have been developed for passive transcellular permeability. Irrespective of which model is used, the rate of passive permeation largely depends on simple molecular descriptors such as the hydrogen bond capacity, lipophilicity, and the size and charge of the molecule (41). For instance, it has been estimated that oral drug absorption via the passive transcellular route is unlikely if the polar surface area (a measure of the hydrogen bonding capacity) of the drug molecule is more than 120Å² (42, 43).

1.3.2 Passive paracellular transport
Transport via water-filled pores between the cells is a process known as paracellular transport. This transport route is generally considered to be passive, although it appears to be more permeable to cationic drugs than to anionic or neutral species (44, 45). Drugs that are relatively hydrophilic and of small to moderate size (e.g., atenolol and furosemide) can permeate the intestinal epithelium via this route in significant amounts, at least in the upper part of the small intestine, where the paracellular route is more leaky than in the lower parts of the small intestine and colon (46). However, this type of drug is usually incompletely absorbed since the paracellular pores represent only 0.01-0.1% of the total surface area of the intestine. Moreover, the apical and basolateral membrane domains are separated by tight junctions, providing a seal between adjacent epithelial cells that further restricts transport via this route (47, 48). The paracellular permeability is dynamically regulated, a fact that has been exploited to enhance drug delivery via this route (49, 50).

**Figure 3.** Schematic representation of routes and mechanisms for drug transport across the intestinal epithelium. 1. passive transcellular and 2. paracellular transport, 3. carrier-mediated efflux, and 4. carrier-mediated active transport. Membrane proteins involved in carrier-mediated transport or efflux may also be localized in the basolateral membrane of the enterocyte.

1.3.3 Carrier-mediated transport
Although a vast number of drugs are transported by means of passive diffusion, recent studies suggest that carrier-mediated transport has an even larger impact than had been believed previously. An increasing number of active transporters such as the ABC-transporters that may have an impact on oral absorption are being identified, although their influence on the in vivo drug absorption is still largely unexplored. The mapping of the human genome identified nearly 1300 genes coding for ion channels and transporters, which might be directly or indirectly involved in the absorption process.
(51), and a qualitative cDNA array analysis of the number of transporters in the human jejunum indicated the presence of mRNA for approximately 200 transporters (52). Langmann et al. (4) provided the mRNA expression profiles for 47 of the 48 known ABC transporters in 20 human tissues, and showed that human tissues vary greatly in these profiles. This variability may have an impact on regional kinetics displayed by different organs within the body, especially since additional variability in ABC-transporter expression may occur during disease or as a response to administered drugs.

Intestinal absorptive cells express a number of carrier systems which play a key role in determining the exposure of cells to a variety of solutes including nutrients (e.g., peptides, amino acids and sugars) and cellular by-products. Transport by means of carriers can be directly or indirectly energy dependent, referred to as active transport, or independent on energy, i.e., facilitated diffusion (53). The process of carrier-mediated transport is saturable, i.e., drugs that are substrates may show non-linear pharmacokinetics.

In order to prevent unwanted entry of compounds into the body, carriers are substrate specific. However, the substrate specificity is not absolute and carrier-mediated transport is available to a limited number of drugs with nutrient-like molecular structures. The most promiscuous drug transporter known to date that enhances oral drug absorption is the H+-coupled oligopeptide transporter PEPT1, which is abundantly expressed in the small intestine (54, 55), and participates in the absorption of β-lactam antibiotics, renin inhibitors and angiotensin converting enzyme (ACE) inhibitors (56).

Organic anion and cation transporters have recently received considerable attention. For instance, the organic cation/carnitine transporter 2 (OCTN2), transports physiologically important carnitine in an Na+-dependent manner, as well as organic cations in an Na+-independent manner (57-59). Most importantly, Tsuji and colleagues provided evidence that primary systemic carnitine deficiency is caused by loss of OCTN2 function (60). In addition, the uptake of L-carnitine was shown to be primarily mediated by this transporter in differentiated Caco-2 cells (61). OCTN2 also plays a pharmacological role, since it mediates the transport of cations such as tetraethylammonium (TEA) and drugs such as pyrilamine, valproate, and verapamil (58).

Members of the organic anion transporting polypeptide (OATP) family are involved in the transport of various endogenous and xenobiotic compounds, such as conjugated metabolites of steroid hormones, thyroid hormones, bile acids, bilirubin, pravastatin, benzylpenicillin, and digoxin. So far, nine members of the OATP family have been reported in humans (62) of which OATP-B is the first OATP member shown to be expressed at the apical membrane of human enterocytes. OATP-B might therefore play a role in the pH-dependent transport of anionic drugs in the human intestine (63).

In the process known as receptor-mediated transcytosis, the solute binds to a receptor on the cell surface where it is internalised by endocytosis, and then a fraction of the internalised vesicle proceeds towards the opposite membrane. This complex route has a very low capacity and is only of relevance for highly potent macromolecular drugs that are active at low concentrations (64).
1.4 The ATP-Binding Cassette (ABC) Superfamily of Transporters

Multidrug resistance is a phenomenon whereby tumor cells in vitro that have been exposed to one cytotoxic agent develop cross-resistance to a range of structurally and functionally unrelated compounds. Multidrug resistance is, in part, the result of an increased expression of efflux proteins such as ABCB1/MDR1 P-glycoprotein, which is a member of the ATP-binding cassette (ABC) superfamily of transporters. These efflux proteins limit the intracellular exposure to xenobiotics by pumping agents out of the cell (65). However, ABC-transporters are also expressed in normal human tissues including the gastrointestinal tract (3, 4, 66, 67).

The human genome contains 48 ABC genes, of which 16 have a known function and 14 are associated with a defined human disease, as reviewed by (65, 68). Physiological functions of the ABC transporters include the transport of lipids, bile acids, peptides and toxic compounds (68). The role of ABC transporters in disease is exemplified by mutations in the ABCC2/MRP2 gene, which causes the Dubin-Johnson syndrome, in which biliary excretion of conjugated bilirubin by ABCC2 is impaired (69). Most importantly, multidrug resistance is a significant obstacle to the success of chemotherapy in many cancers (70). In addition, polymorphisms in the ABCB1 gene result in kinetic alterations on the transporter level that may have an impact on the therapeutic efficacy of many drugs (71, 72). The polymorphism in the ABCB1 gene is further discussed below.

Figure 4. Schematic representation of the localization of ABCB1, ABCC1-3 and ABCG2 in polarized cells in the intestinal epithelium. See text for further details. Redrawn from (73).

Efflux proteins have the potential to affect oral bioavailability by pumping agents out of the enterocyte. Export of these compounds occurs in an active ATP-dependent manner, and can take place against considerable concentration gradients. ATP-hydrolysis provides the energy for this process (74, 75).

Membrane proteins shown to transport clinically relevant drugs belong to the ABC-transporter family, and more specifically, include ABCB1/MDR1 Pgp, multidrug resistance proteins ABCC/MRP1-5, and breast cancer resistance protein ABCG2/BCRP (76). As shown in figure 4, these transporters are localised either to the apical membrane of the intestinal enterocytes (such as ABCB1, ABCC2 and ABCG2), where they function as a total organism defence mechanism by pumping drugs out of the cell back to the intestinal lumen, or to the basolateral membrane of the enterocyte (such as ABCC1 and ABCC3) where they constitute a form of cellular defence (74, 76, 77).
1.4.1 Structure of ABC-transporters

The variation in the subunit structure of ABC-transporters is demonstrated in Figure 5. The basic structure, exemplified by that found in P-glycoprotein (ABCB1), is thought to consist of 12 transmembrane segments and two ATP-binding sites in a protein consisting of about 130 amino acids. ABCG2/BCRP functions as a homodimer i.e., the basic structure is assembled from two equal halves (78, 79).

![Figure 5. Predicted topology of three major classes of mammalian ABC-transporters. The intracellular nucleotide-binding domains NBDs, and the N and C termini of the protein are indicated in this simplified scheme. Redrawn from (65).](image)

How ABC-transporters work is not yet known in detail. For instance, there is still doubt about the position and number of substrate-binding sites in ABC-transporters, such as Pgp (65). Nevertheless, several models have been presented for the transport mechanisms (80). However, a recently published crystallographic structure of the bacterial multidrug efflux pump AcrB reveals a large drug binding cavity with separate drug-binding sites for each of the four substrates studied. If the Pgp binding cavity resembles that of AcrB, this could explain the difficulties encountered when attempting to describe the substrate binding process in Pgp (81).

ABCB1/MDR1 Pgp, ABCC2/MRP2 and, more recently, ABCG2/BCRP have come to be considered as potentially important barriers to the intestinal absorption of a large variety of drugs (76, 82, 83). These transporters were, therefore, selected for a more detailed discussion.

### Table 1. Nomenclature for some ABC transporters that are expressed in the human intestine and studied in Paper III.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Conventional name</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ABCB1</td>
<td>Multidrug resistant gene 1 P-glycoprotein</td>
<td>MDR1 Pgp</td>
</tr>
<tr>
<td>ABCC2</td>
<td>Multidrug resistance associated protein 2</td>
<td>MRP2</td>
</tr>
<tr>
<td>ABCC3</td>
<td>Multidrug resistance associated protein 3</td>
<td>MRP3</td>
</tr>
<tr>
<td>ABCG2</td>
<td>Breast cancer resistance protein</td>
<td>BCRP</td>
</tr>
</tbody>
</table>

* Standardized names adopted by the Human Gene Nomenclature committee
  http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html
1.4.2 ABCB1/MDR1 P-glycoprotein

P-glycoprotein (Pgp), encoded by the ABCB1 gene, was discovered by Juliano and Ling (84) and is the most studied ABC drug efflux transporter to date. Proof-of-concept was provided by Sparreboom et al. (85) who tested the fate of orally and i.v.-administered paclitaxel in wild-type (wt) and mdr1a(−/−) mice. This study showed that Pgp in the intestinal epithelium limits the oral bioavailability of paclitaxel, and that it is likely that a similar protection is afforded to many other orally administered compounds that are Pgp-substrates. It was also concluded that intestinal Pgp contribute to the elimination of parenterally administered substrate drugs by a direct secretion of drug into the intestinal lumen.

The most striking property of ABCB1 Pgp include its broad substrate specificity, and the fact that it transports a large number of structurally diverse drugs used in a range of clinical applications (76). Hydrophobicity, planar aromatic rings and the presence of tertiary amino groups favour substrate interaction with Pgp, but no highly conserved elements of recognition have been found (86-88). Recently, pharmacophore models of Pgp substrate affinity have been proposed, generally containing ring aromatic or hydrophobic functionalities as well as hydrogen bond acceptor functional groups (89, 90). Identification of compounds that are ABCB1/Pgp substrates can aid drug candidate selection and optimization. In this respect, ultimately, the generation of 3D-structures of ABC-transporters would help us to understand how the ABC-transporters work, and ideally, to predict when and whether, and if so, how a drug candidate will interact with the transporter in question.

