Refined in vitro Models for Prediction of Intestinal Drug Transport

Role of pH and Extracellular Additives in the Caco-2 Cell Model

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Abstract

Drug transport across the intestinal epithelium is roughly predicted from permeability values obtained from Caco-2 cell monolayers. This thesis examines the important role of pH and extracellular additives for increasing the reliability and predictivity of the in vitro screening system, Caco-2.

It was shown that the passive transport of ionizable compounds may be biased by a false efflux or uptake component, when applying a physiological pH-gradient across the membrane. pH also affected the amount of compound available at the transporter-binding site. Therefore, pH dependence should be considered in studies of such compounds and of drug-drug interactions involving efflux transporters. It was also shown that proton-dependent apical uptake or basolateral efflux should be studied both with and without a pH gradient over the whole monolayers.

The two extracellular additives, bovine serum albumin (BSA) and the solubilizing agent, Cremophor® EL, also influenced Caco-2 permeabilities. BSA applied to the receiver side increases, and to the donor side decreases drug permeation according to the drug’s protein binding capacity. Thus, the absorptive transport for both passive and active compounds is favoured, giving a physiologically sound improvement of the Caco-2 cell model. Inclusion of BSA increased both the predictivity and quality of permeability studies, particularly of highly lipophilic, BCS class II compounds. Passive and active transport processes could also be distinguished after accounting for unbound concentrations. The overall effect of Cremophor® EL on the permeability to a drug was compound-specific and probably dependent on micellar incorporation. Cremophor® EL can therefore not be recommended.

Neither pH nor BSA affect the functionality of transporters such as P-glycoprotein. However, efflux ratios of ionizable or protein bound drugs are altered in the presence of a pH-gradient or BSA, indicating that an experimental system without protein or pH gradient can over- or underestimate active and passive efflux in drug transport.

Keywords: Caco-2 cells, membrane permeability, drug permeation, drug efflux ratios, drug uptake ratios, Cremophor EL, bovine serum albumin, pH-dependent permeability, drug transport

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Glossary

a-b apical-to-basolateral, transport in the absorptive direction
ABC ATP-binding cassette
ABL aqueous boundary layer
ADME(T) absorption, distribution, metabolism, elimination and toxicity
ATP adenosine triphosphate
ATCC American Type Culture Collection
b-a basolateral-to-apical, transport in the secretory direction
BBB blood-brain barrier
BCRP breast cancer resistance protein
BCS Biopharmaceutics Classification System
BDDCS Biopharmaceutics Drug Disposition Classification System
BSA bovine serum albumin
BSP sulfobromophthalein
Caco-2 cells Human colon adenocarcinoma cells clone 2
CEL Cremophor® EL
C<sub>max</sub> maximum plasma concentration
CMC critical micelle concentration
C<sub>u</sub> concentration of unbound drug
CYP cytochrome P450 enzyme system
DDI drug-drug interaction
DMEM Dulbecco’s Modified Eagle Medium
DMSO dimethyl sulphoxide
DPHT 1,6-Diphenyl-1,3,5-hexatriene
Efflux transport out of the cell
F bioavailability
f<sub>a</sub> fraction of drug absorbed after oral administration to humans
f<sub>u</sub> fraction of unbound drug as percent of total concentration
FCS foetal calf serum
GI gastrointestinal
HBSS Hank’s balanced salt solution
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTS high-throughput screening
MDCK Madin Darby canine kidney cells
MDR multidrug resistance
MES 2-morpholino-ethanesulfonic acid monohydrate
MRP multidrug resistance associated protein
MW molecular weight
OAT organic anion transporter
OATP organic anion transporting polypeptide
OCT organic cation transporter
PAMPA parallel artificial membrane permeation assay
P<sub>app</sub> apparent permeability coefficient
PB protein binding
PBS phosphate-buffered saline
P-gp MDR1 gene product; Permeability-affecting glycoprotein
pH<sub>i</sub> intracellular pH
RT-PCR reverse transcription polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tr>
<td>SLC</td>
<td>solute carrier</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TER</td>
<td>transepithelial electrical resistance</td>
</tr>
<tr>
<td>Uptake</td>
<td>transport into the cell</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström (1 x 10⁻¹⁰ m)</td>
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Papers Discussed

This thesis includes the following papers, which will be referred to in the text by their Roman numerals assigned below:


IV. Neuhoff, S., P. Artursson, and A.-L. Ungell. Advantages and disadvantages of using bovine serum albumin and/or Cremophor® EL as extracellular additives during transport studies of lipophilic compounds across Caco-2 monolayers. *In manuscript.*
1. Introduction

There are several routes by which drugs are commonly administered. However, oral administration is usually preferred by patients and can encourage compliance. In addition, oral formulations normally cost less to produce. Therefore, the majority of therapeutic agents today are administered orally, even when the formulation might be associated with problems such as poor solubility of the drug or acidic or enzymatic degradation of the compound in the gastrointestinal (GI) tract. Only when the drug is sufficiently soluble and stable in the GI fluids can it be absorbed.

The absorptive processes after oral administration can be summarized as the permeation of a compound across tissue membranes (Chap. 1.1.). The rate and extent of absorption determines the rate and extent of distribution by the systemic circulation to the various tissues of the body (Chap. 1.1.1.). The rate of absorption (permeation) is, in turn, determined by the physicochemical properties of the drug molecule, the specificity of the drug for the intestinal transport systems, and the environmental, anatomical and physiological state of the GI tract (Chap. 1.1.2.). In order to predict the absorption of a drug (e.g., permeation through the intestinal epithelium) and to distinguish between the different mechanisms for absorption (Chap. 1.2), several *in vitro* models have been developed (Chap. 1.3), one of which is the Caco-2 cell model (Chap. 1.4.). Currently, the fraction of a drug dose absorbed by humans is frequently predicted from permeability values obtained from Caco-2 cell monolayer systems (Artursson and Karlsson, 1991; Lennernäs, 1998). These systems are regarded as reasonably reliable tool in the area of drug discovery. However, several issues limit the performance of Caco-2 cell monolayers and further research is essential to increase the reliability and predictive ability of these *in vitro* systems.

The work presented in this thesis is part of an effort to refine *in vitro* models for the prediction of intestinal drug transport. One major theme of this thesis is the investigation of the impact of physiological pH gradients in the intestine on the active and passive transport of drugs across the membrane (Chap. 1.5.). Although this approach has been partly examined in the literature already (Palm et al., 1999; Yamashita et al., 2000), a more systematic investigation of the effects on various types of drugs was not available at the beginning of this project.

Another approach to improving the physiological basis of *in vitro* systems for permeability screening is the inclusion of extracellular proteins (Chap. 1.6.). The basolateral application of bovine serum albumin (BSA) in cell culture transport assays had been described in the literature (Raub et al., 1993), but also in this case more systematic studies on different types of drugs had not been performed. During the course of the work presented here, some work has now been published on alterations to drug transport rates due to the inclusion of BSA in cell culture transport assays.
(Aungst et al., 2000; Saha and Kou, 2002; Taub et al., 2002; Walgren and Walle, 1999; Yamashita et al., 2000). However, the extent of these changes have not, to my knowledge, been sufficiently evaluated in the Caco-2 cell model to date.

Structurally different surfactants have been used to enhance drug absorption across various epithelia (van Hoogdalem et al., 1989) and the addition of bile acids or micelle-building solubility enhancers has been studied in Caco-2 cell monolayers (Anderberg et al., 1992; Ingels et al., 2002) (Chap. 1.7.). The application of apical Cremophor® EL (CEL) in combination with basolateral BSA to Caco-2 cell monolayers was a new approach used in this thesis to develop an in vitro system for determining the permeation of poorly soluble compounds and predicting the fraction of the dose absorbed. This method might have the advantage of both increasing throughput capacity and improving the quality of permeability data.

Finally, the interaction of drugs with other drugs (and food) is a current issue in drug development. For instance, multidrug resistance (MDR) plays an important role in cancer treatment, and MDR1-encoded P-glycoprotein (P-gp) in combination with CYP3A4 has been found to be responsible for several drug-drug and food-drug interactions (DDI) (Deferme and Augustijns, 2003; Rau et al., 1997; Rodrigues, 2002) (Chap. 1.8.). However, at the onset of this thesis, there were no publications on the pH-dependence of DDIs and, therefore, this subject was also studied in vitro in the present work.