Overexpression of Pgp in cancer cells confers high levels of resistance to Vinca alkaloids, anthracyclines, taxanes, etoposide and a vast number of other compounds (91, 92). Several studies suggest that the physiological role of Pgp is to protect the organism from toxic compounds. This suggestion is partly based on the fact that Pgp is expressed at barriers involved in drug excretion, including the epithelium lining the gastrointestinal tract (66, 93). In addition, evidence is derived from in vivo knock-out mouse models in which the murine orthologue for Pgp has been deleted or disrupted. These mice are considered to be healthy and reproduce normally in a protected environment, but show altered sensitivity to and excretion of compounds that are ABCB1 Pgp substrates (94, 95).

Variations in the expression levels and the activity of ABCB1-encoded Pgp may have a major impact on the therapeutic efficacy of many drugs. As the functionality of Pgp is defined by the amino acid sequence that is encoded by the ABCB1 allele, the ability to detect relevant MDR1 alleles is of potential importance for the treatment of patients with drugs that are substrates of Pgp (96). The first two naturally occurring ABCB1 polymorphisms were described and correlated with possible clinical effects by Mickley et al. (97). More extensive studies followed, with nineteen polymorphisms being identified in Germany (71, 98) and a further nine in Japan (99, 100).

The most interesting mutation, C3435T, is associated with a lower level of ABCB1 expression in duodenum and higher digoxin plasma levels (71), which were suggested to result from a weaker restriction of absorption. However, this finding was opposed by
recent investigations, which also suggested the importance of the C3435T, but the relationship with the phenotype did not always agree with the report by Hoffmeyer et al. (71). Plasma or serum concentrations of digoxin (101, 102), fexofenadine (103) and efavirenz and nelfinavir (104) were lower in subjects with the mutant T/T allele. However, another recent clinical study (Kurata et al., 2002) supported the observations by Hoffmeyer et al. (71). The discrepancy was proposed to be related to e.g., pharmacokinetic profiles of the probe drug used, ethnic differences and mode of administration (102).

The influence on the oral bioavailability of the pharmaceutical formulation and by physiological factors such as gastric emptying rate, and pH in the gastrointestinal fluids were illustrated in a clinical study where the effects of ABCB1 genotype on the duodenal absorption of digoxin were investigated (102). When digoxin was administered as a conventional tablet, delayed absorption caused by disintegration of the tablet, dissolution of digoxin, and by gastric emptying resulted, and no significant influence of the ABCB1 genotype was observed. In contrast, when digoxin was applied directly in the duodenum as a solution, a marked effect of the genotype-dependent serum digoxin levels was observed. Thus, in subjects expressing the T/T allele, the bioavailability of digoxin was markedly reduced (102).

1.4.3 ABCC2/MRP2
ABCC2 transports a large range of organic anions and endogenous compounds, including glutathione-, glucuronide-, and sulfate conjugates. ABCC2 affects the pharmacokinetics of clinically important drugs including cancer chemotherapeutics (irinotecan, methotrexate, vinblastine), antibiotics (ampicillin, ceftriaxone, and rifampin), and angiotensin-converting enzyme inhibitors, as well as many toxins and their conjugates (105). Many of these substrates are common with ABCB1 and ABCG2 (76). ABCC2 is able to confer resistance against e.g., Vinca alkaloids, anthracyclines, mitoxantrone and cisplatin in vitro, but its role in anticancer drug resistance in patients remains to be verified (106, 107).

ABCC2 is not only acting as a protective barrier in the intestine, but also in the liver, kidney, brain and placenta (106). Most importantly, ABCC2 has an important function in the biliary excretion of endogenous compounds such as conjugates of bilirubin, steroids and leukotrienes (65) thus, patients lacking functional ABCC2 have hyperbilirubinemia (105).

1.4.4 ABCG2/BCRP
ABCG2 was first discovered by Doyle et al. because of its overexpression in a highly doxorubicin-resistant breast cancer cell line (108). The range of drugs to which ABCG2 can confer resistance is somewhat more limited than that for ABCB1, but includes mitoxantrone, topotecan derivatives and anthracyclines, but not Vinca alkaloids and taxanes (108, 109). ABCG2 is a half-size transporter that is presumed to require dimerisation to form a functional unit. ABCG2 is highly expressed in the human small intestine, colon, placenta and in the bile ducts of the liver (3, 110).
ABCG2 was recently shown to transport sulfated conjugates of both steroids and xenobiotics; and estrone-3-sulfate (E1S) and dehydroepiandrosterone sulfate were suggested to be the potential physiological substrates for this transporter (111). In addition, ABCG2 has been demonstrated to be expressed in a variety of stem cells and to be a molecular determinant of the side-population phenotype (112).

1.5 INTESTINAL DRUG METABOLISM

Compounds entering the body can be modified by oxidation, through phase I metabolism e.g., by the CYP450 system, and/or made more water soluble by conjugation with glutathione (GSH), sulfate or glucuronic acid through phase II metabolism.

In a survey on the elimination pathways of over 400 drugs marketed in Europe and the United States, it was shown that P450-mediated metabolism represented 55% of the total elimination of these drugs (113). CYP3A appears to be involved in the metabolism of nearly 50% of all the drugs currently prescribed (114, 115). Hence, alteration in the activity or expression of this enzyme is a key predictor of drug responsiveness and toxicity.

Of the 35 P450 enzymes hitherto described in man, only P450’s in families 1, 2 and 3 appear to be responsible for the metabolism of drugs and are, therefore, potential sites for drug-drug interactions. P450 enzymes from other families are generally involved in endogenous processes, particularly hormone biosynthesis (113).

1.5.1 Cytochrome P450 3A

Among adults, CYP3A4 is the dominant CYP3A isoform in human small intestine (2, 116) and liver (117). CYP3A5 is also found in the liver and intestinal mucosa (118, 119) and other extrahepatic tissues (120, 121), but its expression is clearly polymorphic, with some individuals exhibiting relatively high or low levels of the protein (118, 122). It has been suggested that, for most individuals, CYP3A5 only plays a minor role in the first-pass extraction of CYP3A substrates in vivo (113) and CYP3A7 is primarily a fetal enzyme (123). Recently, human CYP3A43 has been identified and cloned (124), although its contribution to hepatic or extrahepatic CYP3A-dependent drug clearance is believed to be negligible (125).

Many CYP3A-substrates undergo substantial metabolism in the mucosal lining of the small intestine, examples being midazolam (6), felodipine (126, 127), verapamil (128), nifedipine (129), tacrolimus (130), saquinavir (131) and cyclosporine (21). CYP3A constitutes only 30% of total human CYP content (132), but accounts for approximately 70% of the CYP content in the human intestine (1, 133, 134). The situation of CYP3A in the villus tip enterocytes (2) renders a very large surface area over which the enzyme can encounter its substrates, thus providing conditions for an extensive metabolism by CYP3A4 in the intestine.
Interindividual differences in the oral bioavailability and systemic clearance of CYP3A substrates can be attributed, in large part, to variable expression of CYP3A in the mucosal epithelium of the small intestine (6, 119) and in the liver (135, 136). This variation is manifested by differences greater than 10-fold in the in vivo metabolism of drugs that are substrates for CYP3A enzymes e.g., (136). This variation can be further enhanced by inhibition or induction of CYP3A4 and may result in clinically important differences in toxicity and pharmacological response.

Genetic factors appear to be of great importance for the interindividual variability in constitutive expression and activity of CYP3A, but the underlying mechanisms remain largely unknown (137). The functional polymorphism of the CYP3A4 gene has been studied, and different CYP3A4 alleles have been found (138). However, these mutations are rare and only a few of them influence function. Thus, variable expression of other members in the CYP3A family or of the human pregnane X receptor (PXR) might influence the overall variability in CYP3A4. It has been suggested that polymorphic expression of CYP3A5 is a significant contributor to CYP3A-dependent drug metabolism in individuals with a high hepatic expression (139), and a single nucleotide polymorphism has been identified, explaining the lack of CYP3A5 in the majority of Caucasian livers. In contrast, however, a more recent study showed that the contribution of CYP3A5 to hepatic CYP3A activity in Caucasians is insignificant. Furthermore, it was concluded that regulatory factors common to the CYP3A4, CYP3A5, CYP3A43 and PXR genes seem to be important for their expression and for the interindividual variability in CYP3A4 (140).

The vitamin D receptor (VDR) was recently identified as a potentially important regulator of intestinal CYP3A4 (18, 141). Interindividual differences in expression of intestinal CYP3A4 could arise from mutations in the VDR (142) and from differences in intestinal VDR content and circulating 1α, 25-dihydroxy vitamin D3 (D3) (18). VDR, D3, and CYP3A4 have been shown to participate in the enteric defence against colon cancer (143).

1.5.2 Phase II metabolism

Several phase II enzymes in the intestine are expressed at levels that are comparable to or exceed those in the liver. Specific forms of phase II enzymes have been identified in the intestine, including glutathione S-transferase A1-1 which may contribute to variable oral clearance of busulfan (144), and dopamine sulfotransferase SULT1A3 (145). The UDP-glucuronosyltransferases (UGT) catalyse the addition of a β–glucuronic acid moiety to a variety of sites (146) e.g., UGT1A1, 1A8 and 1A10 (147-149).

The conjugates formed through phase II metabolism are often too hydrophilic to diffuse out of the cell and therefore require transporters such as members of the ABCC subfamily, to assist their exit. Thus, conjugative enzymes and efflux transporters such as ABCC2 may act synergistically to reduce intestinal absorption of organic anions and conjugates (105). Transport of typical substrates for ABCC2, such as 2,4-dinitrophenyl-S-glutathione and 17β estradiol-17β-D-glucuronide, has been demonstrated in Caco-2 cells (150, 151).
1.6 Functional Interplay Between ABCB1 and CYP3A4

It is believed that intestinal CYP3A4 and ABCB1 act in a concerted manner to control the absorption of their substrates (131, 152-155). This suggestion is based on a considerable overlap in the substrate specificity of the two proteins and the proximity of their expression in the intestine. Furthermore, it was demonstrated that modulators and substrates of ABCB1 and CYP3A up-regulate these proteins in human colon carcinoma cells in a coregulatory manner (67). At the onset of this thesis, it was hypothesized that ABCB1 effectively recycles its substrates over the apical membrane, thereby maximizing the exposure to metabolising CYP3A4 in the intestine, and decreasing the importance of enzyme quantity, Figure 6 (154). Although ABCB1 and CYP3A4 may be functionally coordinated in order to minimize the exposure of the organism to xenobiotics, they appear to be regulated separately (156).