1.1. Gastrointestinal (GI) drug absorption and bioavailability

Oral administration is currently considered the most convenient and safest drug delivery route. However, an orally administered drug needs to reach the systemic circulation in order to arrive at the site of action in adequate quantities, as determined by the potency of the drug.

The fraction of an oral dose that reaches the systemic circulation in its parent (original) form is called the oral bioavailability \( F \), which can be described by the following equation:

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

where \( f_a \) is the fraction of the administered dose that is absorbed across the apical membrane of the enterocytes, \( f_G \) is the fraction of the absorbed dose that is not metabolized in the gut wall (\( f_G = 1 - E_G \)) and \( f_H \) is the fraction that escapes metabolism and biliary excretion in the liver (\( f_H = 1 - E_H \)). There are many reasons for low oral bioavailability, and some of the losses occur in the GI lumen. For example, the luminal pH can affect the disintegration, dissolution and/or the stability of the dosage form and the drug. Moreover, the intestinal permeability (as a measure of the ability of a compound to cross the rate-limiting barrier) varies according to the specific site in the GI tract. Although first pass metabolism generally occurs in the liver, it has become increasingly recognized that metabolism may also occur within the enterocyte during absorption and should be considered (Fisher et al., 1999; Wu et al.,...
The main processes, which affect the bioavailability of a drug are schematically illustrated in Figure 1.

**Figure 1**: Schematic illustration of factors influencing the route to the systemic circulation after oral drug administration of a solid dosage form. After disintegration, dissolution, enzymatic and chemical degradation and complexation in the GI tract, permeation of the epithelium may influence absorption of drug molecules. Apical efflux and basolateral uptake together with enzymatic degradation in the intestinal epithelium \((E_G)\) and in the liver \((E_H)\) may decrease the fraction of drug molecules entering the systemic circulation \((F)\). This figure is based on figure 2-2. in reference: Rowland and Tozer (1989).

### 1.1.1. Function of the GI tract

The small intestine absorbs the largest amount of digestive water. It also absorbs nutrients, i.e. electrolytes, fat, monosaccharides, amino acids, di- and tripeptides and vitamins, while the major physiological functions of the colon are to store and concentrate feces by absorbing water and electrolytes. The colon lacks digestive enzymes, but the lumen contains hundreds of different microorganism species which are involved in the reductive reactions of food digestion.

The GI tract is constantly exposed to chemicals, either man-made (e.g. drugs, industrial chemicals, pesticides, pollutants) or natural (e.g. alkaloids, secondary plant metabolites, and toxins produced by moulds, plants and animals). Thus, while the intestine must absorb nutrients, it must at the same time avoid absorption of poten-
ially harmful compounds and microorganisms. These dual requirements of absorp-
tion of nutrients on the one hand and exclusion of harmful substances on the other
hand result in the necessity for a selective permeability barrier for transport both into
and out of the intestinal lumen.

1.1.2. Factors affecting GI absorption of drugs

The factors that affect oral drug absorption can be divided into two categories: phys-
icochemical and physiological.

The physicochemical properties of the compound, such as particle or molecular size,
hydrogen bonding, conformation or partition coefficients \((\log P, \log D)\), direct the
pathway of a compound across the intestinal tissue, i.e. whether the compound will
be transported by paracellular or transcellular routes, or transported at all (Goodwin
et al., 1999; Horter and Dressman, 2001; Kulkarni et al., 2002; Lennernäs, 1998;
Ungell, 1997). Properties such as hydrophilicity and lipophilicity, electrostatic po-
tential, and polarizability may influence affinities to transporters and enzymes. Thus,
they direct the degree of transport and metabolism of the substance. Additionally,
the susceptibility of a drug to chemical degradation and stability will determine
whether the compound is intact long enough for absorption to occur (Kerns, 2001).

The composition of intestinal contents, including food, changes in gastric emptying
and intestinal transit can significantly alter the amount of time that compounds re-
side at favorable absorptive sites (Norberg et al., 2003). The composition of intesti-
nal content can provide significant opportunities for the binding or complexation of
drugs, altering the amount of drug available for absorption (Davis et al., 1986; Galia
et al., 1998). The extent of complexation or binding may also depend on the disease
state because of up- or downregulation of protein levels along the intestine. Regional
\(pH\) differences can prevent dissolution of the drug or provide environments for in-
creased degradation (Fallingborg et al., 1989; Galia et al., 1998). Depending on the
\(pKa\) of the drug, the acid microclimate could act either as a barrier to or an enhancer
of drug transport.