Figure 6. A schematic representation of the events occurring after oral administration of drugs, which are substrates for both ABCB1 and CYP3A4. A drug may be directly effluxed by ABCB1 back to the intestinal lumen, and, if the drug is subjected to CYP3A4-mediated metabolism, the formed metabolite(s) may be effluxed by ABCB1 and/or they may proceed through the basolateral membrane.

Several studies have been performed to test the ‘apical recycling’ hypothesis, using cell culture or rat models. Hochman et al. investigated the metabolism of indinavir in D3-induced Caco-2 cell monolayers, and showed that the amount of metabolite formed per molecule of transported indinavir was higher when ABCB1 was active than when it was inhibited (157). When the metabolism of verapamil across excised rat intestine was evaluated at increasing substrate concentrations, it was found that the cellular residence time and intestinal metabolism were higher at low concentrations of verapamil when ABCB1 was active, than at higher concentrations where saturation had occurred (158). Further, the bioavailability for the cystein protease inhibitor K02 (a dual substrate for CYP3A and ABCB1) increased from 3 to 30% in rats dosed with oral ketoconazole, with no corresponding change in intravenous clearance (159). However, in none of the studies was the relative importance of CYP3A and Pgp on intestinal metabolism clarified, since the experiments were performed at saturation levels and the substrates/inhibitors were not specific for CYP3A and/or ABCB1.

Recently, a rat single-pass intestinal perfusion model was used to demonstrate that the extraction ratio of K77, a dual CYP3A/Pgp-substrate, decreased when ABCB1 was inhibited, while it stayed the same for midazolam, an exclusive CYP3A substrate (160).
The rat data were in agreement with in vitro data from CYP3A4-transfected Caco-2 cells (155). In summary, although a number of studies have been performed to investigate the suggested apical recycling by Pgp further studies are needed. Moreover, support for an in vivo link between enterocytic efflux transport and gut wall metabolism in humans still remains to be provided.

1.7 Regulation of ABC-Transporters and CYP450 Enzymes via Nuclear Receptor Pathways

Many drugs in clinical use have been shown to, directly or indirectly, regulate ABC-transporters as well as CYP450 enzymes through nuclear receptor pathways. Importantly, the orphan nuclear pregnane X receptor (PXR) has been shown to coregulate genes for CYP450 enzymes (e.g., CYP3A4) and ABC-transporters (e.g, ABCB1) in the intestine (19), Figure 7.

![Diagram showing cross-talk between PXR and CAR signaling pathways](#)

**Figure 7.** Cross-talk between the PXR and CAR signaling pathways. Both CAR and PXR are modulated by both xenobiotics and endogenous compounds and bind as heterodimers with RXR to response elements in the regulatory regions of genes encoding proteins involved in metabolism and transport. Other regulatory pathways involving nuclear receptors exist in parallel with those described for PXR and CAR.

For instance, PXR is activated by the anti-cancer drug paclitaxel and induce the expression of both CYP3A4 and ABCB1/Pgp. Since paclitaxel is both metabolised by CYP3A4 and transported by Pgp, induction of these proteins leads to its enhanced clearance (19).

Hyperforin, the active component in the anti-depressant Saint John’s wort (SJW), is a highly potent PXR-activator (161), and has lately gained much attention for its involvement in several severe interactions with orally coadministered drugs. Hyperforin induction of ABCB1/Pgp and CYP3A4 in the intestine have been reported to reduce the oral bioavailability of drugs such as e.g., cyclosporine and digoxin (162, 163).
Rifampicin, another PXR-ligand, substantially reduced the plasma concentrations of orally administered digoxin by induction of intestinal ABCB1 (8). Further, the constitutive androstane receptor (CAR), the glucocorticoid receptor (GR) and the vitamin D receptor (VDR) have been reported to be important transcriptional regulators and coregulators of the ABC-transporter and CYP450 gene expression (164-169). Interestingly, the orphan nuclear receptor hepatocyte nuclear factor4α (HNF4α) seems to be directly involved in PXR and CAR-mediated transactivation of CYP3A4, and may also participate in cell type dependent upregulation of CYP3A4 (17).

However, although a great deal is known about the regulation of basal and induced CYP450 and ABCB1 gene expression, comparatively little is known about how various drugs regulate the expression of other ABC-transporters. For instance, the adaptive response by ABCG2/BCRP to drug exposure has yet to be understood. Further, although it is clear that PXR plays a key role in the induction of Pgp, the inductive effects of a single drug may be mediated by more than one mechanism. Therefore, a detailed knowledge of the inductive patterns for each drug is required before one can understand its implications and consequences. The aim of the second part of this thesis was therefore to study potential effects of repeated drug administration on the gene regulation and activity of different ABC-transporters and CYP3A4 in the human intestine.

1.8 DRUG-DRUG INTERACTIONS INVOLVING INDUCTION OF ABCB1 AND CYP3A4 AT THE INTESTINAL LEVEL

Inhibition and induction of CYP enzymes, particular CYP3A4, are the most common causes documented for drug-drug interactions (170). However, there is an increasing awareness that the pharmacokinetics of drugs that are not subject to metabolism, but to carrier-mediated transport mechanisms such as ABCB1, may have a substantial potential for drug-drug interactions (171). The pharmacokinetic consequences of ABCB1 induction are similar to those observed for induction of CYP3A4, that is, induction of ABCB1 results in a decrease in systemic exposure. Thus, data on how drugs regulate transporter expression are helpful in predicting pharmacokinetics and drug-drug interactions at the transporter level (172, 173).

In the section below, the discussion will focus on drug-drug interactions involving induction of ABCB1. This is followed by a presentation of drug-drug interactions based on induction of CYP3A4 and, finally, interactions involving induction of both ABCB1 and CYP3A4 are considered.

1.8.1 Drug-drug interactions caused by induction of ABCB1

Greiner et al. provided compelling evidence that ABCB1 induction can be the cause of drug-drug interactions in a clinical study comparing the pharmacokinetics of digoxin before and after ten days pre-treatment with rifampin (600 mg daily) in eight healthy volunteers (8). The plasma AUC value of oral digoxin was significantly lower during rifampin treatment while the effect was less pronounced after intravenous administration. The renal clearance and half-life of digoxin were unaltered by rifampin. In addition, the ABCB1 content in duodenal biopsies was increased by a factor of 3.5 by
rifampin, an increase which correlated with the AUC value after oral but not iv administration of digoxin (8). Digoxin is mainly eliminated by renal excretion in humans, and administered orally at a very low dose (0.5-1 mg). It was therefore concluded that the decreased plasma concentration of digoxin was caused by a reduced absorption and bioavailability of digoxin, arising from induction of ABCB1 at the intestinal level.

The effects of rifampin of nine days pretreatment (600 mg daily) on the pharmacokinetics of the P-glycoprotein substrate talinolol, a β1-blocker without appreciable metabolism, but extensive intestinal secretion, was studied in healthy volunteers (174). The AUC values for intravenously and orally administered talinolol were significantly lower (than their control levels during rifampin treatment. A 4-fold increase in the expression of duodenal P-glycoprotein was observed, which was significantly correlated with the total clearance of talinolol. On the basis of these results, Westphal et al. concluded that the talinolol-rifampin interaction is attributable, mainly, to the combination of a decrease in absorption and an increase in elimination from induced Pgp expression in the intestine (174).

The bioavailability of fexofenadine was also decreased by rifampin pre-treatment (600 mg daily for six days) in twenty volunteers (13). The authors assumed that the metabolism of fexofendine is negligible in humans, and suggested that the decrease in the plasma concentration of fexofenadine relied on an induction of intestinal Pgp. However, the basis for this interaction have been claimed to be more complex, involving factors such as metabolism and hepatic uptake by rifampin-induced CYP and OATP, respectively (170, 175, 176).

St John’s wort (SJW) is one of the most popular herbal drugs for treating mild depression without a prescription. Since 1999, there has been a growing concern regarding the clinical significance of drug interactions involving SJW. Hence, the Food and Drug Administration (177) and the Medical Products Agency (178), recently issued a warning about the use of SJW in combination with other drugs. For instance, Durr et al. showed that administration of SJW for 14 days in healthy volunteers resulted in a 18% decrease in digoxin plasma AUC after a single dose (12). The decrease in AUC was accompanied by an increase in the duodenal expression of Pgp. Further, the coadministration of SJW and the HIV protease inhibitor indinavir reduced the systemic exposure of indinavir by nearly 60% (14), the induction of intestinal Pgp by SJW was assumed to play a role in this.

Interestingly, SJW also displayed inhibitory properties. The effect of SJW on Pgp-activity in vivo was recently examined using fexofenadine as a selective Pgp probe. A single dose of SJW resulted in a significant inhibition of intestinal Pgp, while this effect was not observed following repeated administration of SJW (179).

1.8.2 Drug-drug interactions caused by induction of CYP3A4

Midazolam is an established in vivo probe drug for CYP3A4 activity, and there are several examples of interactions involving this compound (180). For instance, two weeks of treatment with SJW (300 mg three times a day) in healthy volunteers
increased the oral clearance of midazolam by more than 50%, while a 20% increase was observed after intravenous administration. Since midazolam is not a substrate for ABCB1, it was concluded that the increase in clearance was caused by induced CYP3A4-levels (181).

**Figure 8.** The molecular basis for a drug-drug interaction involving induction of CYP3A and/or ABCB1. Redrawn from (182).

The effect of enzyme induction on prehepatic and hepatic metabolism of R/S-verapamil after simultaneous oral and intravenous administration of rifampin for 11 days (600 mg daily) was examined by Fromm et al. (128). Rifampin increased the apparent oral clearance of S-verapamil 32-fold and decreased its bioavailability 25-fold. It was concluded that prehepatic metabolism, presumably in the intestinal epithelium, was preferentially induced compared with hepatic metabolism. Other orally administered CYP3A4 substrates that show a similar reduction in AUC during rifampin treatment include nifedipine (129), buspirone (183), and tamoxifen (184).

Certain drugs act as both inhibitors and inducers, an example being ritonavir, for which interactions with CYP3A substrates will be time-dependent. Initial exposure to ritonavir inhibited CYP3A, but as the duration of exposure proceeded, CYP3A was induced. Thus, the clinical outcome after administration of ritonavir is very complex and varies amongst individuals (185).

### 1.8.3 Drug-drug interactions caused by induction of ABCB1 and CYP3A4

Owing to the overlap substrate specificities of ABCB1 and CYP3A4, and because many inducers affect both proteins, many drug-drug interactions may involve both enzyme and transporter systems. The interaction between rifampin and cyclosporine resulted in an increased clearance and decreased bioavailability of cyclosporine A (7). Since cyclosporin A is a substrate for both ABCB1 and CYP3A4, and rifampin is an inducer of both proteins, the basis for the interaction is most likely a combined effect of induced levels of both ABCB1 and CYP3A4. Similarly, coadministration of SJW with cyclosporine has been found to reduce the level of cyclosporine in the blood by 30% to 60% after oral administration; and a number of cases of organ transplant rejection have been associated with this interaction (186, 187).

The effects of administration of SJW for 12 days (300 mg three times a day) on the disposition of three in vivo probe drugs were explored in 21 volunteers (Dresser et al., 2003). Midazolam was used after oral and intravenous administration to assess CYP3A activity in both the intestine and the liver. The disposition of fexofenadine after an oral
dose provided a measure of ABCB1 function; and the oral plasma concentration-time profile of cyclosporine was considered to reflect both CYP3A and ABCB1 activity. SJW markedly affected the disposition of all three drugs. The effect of induction measured as oral clearance was more pronounced on CYP3A activity (midazolam) than on ABCB1 activity (fexofenadine). Despite the fact that the disposition of cyclosporine involves both CYP3A and ABCB1, the change in oral clearance of cyclosporine appeared to be more closely related to the increase in ABCB1-function. The lower than expected effect of SJW on the oral clearance of cyclosporine (as compared with the CYP3A-substrate midazolam) suggested that ABCB1 was the rate-limiting step for the overall intestinal uptake an metabolism (188). In conclusion, one can say that the quantitative aspects of induction of CYP3A4 and ABCB1 are complex and vary from one drug to another.

1.8.4 Clinical relevance

According to the Food and Drug Administration industry guidelines (189), a drug interaction is associated with clinically significant induction when there is a greater than 30% decrease in plasma drug concentrations and this decrease has the potential to alter the drug response. However, although many drug-drug interactions are of clinical relevance, it is important to keep in mind that the usual outcome is that the pharmacokinetic disposition and clinical activity of each drug proceed independently of each other. In the process of deciding what interactions are of real concern in the course of drug therapy, some general guidelines have been applied. That is, drug interactions are more likely to be important when: 1) drug A produces a very large change in the kinetics and plasma levels of drug B, that is, drug A is a potent inhibitor or inducer (e.g., rifampin or ritonavir coadministered with CYP3A-substrates); 2) the therapeutic index of drug B is narrow (e.g., digoxin) (185). Furthermore, the intrinsic pharmacokinetic properties of the drugs involved influence the potential consequences of an interaction.

As discussed in previous sections, the overlap in substrate specificities between ABCB1 and CYP3A4, along with the fact that many inducers affect both proteins, means that many drug-drug interactions may involve both enzyme and transporter systems. However, there is still no straightforward method by which these two systems can be quantified owing to the complexity involved when both intestinal and hepatic CYP3A4 and Pgp and potentially also other ABC-transporters, are being considered. Care should be taken when evaluating the underlying mechanism of drug-drug interactions until the relative contribution of Pgp-transport and CYP3A4-metabolism to the overall interaction can be quantitatively estimated.

Recently, it was argued that the role of transporters as an intestinal barrier to oral bioavailability may have been overemphasized, especially when evaluated in in vitro systems such as the Caco-2 cell model e.g., (170). This argument was motivated by the fact that high secretory efflux over cell monolayers in vitro do not necessarily correspond to an unsatisfactory oral bioavailability in vivo. For instance, although digoxin and cyclosporine show high efflux ratios in vitro, they show sufficiently high oral bioavailability in vivo.
In summary, CYP3A mediated metabolism as an intestinal barrier to oral drug bioavailability and as a cause of important drug-drug interactions is well established. However, although the importance of transporters as an intestinal barrier to oral bioavailability may have been overemphasized, the significance of efflux proteins such as Pgp as a cause for drug-drug interactions is being appreciated and should be considered during drug development. Similarly, the interplay between ABCB1 and CYP3A4 remains a complex issue to study; new methods are required to assist with this. Moreover, ABC-transporters localised to other sites in the body than the intestine, including the liver, kidney, and the blood-brain-barrier and the maternal-fetal barrier, may also have a major impact on the disposition of drugs (65, 170). Studies on the role of ABC-transporters as a cause for drug-drug interactions and reduced drug bioavailability are therefore warranted not only in the intestine, but also in other major organs.

1.9 METHODS FOR STUDYING INTESTINAL DRUG TRANSPORT AND METABOLISM

Studies of intestinal drug absorption and metabolism can be performed using models of increasing complexity in the order in vitro < in situ < in vivo. Detailed mechanistic studies are usually easier to interpret when using the less complex in vitro models and require a much smaller amount of drug, than evaluations done in vivo. However, once a mechanism has been revealed in vitro, its relevance in an in vivo situation must still be shown (190).

1.9.1 Methods for studying intestinal drug transport

In the characterization of intestinal drug transport, a number of experimental approaches can be applied. Simple uptake/efflux studies can be performed in whole cells either adherent or in suspension or in membrane vesicles for a rapid screening of substrates and/or inhibitors involved in drug efflux interactions (191). In such assays, fluorescent probes, that are substrates for well-studied ABC-transporters such as ABCB1 are often used. Examples of such probes are rhodamine-123 (192) or calcein-AM (193). Monitoring ATPase activity in cell membrane preparations or purified membrane proteins represents another method of identifying compounds that interact with ATP-consuming ABC-transporters. This method is readily adapted to high throughput format, and ABCB1 membrane preparations are commercially available for this purpose (193).

![Figure 9](image_url)

**Figure 9.** Schematic representation of an epithelial cell monolayer grown on a permeable support. Cell monolayers used in Paper I-III were cultured on permeable supports coated with a laminin-rich extracellular matrix. The dotted line represents the surfaces of the experimental medium in the apical and basolateral chambers (194).
Drug permeability studies in polarized cell monolayers have been extensively used to determine the effect of drug efflux on the permeability of a drug. A major advantage with this approach is the ability to study the directionality of the efflux. Popular epithelial expression systems for studies of drug transport by a selected ABC-transporter include MDCK and LLC-PK1 cells, which are canine and porcine kidney epithelial cell lines, respectively. These cells require relatively short time in culture and grow as monolayers, thus enabling drug transport studies in both absorptive and secretory directions. MDCK and LLC-PK1 cells transfected with efflux transporters have been used for over a decade, e.g., (195, 196). Double-transfectants with one uptake- and one efflux transporter were recently demonstrated (197, 198). These systems allow for the study of the interplay between uptake and efflux transporters. Wild-type cells are used as controls for the transfected variant. A commonly used alternative to the epithelial expression systems is the Caco-2 cell line, which originates from human colon carcinoma (199) and spontaneously differentiate into tight monolayers. Caco-2 cells express ABC-transporters in amounts comparable to the human jejunum (3), at least at the mRNA level.

In situ intestinal perfusion techniques allow simultaneous measurements of drug transport, secretion and metabolism to be made. Perfusions are generally performed in anaesthetized experimental animals such as rats. An intestinal segment is isolated by surgery, tubes are connected and the segment is perfused with a buffer containing the drug. Since the blood supply is intact in this model, the viability of the tissue is high. In situ perfusions of rat jejunum has been used for simultaneous determinations of passive permeability, carrier-mediated transport, for instance by ABCB1, and metabolism (200-202).

Current transgenic animal models for assessing drug efflux transporter activity include single, double and triple knock-out mouse models (94, 203, 204). These animals are obtained by the removal or silencing of a gene through homologous recombination. In a seminal study by Sparrebom et al. (85), wild-type (wt) and mdr1a(−/−) mice were used to show that Pgp in the intestinal epithelium limits the oral bioavailability of paclitaxel. Although knock-out models are invaluable tools in transporter science (205), removal or silencing of one or more genes will certainly have the potential to affect the organism in different ways. For instance, Schuetz et al. (203) demonstrated compensatory effects in hepatic P-450s and mdr1b following deletion of mdr1a. This must be taken into account when interpreting data obtained from knock-out models.

1.9.2 Methods for studying intestinal drug metabolism
In the screening of phase I biotransformation of a new drug, the preferable sequence is to start with microsomes, then progress to CYP supersomes, followed by the use of stable or transfected cell lines and excised tissue (206). Methods with a higher degree of complexity include in situ perfusion techniques performed in anaesthetized animals (200), and transgenic animal models (207, 208).

Subcellular fractions, supersomes (209) and other sources of artificially expressed human CYP are commercially available and have become an integrated part of the drug discovery process. By using different subcellular fractions it is possible to differentiate
between phase I and II reactions (206). Since microsomes are derived from the endoplasmatic reticulum they are very rich in drug metabolising enzymes such as oxidases like CYPs, and are therefore used for the evaluation of phase I metabolism. Intestinal microsomes can be prepared from different segments of the intestine, thereby enabling regional characterization (119). The cytosolic fraction may used for evaluating certain phase II reactions catalysed by soluble enzymes (206).

A disadvantage with subcellular fractions is that they contain a mixture of enzymes, so it may be difficult to determine which enzyme that catalyses a particular reaction. Thus, the contribution of individual CYPs can be investigated in the presence of specific inhibitors (210), or by using expression systems such as supersomes (209). Supersomes consist of the microsomal fraction of insect cells transfected with the drug metabolizing enzymes of choice. Insect cells lack endogenous CYP expression, and nontransfected insect cells can therefore be used as controls.

Below, the two methods used for the drug transport and metabolism studies in this thesis are presented.

1.9.3 Cell culture models for the assessment of passive drug transport and drug efflux

Caco-2 cells grown as monolayers on permeable supports are extensively used in studies of both passive and active drug transport. Studies in this cell culture model are easily performed under controlled conditions, which makes it possible to extract detailed mechanistic information on the various transport processes (50, 194). Since Caco-2 cells are unusually well differentiated, they are functionally similar to the human small intestinal enterocyte in many respects, despite the fact that they originate from a human colorectal carcinoma (30, 199, 211). A variety of transport systems and enzymes are expressed in Caco-2 cells (3, 52, 212). Thus, the Caco-2 cell model may be considered as a multifunctional transport model that allows the study of the interplay between different e.g., ABC-transporters, and also of the relative active and passive contributions to the overall transport.