The type of delivery system chosen for drug administration will influence where and
when the compound will be available for absorption. Dissolution and release rates
are factors affecting absorption, since they determine the amount of the drug avail-
able for absorption and indirectly at the site of action. Additives, e.g. in the formula-
tion enhancing solubility, can influence passive and active transport processes.

The rate-limiting step in the absorption of many orally administered drugs is the
permeation of the drug across the intestinal wall (Jackson, 1987; Powell, 1981), a
single continuous monolayer of columnar cells forming the so called epithelium of
the small intestine (Mackay et al., 1991; Madara and Trier, 1994). Absorptive cells
make up over 90% of the epithelial cell population that lines the gut lumen (Kari,
1989). They are formed in the crypts and differentiate during migration to the tips of
the villi. The most distinct feature of these cells is their apical brush border mem-
brane. The apical membrane of an absorptive cell is enlarged by microvilli, increas-
ing the surface area of the apical membrane (Madara and Trier, 1994). The absorp-
tive surface area of the colon is lower than the surface area of the small intestine,
since the absorptive cells of the large intestine process microvilli but are not organized on a villus (Mackay et al., 1991). Compared to the colon, the large surface area and the leakier epithelium make the small intestine the major site of drug absorption.

In general, GI absorption of a compound is often predominantly limited by the dissolution rate/solubility of the drug or the permeability of the apical membrane of the enterocyte to the drug. The Biopharmaceutics Classification System (BCS), which scales these two parameters, provides a basis for predicting oral drug absorption and for in vitro/in vivo correlations (Amidon et al., 1995; Food and Drug Administration, 2000).

The Biopharmaceutics Drug Disposition Classification System (BDDCS) is a modified version of the BCS, which aims to predict overall drug disposition by including the major route of elimination, described by the relative amount of metabolites to the parental drug, and the effect of transporters on oral drug absorption (Wu and Benet, 2005).

This thesis is focused on permeability to drugs as the rate-limiting step to enter the systemic circulation. Hence, a fundamental understanding of drug transport across the main rate-limiting barrier, the intestinal mucosa, is required. The mechanisms of intestinal drug transport are, therefore, discussed next.

1.2. Mechanisms of intestinal drug transport

The aqueous boundary layer (ABL, also called ‘unstirred water layer’) is the aqueous region adjacent to the membrane surface which acts as a diffusion barrier. The permeation of rapidly transported drugs is dependent on the barrier properties of the ABL while the permeation of more slowly permeating drugs is unaffected (Karlsson and Artursson, 1991). The mucus layer contains more than 90% water and usually 0.5-5% mucin (Carlstedt et al., 1985). After the drug has passed the ABL and the mucus, it is exposed to the acid microclimate at the luminal surface of the epithelium. The drug molecule can then be transported across the intestinal epithelium. Figure 2 schematically shows the drug absorption routes across the intestinal epithelium and the intestinal barriers for drug absorption in vivo. In general, there are two distinct pathways by which molecules, such as drugs, can cross the intestinal epithelium; either through absorptive cells (transcellular, case 1) or between cells via tight junctions (paracellular, case 2). The drug can, in the process, be metabolized (extra- and/or intracellularly, case 3) and can be exposed to carrier-mediated transport processes (uptake, efflux, antiport, symport, cases 4 and 5) or transcytosis and endocytosis (variations of the passive transcellular pathway, case 6). Transport by the transcytosis route is very limited and is only relevant for some macromolecules (Gullberg, 2005). This pathway has not been studied in this thesis and therefore is not discussed further.
1.2.1. Paracellular transport

In general, the tightness of the tight junctional complex and the concentration gradient of the solution are the rate-limiting parameters which regulate passive diffusion via the paracellular pathway. It is believed that the narrow pores of the tight junctional complex carry a net negative charge and, thus, it appears to be more permeable to cationic drugs than to anionic and neutral species (Adson et al., 1994; Karlsson et al., 1999; Pade and Stavchansky, 1997; Powell, 1981). The tight junction permeability can be influenced by factors such as intra- and extracellular Ca²⁺ ion concentration, osmolarity, protein kinase activators and inhibitors, and Na⁺ ion linked nutrient absorption and peptide hormones (Hochman and Artursson, 1994). Thus, transport via the paracellular pathway depends mainly on the size (molecular weight (MW) or volume) and the net charge of the compound (Tavelin, 2003).