However, the simultaneous expression of several parallel transport and metabolism processes is also a drawback since: 1) the expression levels in the cell lines do not always mimic the in vivo counterparts, and 2) the study of a single transport or metabolic pathway in a multifunctional system require that specific substrates/inhibitors are available, which is not always the case. Therefore, when various transporters are to be studied in isolation, they are usually expressed in cell lines or other expression systems with a lower background than, for instance, Caco-2 cells.

1.9.4 Cell culture models for simultaneous studies of drug transport and CYP3A4 metabolism

In principle, three approaches have been used to develop intestinal cell models for studies of CYP3A4-mediated drug transport and metabolism: 1) use of the CYP3A5 expressing Caco-2 clone TC7 as a substitute for CYP3A4 expression (213) 2) development of CYP3A4-expressing Caco-2 cells using heterologous expression techniques (214-216)
and 3) 1α,25-dihydroxy vitamin D₃-mediated induction of CYP3A4 in the Caco-2 cell line (157, 217). The third approach is the most physiological system, in which endogenous CYP3A4 is hormonally upregulated and therefore that system was selected for the studies in this thesis.

In its original version, the D3-induced Caco-2 cell model is based on a specific Caco-2 clone, which requires 14 days growth in a complex differentiation medium, in which D3 is the supplement responsible for the upregulated CYP3A4 expression and activity (217). It was reasoned that a more accessible model, based on a widely available cell line such as the parental Caco-2 cells, would find wider application.

1.9.5 In vivo methods for studying transport and metabolism

Direct measurements of intestinal absorption, metabolism and secretion of drugs in humans in vivo may be performed using regional perfusion techniques. Perfusions of the proximal part of the human jejunum have now been successfully performed using a large number of drugs by a few laboratories e.g., (218-220). By collecting shed enterocytes during the single-pass perfusion, analysis of the expression and function of human transporters and enzymes may be combined with the determination of intestinal transport and presystemic metabolism (219, 221).

**Figure 10.** The Loc-I-Gut® instrument consists of a multilumen tube that is introduced from the mouth. Two ballons occlude a 10 cm long intestinal segment, which is perfused with a buffered solution of the drug. Redrawn from Lennernas et al. (218).

In this thesis, the Loc-I-Gut® technique (218), shown in Figure 10, was used to investigate the effect of repeated oral administration of St John’s wort (SJW) on jejunal transport and presystemic metabolism of R/S-verapamil. By combining measurements of jejunal permeability and of plasma concentrations of drug and metabolite before and after SJW administration, conclusions could be drawn on the potential interaction between St John’s wort and R/S-verapamil.
2. AIMS OF THE THESIS

The overall aim of this thesis was to study the role of intestinal barriers to oral drug bioavailability. Specific aims were to:

- establish and characterize a cell culture model of the human intestinal epithelium for use in studies of ABCB1-mediated drug efflux and CYP3A4-mediated drug metabolism in vitro (Paper I)

- use the new cell culture model to study enantioselective efflux and CYP3A4-mediated metabolism of R/S-verapamil (Paper II)

- study the effects of repeated drug administration on the gene regulation of ABC-transporters and CYP3A4 in human intestinal cell lines in vitro (Paper III)

- explore the effects of repeated oral administration of the herbal drug St John’s wort on the intestinal absorption and presystemic metabolism of R/S-verapamil in humans (Paper IV)
3. MATERIALS AND METHODS

3.1 DRUGS AND RADIONUCLIC MARKERS

Testosterone, 6β-hydroxytestosterone, clotrimazole, 9-cis-retinoic acid, dexamethasone, rifampicin, verapamil, R-methoxyverapamil, tetrahexylammonium, 17β-estradiol, hyperforin, digoxin and mitoxantrone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1α,25-dihydroxy vitamin D3 (D3) was purchased from Solvay Duphar (Weesp, the Netherlands). Phenobarbital was obtained through Apoteket AB, Sweden. The hydrochloride salts of racemic verapamil and racemic norverapamil, respectively were kindly provided by Knoll AG (Darmstadt, Germany). GF120918 was a generous gift from Glaxo Wellcome (Hertfordshire, UK). Androstenedione and 2β-hydroxytestosterone were kindly provided by Dr. Tommy B Andersson (AstraZeneca, Möln达尔, Sweden), and the recombinant human growth hormone was a gift from Dr. Jonas Fransson (Pharmacia Corp., Stockholm, Sweden). [14C]celiprolol (37.3 μCi mg⁻¹) and [14C]mannitol (52 mCi mmol⁻¹) were purchased from Rhone-Poulenc Rorer Pharmaceutical Inc. (Collegeville, PA, USA), and New England Nuclear (Boston, MA, USA), respectively. [3H]digoxin (17.0 Ci/mmol) and [3H]mitoxantrone (3.0 Ci/mmol), were obtained from Perkin Elmer Life Sciences (Sweden) and Biochemicals Larodan (Sweden), respectively.

In the in vivo study (Paper IV), racemic verapamil hydrochloride (120 mg/L, 244 μM, Knoll AG, Darmstadt, Germany) and St John’s wort tablets (300 mg, Movina®, Boehringer Ingelheim, Germany) were used. A low perfusate concentration (10 mg/L, 53 μM) of antipyrine (Astra Läkemedel AB, Södertälje, Sweden) was used as a marker for passive transcellular diffusion in all of the perfusion experiments. 14C labelled polyethylene glycol 4000 (14C-PEG 4000) (2.5 μCi/L, Amersham Pharmacia Biotech, Little Chalfont, England) was used as a non-absorbable volume marker.

3.2 CELL CULTURE (PAPER I-III)

Three cell lines (BN, LG and WT) originating from biopsies taken from healthy human duodenum were generous gifts from Dr Gerald Pang, cultured according to (222) and used at passage numbers 17-30. The parental population of Caco-2 cells, Caco-2p (199), and the morphologically well-differentiated Caco-2 clone, BB-1 (223), were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA), while the CYP3A-expressing Caco-2 clone TC7 was a generous gift from Dr. Alain Zweibaum (224). The Caco-2 cells were cultured as described in detail previously (211, 225).

Caco-2 cells were used at passage intervals 20-30 and 92-105, while TC7 and BB-1 were used at passage intervals 22-30 and 48-58, respectively. In Paper I and II, the cell lines were seeded at a density of 400 000 cells/cm² onto Transwell cell culture inserts (filter diameter 24 mm, mean pore size 0.45 μm, Costar, The Netherlands) coated with a mixture of extracellular matrix proteins (Matrigel, 15 μg/cm²). At confluency, the culture medium was supplemented with 0.5 μM of D3 (217).

The LS180 cell line (226) was also obtained from ATCC, cultured at 37°C, 10% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 1% sodium pyruvate, 1% L-glutamine, and 1% non-essential amino acids and used at
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passage 39 to 43. In Paper III, Caco-2p and LS180 cells were seeded at a density of 150,000 cells/cm² in plastic culture dishes (12-well Transwell plates) coated with Matrigel 15 μg/cm². The cell culture medium was replaced with fresh medium ± inducer every second day and always 24 h before an experiment. The exposure to the drugs took place at concentrations recommended previously in published cell culture studies (67, 167, 227).

3.3 REAL-TIME QUANTITATIVE PCR ANALYSIS (PAPER I AND III)

Total-RNA was isolated using the RNeasy mini kit (QIAGEN) according to the instructions provided by the manufacturer, with an additional on-column DNase treatment step. RNA was quantified using the RiboGreen reagent from Molecular Probes. Quantitative real-time PCR assay of transcripts was carried out using gene-specific double fluorescence labelled probes and the TaqMan® Universal PCR Master Mix in a 7700 Sequence Detector (PE Applied Biosystems, Norwalk, CT). 6-Carboxy fluorescein (FAM) was used as the 5’ fluorescent reporter while quenching tetramethylrhodamine (TAMRA) or non-quenching major groove binding (MGB) probes were added to the 3’ end. Gene-specific primers for ABCB1, ABCC2, ABCC3, ABCG2, CYP3A4, CYP3A5 and CYP2B6 (FAM) were obtained from PE Applied Biosystems (Foster City, CA). For primers and probe sequences, see Paper I and III. To account for variations in the amount of RNA added to each reaction and for variations in the efficiency of the reverse transcription, data were normalized against the number of human acidic ribosomal phosphoprotein (huPO) or 18S rRNA transcripts in each sample. The relative fold of induction was calculated using the comparative CT method (‘‘CT, Paper I) or the standard curve method (Paper III), using huPO or 18S rRNA, respectively, as the endogenous control reference in Paper I and III, respectively. The untreated samples were designated the calibrator and assigned the value of 1x for each of the CYP or ABC-transporter targets. The quantity of each mRNA in each treated sample was given relative to the calibrator sample.

3.4 IMMUNOBLOT ANALYSIS (PAPER I)

In Paper I, cell lysates from Caco-2 and TC7 cell monolayers were resuspended in a sodium dodecyl sulfate reducing buffer and heated for 5 min at 95 ºC. The samples underwent electrophoresis on a 12% SDS-polyacrylamide gel and were transferred to nitrocellulose membranes, which were sequentially incubated with a monoclonal antibody to human CYP3A4 (Xenotech LLC, Cambridge, KS, USA) and an alkaline phosphatase conjugated anti-rabbit IgG. The immunoblots were then visualised using the CDP-Star™ Western Blot Chemiluminescence Reagent (NEN™ Life Science Products, Inc.).

3.5 IN VITRO DRUG TRANSPORT AND EFFLUX STUDIES (PAPER I AND II)

The in vitro drug transport experiments in Papers I and II were performed as described previously (211, 225). Briefly, the cell monolayers were kept at 37°C and agitated on a plate shaker throughout the experiment. Samples were taken from the receiver chamber.
at given times and the extracted volume was compensated with inhibitor solution. At the end of the experiment a sample was drawn from the donor chamber. Drugs were dissolved in Hanks’ Balanced Salt Solution (HBSS, pH 7.4). The integrity of the monolayers was routinely checked with the hydrophilic integrity marker $[^{14}\text{C}]$-mannitol and by measuring the transepithelial electrical resistance. In order to detect any significant carrier-mediated transport of the compound, and to evaluate any possible effects of D3 on transport protein functionality, the R/S-verapamil and $[^{14}\text{C}]$celiprolol transport rates were determined in both apical-to-basolateral (a-b) and basolateral-to-apical (b-a) directions in the untreated (control) and D3-treated monolayers. The efflux activity was expressed as the ratio b-a/a-b. Inhibition of carrier-mediated transport across both control and D3-treated Caco-2 cell monolayers was investigated after the addition of inhibitors to the apical and basolateral side.