The paracellular transport route is dynamically regulated. It varies not only along the intestine, but also along the crypt-villus axis (Figure 3). In general, the paracellular route is leakier in the upper part of the small intestine than in the lower parts of the small intestine and colon (Artursson et al., 1993; Taylor et al., 1985). The paracellular pathway constitutes less than 0.1% (estimated 0.01-0.1%) of the total surface area of the intestinal tract and is mainly used by small molecules of hydrophilic character, e.g. mannitol. Drugs transported by this route are often incompletely absorbed (Lennernäs, 1998; Nellans, 1991).

Although the paracellular transport route is generally considered to be passive, the pathway has been shown to be saturable (Gan et al., 1998; Lee and Thakker, 1999) and indirectly linked to intracellular processes (Zhou et al., 1999) or processes in-
volving the tight junction complex (Lee et al., 2002). Thus, the complexity and the dynamics of regulation of this pathway are still under investigation.

Compounds which are believed to be significantly absorbed by the paracellular route include: furosemide (Flanagan et al., 2002), atenolol (Adson et al., 1995), cimetidine (Nagahara et al., 2004; Zhou et al., 1999), glycinamide (Hubatsch et al., 2004), ranitidine and famotidine (Lee and Thakker, 1999). These drugs are all of moderate MW and are relatively hydrophilic.

1.2.2. Passive transcellular transport

Molecules using the transcellular pathway can be transported either passively or by carrier-mediated (active) transport. Generally the passive transcellular pathway is restricted to molecules with a lipophilic character and the concentration gradient is the driving force of this pathway. Although a degree of lipophilicity is necessary to enter the lipid membrane, compounds that are too lipophilic can be trapped in the membrane. Thus, the membrane composition as well as the physicochemical properties of the drug characterize the importance of this transport route for drug absorption.

The membrane composition (phospholipids, proteins, cholesterol) varies between different cell types and is altered during the cell cycle. It may, therefore, influence the membrane properties. Even the apical membrane contains, for instance, lower levels of phosphatidylcholine and higher levels of glycosphingolipids than the basolateral membrane of epithelial cells (van Meer, 1989). The apical membrane is believed to be less permeable than the basolateral membrane (Fromm and Hierholzer, 2000; Hayton, 1980) due to less membrane fluidity and increased thickness as a result of different membrane compositions (Lande et al., 1994). In general, the apical membrane is considered the rate-limiting barrier to passive transcellular transport in polarized cells.

Since it is assumed that the apical membrane is the rate-limiting barrier, a satisfactory description of the process can often be generated by only considering passive transport across this membrane. Thus, experimental and theoretical models describing drug transport only by this mechanism have recently gained attention, e.g. PAMPA (Avdeef et al., 2005) and in silico predictions (Palm, 1998).

The structural requirements of the transcellular route can be simplified by the use of physicochemical properties of the compound such as logP or logD, MW etc. Also other properties like polar surface area and hydrogen bonding have lately been used to describe the molecule. The simplest prediction model for passive transcellular transport is probably the ‘Rule of 5’ (Lipinski, 2003; Lipinski et al., 2001). Famous outliers for the ‘Rule of 5’ are, for instance, cyclosporine A and digoxin, both compounds known for their relevant interactions with active transport systems.

The anatomical and physiological state at the site of absorption will affect drug absorption. For a drug with slow passive transcellular permeation, the absorptive surface area is the rate-limiting step (Figure 3). The longer residence time in the intestinal fluids permits the drug to diffuse down the crypt-villus axis (Artursson et
al., 1996). This not only increases the surface area, but also changes the cell composition, which can in turn lead to an altered route of absorption for the drug.

Figure 3: Light micrograph of rat jejunum, approximately x50 magnification (Åsa Sjöberg, AstraZeneca AB, Mölndal). The absorptive surface area is more accessible for compounds with a long residence time. In vitro models have been developed to reflect the different regions of the small intestine: Caco-2 cells mimic the villus tip and 2/4/A1 cells mimic the crypt base, as seen in the difference of the average pore radius (Tavelin, 2003). Inset: Caco-2 cells grown on filter support for 21 days. The tissue and the cell monolayer were stained with Periodic-acid Schiff (purpur membrane, mucin, glycogen etc.) and Mayer’s hematoxylin solution (blue nuclei).

1.2.3. Active transcellular transport

Carrier-mediated transport processes can be specific and highly selective for a number of chemical structures (e.g. D-glucose, vitamins) and they operate in parallel with passive transcellular transport. The transport system can be saturated with high substrate concentrations, which occupy all binding sites. Additionally, other structurally similar compounds, which bind to the carrier proteins, may cause competitive inhibition. Mechanism-based inhibition, e.g. via allosteric inhibition or energy depletion, is also possible.

Figure 4 gives an overview of passive absorptive transport and active transport across a cell monolayer combined to the overall transcellular transport of a compound. The active transporter can work as an uptake (influx) transporter, which transports the compound into the cell (white circle with grey triangle; cases 1 and 2), or an efflux transporter, which transports the compound out of the cell (grey circle with white triangle; cases 3 and 4). However, unless a compound can passively gain intracellular access, it is not possible to simply investigate whether it is a substrate for efflux transporters. Transporters can be located in the apical and/or basolateral membrane. Their location will determine the effect on the overall absorption. For instance, apical uptake and a basolateral efflux transporter can work in serial in the direction of the concentration gradient in the absorptive direction (case 5) and apical
Efflux or basolateral uptake mechanisms work against this concentration gradient (case 6).

**Figure 4:** Scheme of the passive (a) and active (b) transcellular transport mechanisms (1-8) through the intestinal epithelial membrane, leading to the total (c) observed transport. (1) Passive transcellular diffusion supported by apical uptake. (2) Passive transcellular diffusion reduced by basolateral uptake. (3) Passive transcellular diffusion reduced by apical efflux. (4) Passive transcellular diffusion followed by basolateral efflux. (5) Passive transcellular diffusion supported by apical uptake and basolateral efflux. (6) Passive transcellular diffusion reduced by apical efflux and basolateral uptake. (7) Apical and basolateral efflux. (8) Apical and basolateral uptake. Key: White circles demonstrate uptake transporter, grey circles demonstrate efflux transporter. The triangle inside the circle indicates the working direction of the transporter.

Gut epithelial metabolism can also affect the transport of a compound. The product of such phase I and phase II metabolism can be transported out of the cell by apical and basolateral efflux transporters (case 7). Finally, apical and basolateral uptake transporters can work together, for example in transporting compounds which the cell needs to have fast access to and concentration control over (case 8). Similarly, as for the absorptive transport direction, active transporters could have the opposite effect on transport in the secretory direction.

Active transport against the concentration gradient of the compound requires energy. This energy can be supplied in two ways: (i) By hydrolysis of a high-energy compound such as adenosine triphosphate (ATP). Transporters that use this energy source have an ATP-binding site and are therefore called ABC-transporters. (ii) Via the coupled transport of another molecule, e.g. proton or sodium-ion. These symport or antiport transporters use the concentration gradient of the co-transported compound as a driving force for their own transport and belong in general to the group of solute carriers (SLC). Thus, some exchangers (Figure 2) are able to change their transport direction with an altered driving force.

Since bulk pH and temperature can, for instance, affect membrane fluidity, membrane composition and ion diffusion, all active transport processes are dependent on these variables as well.

1.3. Permeability screening in drug discovery

High-throughput combinatorial chemistry has led to an increased number of possible new drug candidates and has spurred the development of high-throughput screening (HTS) methods for profiling pharmacokinetic drug properties such as absorption,
distribution, metabolism, elimination and toxicity (ADMET). Several HTS assays for the assessment of membrane permeability to drugs are based on cell lines such as Caco-2 and MDCK (Artursson, 1990; Balimane and Chong, 2005; Irvine et al., 1999; Lentz et al., 2000; Li, 2001; Liang et al., 2000; Yamashita et al., 2002).