The uptake and efflux of $^3\text{H}$-digoxin and $^3\text{H}$-mitoxantrone in Paper III were studied in Caco-2 and LS180 cells grown in Matrigel-coated wells and exposed to hyperforin, GF120918 or vehicle for 5 days. After an initial 30 min washing step at 37°C, the cells were incubated for 30 min at 37°C with the respective drug solution. The cells were then placed on ice and rinsed three times in ice-cold PBS. Efflux medium containing unlabelled drug was added, and at regular time intervals, samples were removed. Finally, the cells were lysed and samples were taken again for scintillation counting and for protein analysis.

### 3.6 IN VITRO DRUG METABOLISM STUDIES (PAPER I AND II)

In Paper I, the formation of 6β-hydroxy testosterone from testosterone was used as a measure of CYP3A activity in filter-grown Caco-2 and TC7 cell monolayers after exposure to D3.26 The experiments were performed at 37°C in HBSS, pH 7.4. Uninduced cell monolayers were used as the controls. The metabolite 6β-OH-testosterone was analysed by a reversed phase HPLC method modified from Kostrubsky et al. (228).

In Paper II, the level of CYP3A4-mediated metabolism of R/S-verapamil was determined in CYP3A4-expressing (D3-treated) Caco-2 cell monolayers, using untreated cells as controls. The cell monolayers were washed and equilibrated 15 min in pre-warmed HBSS, pH 7.4. Thereafter, racemic verapamil dissolved in HBSS was added to the apical side of the monolayer, while HBSS without the drug was added to the receiver side. The monolayers were incubated at 37°C for up to 240 min depending on whether time- or concentration-dependent metabolic studies were performed. The concentrations of verapamil and the metabolite norverapamil in apical, basolateral and intracellular samples were quantified by an enantioselective HPLC-analysis described below.

### 3.7 IN VIVO SINGLE-PASS PERFUSION OF THE HUMAN JEJUNUM (PAPER IV)

The study described in Paper IV was performed at the Clinical Research Department, University Hospital, Uppsala, Sweden and was approved by both the Ethics Committee of the Medical Faculty, Uppsala University and the Swedish Medical Product Agency,
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Uppsala, Sweden. The study consisted of two sequential parts, a control and a treatment phase. In the control phase, a regional single-pass perfusion of the proximal jejunum with R/S-verapamil was performed in eight healthy male volunteers using the Loc-I-Gut® perfusion instrument, figure 10. The subjects had fasted overnight (10 hours) when they arrived at the clinic. After local anesthesia with lidocaine (Xylocain®, AstraZeneca, Södertälje, Sweden) in the upper throat, the tube was introduced orally and positioned in the proximal part of the jejunum by fluoroscopic control. The two balloons were inflated with approximately 26-30 ml of air, creating a 10 cm long jejunal segment. To prevent nausea, gastric juice was drained by a separate tube positioned in the stomach. The jejunal segment was continuously perfused with the test solution containing 120 mg/L racemic verapamil at a flow rate of 2.0 mL/min. The total dose of verapamil given in each 100 minute perfusion experiment was 24 mg. Perfusate leaving the jejunal segment was collected and the drug absorption process was terminated by rinsing the segment with isotonic saline immediately after the perfusion. Venous blood samples were collected immediately before and at intervals until 12 hrs after the start of the perfusion.

The treatment phase was initiated following a washout period of four weeks. Each subject was treated orally with St John’s wort for 14 days (300 mg three times a day) before undergoing a procedure identical to that described for the control phase. In order to prevent any acute inhibitory effects of SJW during the absorption phase, the last dose of St John’s wort was scheduled for the day before the second jejunal perfusion experiment.

3.8 ENANTIOSELECTIVE HPLC ANALYSIS OF R/S-VERAPAMIL AND R/S-NORVERAPAMIL

3.8.1 In vitro cell culture samples and jejunal perfusates (Paper II and IV)
R/S-verapamil and R/S-norverapamil in samples from in vitro transport and metabolism experiments in HBSS, pH 7.4 (Paper II) and from jejunal perfusion experiments in perfusion buffer, pH 6.5 (Paper IV) were quantified by enantioselective reversed phase HPLC with fluorescence detection using a modified method from Sandström et al. (229). The analytical column was a Chiral-AGP® (150 x 4.0 mm I.D.). R- and S-verapamil and R- and S-norverapamil were eluted with phosphate buffer (pH 6.5; ionic strength 0.01)/acetonitrile/methanol (74/18/8 v/v) at a flow rate of 1.0 mL/min. To determine intracellular amounts of verapamil and norverapamil in Caco-2 cell samples, intact cell monolayers were washed in ice-cold PBS, cut and frozen. R/S-verapamil and norverapamil were extracted in ethanol and R-methoxyverapamil was added as an internal standard. After reconstitution in the mobile phase, samples were analyzed as above.

3.8.2 Plasma samples (Paper IV)
In Paper IV, the plasma sample preparation was performed using liquid-liquid extraction as described by Hedeland et al. (230). Analysis was performed with LC-MS/MS in the Selected Reaction Monitoring (SRM) mode, using a Chiral-AGP® (150 x
4.0 mm I.D) analytical column, and a mobile phase consisting of (pH 7.4 aqueous ammonium acetate buffer/acetonitrile (85/15 v/v). The LC-MS/MS system consisted of an Agilent 1100 liquid chromatograph and a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK).

3.9 DATA ANALYSIS

3.9.1 Apparent permeability coefficients (Paper I and II)

The apparent permeability coefficient (Papp, cm/s) of mannitol and celiprolol was determined according to the following equation:

$$P_{app} = \frac{K \cdot V_r}{A}$$  \hspace{1cm} (2)

where K is the time-dependent steady state rate of change in concentration in the receiver chamber (Ct/C0), Ct is the concentration in the receiver compartment at the end of each time interval, C0 is the initial concentration in the apical chamber for each time interval (mole/ml), Vr is the volume of the receiver chamber (ml) and A is the surface area of the filter membrane (cm²). For rapidly transported compounds such as verapamil and norverapamil, for which sink conditions could not be maintained during the experiments in Paper II, the apparent permeability coefficient (Papp) was calculated using the non-sink condition analysis described previously (231).

3.9.2 Absorption variables from jejunal perfusate data (Paper IV)

Calculations were performed from steady-state concentrations in the outlet jejunal perfusate (Paper IV). The concentration of each compound in the perfusate leaving the segment was corrected for water flux in the subsequent calculations of the fraction of drug absorbed (fabs), and the effective permeability (Peff). The fraction of the drug absorbed in the segment during the perfusion (f_{abs}) was calculated according to (232):

$$f_{abs} = 1 - \frac{C_{in} \cdot PEG_{in}}{C_{out} \cdot PEG_{out}}$$  \hspace{1cm} (3)

where C_{in} and C_{out} are the concentrations entering and leaving the jejunal segment, respectively, while PEG_{in} and PEG_{out} are the concentrations of 14C-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

$$P_{eff} = \frac{C_{in} - C_{out}}{C_{out}} \cdot \frac{Q_{in}}{2\pi r L}$$  \hspace{1cm} (4)
where the cylindrical area representing the jejunal segment (2πrL) was calculated using the intestinal radius (r = 1.75 cm). The calculation of $P_{eff}$ is based on the disappearance of drug from the jejunal segment, and is therefore not dependent on the mechanisms involved in the drug transport across the intestinal epithelium (26). The appearance of each enantiomer of the metabolite in the perfusate was expressed as the appearance ratio (Ar) for each enantiomer.

$$Ar = \frac{C_{out}(\text{norverapamil})}{C_{in}(\text{verapamil}) \cdot f_{abs}}$$ (5)

where $C_{out}$ (norverapamil) is the concentration of R- or S-norverapamil in the perfusate leaving the segment, and the product $C_{in}$ (verapamil) $f_{abs}$ represents the amount of verapamil enantiomer absorbed in the segment (233).

3.9.3 Pharmacokinetic analysis of plasma data (paper IV)

In Paper IV, the area under the plasma concentration time curve from time zero to infinity ($AUC_{0-\infty}$) corresponding to the enantiomers of verapamil and norverapamil was calculated for each subject using the linear and the logarithmic trapezoidal rules for ascending and descending plasma concentrations, respectively, up to the last time point. The plasma AUC beyond the last point in time to infinity was estimated by dividing the predicted plasma concentration at the last point by the calculated terminal elimination rate constant, $k$. The linear trapezoidal method was used to calculate the plasma AUC up to 100 minutes ($AUC_{0-100}$). The (apparent) terminal half-life ($t_{1/2}$) was obtained from $k$. The maximum plasma concentration ($C_{max}$) of verapamil and norverapamil, and the time at which it occurred ($t_{max}$), were obtained by visual inspection.

3.9.4 Statistical analysis

The differences between two mean values were analyzed with either a two-tailed paired or unpaired Student’s t-test, as appropriate. For multiple comparisons, a one-way analysis of variance was conducted, followed by Tukey’s, Dunnett’s or Newmann-Keuls’ post test, as appropriate. After a variance stabilizing logarithmic transformation, the plasma pharmacokinetic data in Paper IV was analyzed by analysis of variance using the SAS statistical package (SAS Institute Inc., PROC GLM). Differences between the control and treatment phase and the enantiomers of norverapamil and verapamil, were estimated and then transformed back to the original scale. Values are expressed as means ± one standard deviation (SD) if not stated otherwise. P-values of < 0.05 were considered to be significant.
4. RESULTS AND DISCUSSION

4.1 CYP3A4, CYP3A5, AND MDR1/ABCB1 IN HUMAN SMALL AND LARGE INTESTINAL CELL LINES

The aim of Paper I was to develop a cell culture model that would allow investigation of the relative contribution of and interplay between CYP3A, preferably CYP3A4, and MDR1 (ABCB1) in studies of intestinal drug uptake and gut wall metabolism.

At the onset of the work conducted for this thesis, the 1α,25-dihydroxy vitamin D₃ (D₃)-induced Caco-2 cell model presented by Schmiedlin-Ren et al. (217) had not been fully characterized and could, therefore, be improved upon. For instance, Caco-2 cells originate from the colon, where the expression of CYP is lower than in the small intestine and may be differently regulated (157). In an attempt to obtain a cell model with higher CYP3A4 expression initial studies on three cell lines isolated from the human duodenum were performed (222). These cell lines were the first monolayer-forming intestinal epithelial cell lines to be obtained from healthy human intestine. Analysis of the CYP3A4, CYP3A5 and MDR1 mRNA expression in the duodenal cell lines and in three cell lines of human colonic origin revealed that the two duodenal cell lines BN and LG expressed CYP3A4 and two colonic Caco-2 cell lines (parental Caco-2, Caco-2p, and Caco-2 TC7) expressed CYP3A5. These four cell lines were investigated to determine their monolayer formation on ECM-coated permeable supports.

The two duodenal cell lines BN and LG formed cell layers with extremely high and/or variable TEER values. Transmission electron microscopy (TEM) photographs showed that the BN cells formed tight monolayers of cuboid, partially differentiated epithelial cells with a few small patches in which bilayers were formed. In contrast, LG cells formed continuous bi- to multilayers, making this cell line unsuitable for transport studies.

Cell lines forming monolayers with constitutive expression of CYP3A and MDR1 were induced with D₃. CYP3A in the duodenal cell line BN was not induced by D₃, and was therefore excluded from further studies. One reason for the low expression of CYP3A4 may be the crypt origin of these cells since, the expression of CYP3A4 follows a gradient along the crypt-villus axis from low expression in the mid-crypts to high expression on the villus tips (234). However, exposure of both Caco-2p and TC7 cells to D₃ in monolayers resulted in strongly induced CYP3A4 expression, both at the mRNA and protein levels. In addition, MDR1/ABCB1 mRNA levels were increased by D₃.

CYP3A5 has been shown to display a comparable catalytic activity towards several CYP3A4 substrates (235), and the two isoenzymes may also produce different product ratios from the same substrates (1). Thus, expression of CYP3A5 is not ideal in a cell model designed for studies of CYP3A4 metabolism. Preliminary studies of a series of Caco-2 clones revealed that the different clones (TC7, BBBe-1, clone 12, and Caco-2p of low and high passage numbers) displayed highly variable expression of CYP3A4 and CYP3A5 mRNA both before and after D₃ treatment.
For instance, the strongest D3-induction of CYP3A4 was observed in Caco-2p at high passage numbers (used throughout Paper I-III). Unexpectedly, CYP3A5 mRNA was markedly induced in both Caco-2p and TC7 cells. Since the increase of CYP3A5 was less pronounced than that of CYP3A4 in the high passage Caco-2p cells, we identified this cell line as having the most advantageous CYP3A4/CYP3A5 ratio.

Figure 11. Concentration-dependent CYP3A metabolic activity in a) Caco-2 and b) TC7 control (■) and D3-treated (♦) cell monolayers measured as 6β-hydroxylation of testosterone. Cell monolayers were incubated for 120 min in HBSS containing 0-200 μM testosterone. Samples from the apical and basolateral sides were pooled. Each value represents the mean ± SD of three cultures.

Human CYP3A5 has not generally been considered to be an inducible enzyme (118, 236), and there is now evidence that CYP3A5 lacks the distal PXR response element responsible for enhanced transcription of CYP3A4 (237). However, Schuetz and colleagues were able to induce CYP3A5 in the human intestinal cell line, LS180, using clotrimazole and reserpine as inducing agents (67). The discrepancy regarding CYP3A5 induction might be due to tissue-specific regulation (17, 238).

Certain Caco-2 cell clones have been reported to partially loose their integrity after D3 treatment (217, 239), which may bias the interpretation of transport studies in D3 induced cells. Such a large reduction in integrity will seriously affect the permeability of the Caco-2 cell monolayers to low permeability drugs (240). In Paper II, it was found that the D3-treated Caco-2p cell line retained monolayer integrity, suggesting that this polyclonal cell line is superior for transport studies in CYP3A4-expressing cell monolayers. Clone-specific effects (e.g., a loss of integrity after D3 induction) would be evened out in a polyclonal cell population, while they would be readily detectable in isolated subclones.
Figure 12. Effect of D3 treatment on Pgp activity in the Caco-2 cell line. The cumulative fraction of celiprolol transported across a) control and b) D3 treated Caco-2 monolayers was determined in the apical-to-basolateral (●) and the basolateral-to-apical (□) directions, respectively. D3 treatment resulted in a small increase in Pgp activity. Each value represents the mean ± SD of three cultures.

Caco-2p and TC7 both expressed functional CYP3A4 after D3 induction (Fig 12). Thus, after D3 induction, the rate of 6β-hydroxylation of testosterone in Caco-2p cell monolayers increased from below the level of detection to 149±12 pmol/min/mg protein, Figure 11a and in TC7 cells from 3 to 86±4 pmol/min/mg protein, Figure 11b. The 6β-hydroxytestosterone formation rate was comparable to results reported previously for D3 induced Caco-2 cells or stable CYP3A4 expression systems (157, 214). Functional MDR1 Pgp expression in Caco-2p cells, displaying the most advantageous CYP3A4/CYP3A5 ratio, was indicated by celiprolol permeability being five-fold higher in the secretory direction than in the absorptive direction. This efflux ratio was only slightly affected by the D3-treatment, Figure 12.

In conclusion, the absence of CYP3A4 function in uninduced Caco-2p cells as compared to TC7 cells makes the Caco-2 cell line more suitable for studies of intestinal CYP3A4 metabolism, since the CYP3A4 expression can be turned on and off. Caco-2p cells also showed less variability in CYP3A4 activity than the TC7 cells. D3-induced Caco-2p cells were therefore selected for the studies of concomitant drug transport and metabolism.

4.2. ENANTIOSELECTIVE TRANSPORT AND CYP3A4-MEDIATED METABOLISM OF R/S-VERAPAMIL IN CACO-2 CELL MONOLAYERS

In Paper II, the cell culture model established in Paper I was used to simultaneously investigate the enantioselective aspects of both transport and metabolism in Caco-2 cells, applying clinically relevant concentrations of a commonly used and well-studied drug, R/S-verapamil. Verapamil was chosen as the model drug, since at the onset of the study, the following information was available: After oral administration, the
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The absorption of both enantiomers of verapamil in the human intestine was complete and weakly concentration dependent (229, 233). First-pass gut wall metabolism of verapamil was reported to be responsible for as much as 50% of the total first-pass extraction (128, 219, 233), involving a number of enantiospecific human CYP450 enzymes (241, 242). Norverapamil, one of the major metabolites, is formed predominantly via CYP3A4 (243). Thus, this extensive information about the in vivo behaviour of this drug made comparison between cell culture, rat and human possible.

In both untreated and D3-treated Caco-2 cells, the permeability to verapamil and norverapamil was high and only weakly concentration dependent. The permeability was neither enantioslective, nor affected by the D3-induced CYP3A4-activity in the cells. Studies on directional transport resulted in low (less than two) b-a/a-b efflux ratios in D3-treated cells for both R- and S-verapamil, illustrating that carrier-mediated transport is of minor quantitative importance in the absorption of verapamil in the cell cultures too. The efflux ratios were brought close to unity when inhibitors of Pgp, MRP, as well as OCT were applied. Similarly, the transport of R- and S-norverapamil was predominantly passive, but due to lack of material, no inhibition studies were performed with this compound.

The passive permeability to verapamil in human jejunum has been shown to be about three times higher than that observed in Caco-2 cell monolayers, Figure 13. One major reason for the very high permeability in unanesthetized humans is the presence of the systemic blood flow, which makes a significant contribution to the “sink conditions”. In addition, the intestinal motility in humans in vivo reduces the aqueous boundary layer significantly. This hypothesis is supported by experiments performed after Paper II had been concluded. In this study, the verapamil transport experiments were repeated, using the highest possible stirring rate and 4% of human serum albumin as a ‘sink’ in the receiver chamber. This modification resulted in a several-fold increase in verapamil permeability (Lucia Lazarova, unpublished results).

![Figure 13. Permeability to R- and S-verapamil (VL) in Caco-2 cell monolayers in comparison to data from the literature on human and rat jejunal permeability at a) 8 μM and b) 80 μM racemic verapamil. Values from Caco-2 experiments are shown as the mean ± SD from experiments performed in triplicate. Human and rat permeability data were obtained from human in vivo and rat in situ jejunal perfusions (229, 244).](image-url)
In contrast, the permeability to verapamil in Caco-2 cell monolayers and in rat jejunum were of similar magnitude, although, at some concentrations, the Caco-2 cell permeability was more than twice as high (Figure 13). The relationship between Caco-2 and rat permeability has been demonstrated to be valid for several other rapidly transported compounds (245).

CYP3A4-mediated demethylation of R/S-verapamil to R- and S-norverapamil in D3-treated Caco-2 cell monolayers was enantioselective, i.e., S-norverapamil formation was higher than that of R-norverapamil, Figure 15, and the levels of R- and S-norverapamil formed constituted 2.8% and 3.4% of the apically applied dose, respectively. The distribution of verapamil and the metabolite formed was comparable for the apical and basolateral sides, which is in accordance with previously reported data (246). The conversion of verapamil to norverapamil was regarded as a CYP3A4-specific reaction, since norverapamil was not formed in untreated Caco-2 cells lacking CYP3A4-activity (247). The CYP3A4-mediated metabolism of verapamil in the human jejunum (229) was significantly higher than that in Caco-2 cells, illustrating a higher level of CYP3A4 expression (3) and activity in vivo (128, 219).

In conclusion, this study shows that the enantioselectivity in the CYP3A4-catalyzed reactions clearly demonstrated a qualitative correlation between the Caco-2 cell model and the human jejunum. The evaluation of this cell culture model against human and rat jejunal data makes it particularly useful in studies of drug transport and metabolism in the preclinical evaluation of new drugs. Since passive transport mechanisms dominate for both verapamil and norverapamil at the clinical concentrations applied in this investigation, it was not possible to study the interplay between drug efflux and metabolism in greater depth.

Figure 14. Concentration-dependent demethylation of R/S-verapamil to a) R- and b) S-norverapamil (NV) in 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.
4.3 **Drug-induced gene regulation and function of ABC-transporters in human intestinal epithelial cell lines**

In Paper III, the gene regulation and function of the four ABC-transporters expressed in the human jejunum (ABCB1, ABCC2, ABCC3, and ABCG2) were studied. The effects of repeated exposure to a panel of drugs that are known to interact with a variety of nuclear receptors such as PXR, VDR and CAR, were evaluated in the two human intestinal epithelial cell lines, Caco-2 and LS180. These two cell lines differ in that Caco-2 cells express VDR and CAR (164, 248), but not PXR (18), whilst LS180 cells express VDR and PXR (18), but not CAR (164). Studies of the regulation of CYP3A4 and CYP2B6, known to be coregulated through cross-talk between PXR and CAR signaling pathways (167, 169), were used as a control of the transcriptional regulation in the two cell lines.

**ABC-transporter and CYP expression in untreated intestinal epithelial cell lines**

ABCB1, ABCC2, ABCC3 and ABCG2 were expressed in both Caco-2 and LS180 cell lines. For Caco-2 cells, an increase in transcript levels was observed with time in cell culture for ABCC2 and ABCC3, while the expression of ABCB1 and ABCG2 remained relatively constant over time, Figure 15. The expression of the four transporters was much lower in LS180 cells and no clear time dependence was observed. None of the two investigated CYPs were found in the untreated cell lines.

**CYP450 expression in intestinal epithelial cells after repeated drug exposure**

D3-treatment, previously shown to have a pronounced effect on the expression CYP3A4 in intestinal epithelial cells, served as a positive control for the induction capacity in the two cell lines (217, 249), Paper I. CYP3A4 was strongly induced by D3 (a ligand for VDR) in both cell lines, and in LS180 cells also by the PXR activators rifampicin and hyperforin. These results were in agreement with the presence of VDR in both cell lines, and the presence and absence of PXR in LS180 and Caco-2 cells, respectively (18, 248, 250). CYP2B6 was upregulated by the CAR-activator phenobarbital (251) in Caco-2, but not in LS180 cells, which is in agreement with the presence and absence, respectively, of CAR.

**Figure 15.** Changes in ABCB1, ABCC2, ABCC3 and ABCG2 transcript levels with time after seeding in untreated Caco-2p (left), and right in LS180 cells. Data are shown as mean values ± S.D.
Effect of drug exposure time on ABC-transporter expression

In general, ABCB1 and ABCG2 but not ABCC2 and ABCC3 were upregulated following repeated exposure to drugs that activated the various transcriptional pathways. Phenobarbital, which has been shown to activate both CAR and PXR pathways (167, 169), increased the expression of both ABCB1 and ABCG2 significantly in Caco-2 cells. Since CAR has been shown to regulate the basal expression of ABCB1, the upregulation of ABCB1 by phenobarbital could be mediated by CAR. This is supported by that phenobarbital upregulated CYP2B6 transcript levels in Caco-2 cells. LS180 cells exposed to phenobarbital displayed a specific 10- to 30-fold increase in ABCB1 transcript levels, an effect almost certainly mediated by PXR. The absence of CAR in LS180 cells was supported by the lack of effect on CYP2B6 by phenobarbital (not shown). In Caco-2 cells, the ABCB1 transcript levels were also significantly increased by the clinically used drugs verapamil (substrate/inhibitor for ABCB1/Pgp and ABCC/MRP), and GF120918 (inhibitor of ABCB1/Pgp and ABCG2/BCRP). In general, the effects of the PXR-activators hyperforin and rifampicin were modest in Caco-2 cells (except for the 3-fold induction of ABCG2), while both compounds were potent inducers of ABCB1 in LS180. These results are compatible with PXR being expressed in LS180 but not in Caco-2 cells (18, 250). Nevertheless, hyperforin mediated an up to 3-fold increase in the ABCG2 expression in Caco-2 cells, presumably via some pathway other than PXR.

Interestingly, long-term repeated exposure to dexamethasone (a ligand for PXR and GR) (252) selectively induced ABCG2 8-fold in Caco-2 cells. Dexamethasone is a well-known mediator of cellular differentiation (253) and, although, it is not yet known if dexamethasone is a substrate for ABCG2, it can be speculated that continuous and long-term exposure of Caco-2 cells to dexamethasone might trigger quiescent ABCG2 activity in less differentiated Caco-2 cells as a protective barrier against the differentiating effects of dexamethasone, in analogy with that found in stem cells (112).

Effects of drug exposure on ABC-transporter function

Since it was mainly ABCB1 and ABCG2 transcript levels that were upregulated, selected functional studies were performed using digoxin and mitoxantrone as substrates for ABCB1/Pgp (254) and ABCG2/BCRP (255), respectively. ABCB1/Pgp and ABCG2/BCRP function was upregulated in Caco-2 cells exposed to GF120918 and hyperforin, as observed by an increased efflux of 3H-digoxin and 3H-mitoxantrone, respectively, Figure 16. In contrast, no effects on drug efflux were observed in the LS180 cells (not shown). Digoxin is specifically transported by ABCB1, and a good correlation exists between ABCB1 transcript, protein and transporter function (254). Thus, the increased efflux of digoxin in Caco-2 cells was presumably caused by an increased expression of functional ABCB1.
Results and discussion

In conclusion, it is not only ABCB1 but also ABCG2 that is upregulated by repeated exposure to drugs that interact with common transcriptional pathways. These findings formed a platform for further investigations of drug-induced changes in the expression and function of ABC-transporters in vitro and in vivo. Thus, it was considered worthwhile to investigate the effects of hyperforin on the oral bioavailability of verapamil in humans. This study has been conducted and the results are presented in Paper IV.

4.4 ST JOHN’S WORT DECREASES THE BIOAVAILABILITY OF R- AND S-VERAPAMIL THROUGH INDUCTION OF THE FIRST-PASS METABOLISM

In Paper IV, a jejunal perfusion technique was used to investigate the effect of repeated administration of a widely used herbal drug, St John’s wort (SJW), on the systemic exposure of the enantiomers of verapamil and one of its major metabolites, norverapamil. Single-pass perfusions with 120 mg/L R/S-verapamil were performed in eight healthy
male volunteers before and after 14 days of oral treatment with St John’s wort (300 mg three times a day).

Following repeated administration of SJW, the area under the plasma concentration-time curve (AUC) for R- and S-verapamil decreased by 78 and 80%, respectively, Figure 18. The corresponding decrease in the maximum concentration (C\text{max}) was 76 and 78%, Figure 19, respectively, while the terminal half-life was unchanged. S-verapamil was more extensively metabolized than R-verapamil and the AUC for R-verapamil was six times higher than that of S-verapamil in the control phase. This is in excellent agreement with data reported previously (128). Interestingly, SJW did not appear to preferentially induce the metabolism of any of the enantiomers, which makes sense if one consider that the AUC is expected to decrease after induction, irrespective of whether the drug has a high or low extraction ratio (256).

Verapamil undergoes extensive presystemic and systemic metabolism, mainly mediated by CYP3A4. Hyperforin, the active component in SJW, is a potent activator of PXR, which has been shown to mediate the induction of intestinal and hepatic CYP3A4 and ABCB1 (161, 250). Norverapamil is mainly formed via CYP3A4 (241, 257) and it can be speculated that the strongest inducing effect was evoked in intestinal CYP3A4 since the enterocytes are exposed to higher concentrations of SJW than are the hepatocytes. Based on these facts, it was concluded that SJW mediates its effects on the verapamil bioavailability through induction of intestinal CYP3A4. Similarly, the PXR-ligand and CYP3A4-inducer rifampicin decreased the bioavailability of R- and S-verapamil mainly by increasing the gut wall extraction ratio (128).

The results from this study also show that induction of ABCB1 or other intestinal transporters is a less likely explanation for the reduction in oral bioavailability of verapamil, since the jejunal permeability and the terminal half-life were unchanged for both enantiomers. This is in agreement with previous studies, which concluded that the
human intestinal permeability of both R- and S-verapamil was unaffected by concomitant administration of ketoconazole, an inhibitor of CYP3A4 and ABCB1 (233). In addition, although a small but significant increase in the secretion of S-norverapamil was detected after treatment with SJW, the effect was modest and may just mirror an increased metabolic activity in the enterocytes.

St John’s wort decreased the plasma levels of the metabolite R/S-norverapamil. Since the pharmacokinetics of norverapamil are rate limited by elimination, it is plausible that this route too is upregulated by SJW. The observed effect of SJW on norverapamil pharmacokinetics was less pronounced than that on verapamil, which could be explained by an increase in the elimination rate partly counteracted by an increase in the formation rate. The metabolism of R/S-norverapamil in vivo has not been well characterized, but in vitro induction studies indicate that both CYPs and phase II enzymes may be involved (258, 259).

Figure 19. Individual AUC_{0-\infty} values for R-verapamil (left) and (right) S-verapamil after a 100 minutes jejunal perfusion with racemic verapamil before (Control) and after 14 days of treatment with St John’s wort (+ SJW), 300 mg three times a day.

In summary, repeated administration of St John’s wort significantly decreased the bioavailability of verapamil. It was concluded that induction of intestinal CYP3A4 played a major role in causing this effect. In contrast, the absorption of the drug was not affected by SJW-treatment, suggesting that intestinal transporters such as ABCB1, play a minor role in limiting the oral bioavailability of verapamil. St John’s wort also affected the pharmacokinetics of norverapamil, which is in line with an upregulation of the CYP3A4 metabolism.
5. CONCLUDING REMARKS

Two intestinal barriers to oral drug bioavailability have been studied in this thesis: drug efflux proteins of the ABC-transporter family, in particular ABCB1, and the drug-metabolizing enzyme CYP3A4. The main conclusions from this investigation are:

- A cell culture model of the human intestinal epithelium that expressed both functional Pgp and CYP3A4 was established. The cell culture model was based on the generally available parental Caco-2 cell line, which, in contrast to normal duodenal cells formed tight cell monolayers that were suitable for drug transport studies.

- The new cell culture model was successfully applied in an investigation of the stereoselective transport and metabolism of verapamil. The results in the cell culture model, which indicated that active efflux and metabolism play a minor role as barriers to the intestinal epithelial permeability to verapamil, were in agreement with those in the rat and human jejunum. This makes this model potentially useful in studies of drug transport and metabolism during the preclinical evaluation of new drugs.

- The effects of repeated drug exposure on the regulation of the ABCB1, ABCC2, ABCC3 and ABCG2 transporters follow complex patterns in intestinal epithelial cell lines, suggesting that both parallel and diverse nuclear receptor pathways participate in the regulation. Not only ABCB1 but also other ABC-transporters, in particular ABCG2 were upregulated, and the effects were observed on both the transcriptional and the functional level. Results from these experiments also indicate that GR and/or CAR might regulate ABCG2 expression.

- Repeated oral administration of the herbal drug St John’s wort to healthy volunteers decreased the bioavailability of verapamil, providing evidence for a clinically significant herb-drug interaction. The predominant cause of the decreased oral bioavailability was induction of intestinal CYP3A4, since the oral absorption of verapamil was not affected by treatment with St John’s wort. It was concluded, therefore, that whilst metabolism plays a major role in limiting the oral bioavailability of verapamil, the role of active efflux was far less significant.
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7. REFERENCES


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A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy.*

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