The methods for studying the epithelial absorption mechanisms for a drug should be sufficiently simple to gain results that are easy to interpret in order to obtain, for instance, clear cut-off values and, thus, to create guidelines and decision trees for the development process. At the same time, however, they should represent the important features which the investigated drug would have to face at the in vivo barrier under physiological conditions, e.g. pH gradients and protein binding. In addition, the methods should require small amounts of the test compound and other expensive materials. The methods should also be reproducible, rapid and not labour intensive.

In vitro permeability methods can be developed as screening tools for low throughput as well as automated high throughput systems. Cell-based permeability methods can help not only to select a drug with a particular property from a library of drugs, but also to gain information on the mechanisms for structurally closely related compounds. Thus, depending on the question to be answered in the stage of the development of the drug, a useful in vitro system has to be adaptable (Quaroni and Hochman, 1996).

Some immortalized epithelial cell lines can be cultured on porous filter supports to form confluent monolayers with reproducible (relative) protein expression and polarity (Artursson, 1990; Pinto et al., 1983) (Figure 5). Hence, these cells can be used for automatic screening assays as well as manual and mechanistic studies. A general aim of this thesis was to improve the performance of in vitro cell culture models for mechanistic studies. Such more demanding studies can be used to follow up unclear results from HTS studies of drug permeation. They can also be used as guidelines for improvement of the experimental set-up and, thus, the outcome (increased quality and throughput) of HTS.

The species and the organ from which the cell line originates is important. While a human cell line originating from colon carcinomas like Caco-2, HT29 and T84 can be an obvious first choice for predicting absorption mechanisms in the GI tract, a transformed canine cell line from the kidneys such as MDCK II, which has been transfected with a human gene encoding for instance an efflux transporter such as P-gp (MDCKII-MDR1), can be an appropriate alternative in studies of P-gp efflux. However, the most reliable conclusions might be drawn from combined results of both assays.
Since the human anatomy is very complex, a simple system will always only represent and open a small window to the overall picture. The results from in vitro experiments have to be put in reasonable perspective; they should never be seen as a replacement for in vivo experiments. However, in vitro systems significantly reduce the number and increase the quality of in vivo experiments in drug discovery.

1.3.1. Factors affecting data from cell culture assays

The assay conditions are crucial, independent of which cell line is used, since they will determine if for instance a mechanism is functional or not. When investigating a pH gradient-dependent transporter or an energy-dependent transporter, we have to supply the cells with a pH gradient and the necessary energy. This might be problematic using cells in suspension (membrane vesicles, oocytes) or cells grown on plastic, e.g. in culture flasks.

The assay conditions also determine the drug concentration, which can be altered by parameters like temperature, pH, co-solvent and metabolism. Since the rate of permeation is determined by the rate of transport, i.e. amount of compound transported per time, across a defined surface area relative to this concentration (see Equation 6), all calculated permeability values depend on this donor concentration and the accuracy of its determination.

The accuracy of the determination of the drug concentration will also affect the calculated recovery for the experiment and the calculated mass balance during a time slot in the experiment. Loss in recovery can be due to low solubility of the compound, binding to the equipment (plastic tips, filter material, cell culture dishes etc.) or to the lipid membrane, and metabolism, which can often be experienced for lipophilic compounds. Parameters like pH, temperature and co-solvents, which might alter (increase) the solubility by changing the hydration shell of the molecule, can also affect the cells, the buffer capacity and even the equipment. In order to obtain a better overview of the transport of the compound by time, it can be useful to investigate changes in the mass balance at different time intervals. This is particularly important if the compound concentration is altered during the experiment, e.g. in metabolism studies.

The stirring rate will determine the thickness of the aqueous boundary layer adjacent to the epithelial membrane and, for mucus-producing cell lines, the stability of the mucus layer.

The ‘sink’ conditions will determine how close the system is to the equilibrium situation, which all closed systems strive for. Thus, sampling time points and sample volumes alter the ‘sink’ conditions. For efflux experiments, the cells will be loaded under equilibrium conditions in order to define an accurate (‘start’) donor concentration, i.e. concentration in the cell. However, the experiment itself should be performed preferably under ‘sink’ conditions to avoid back-diffusion (in this case into the cell) and to maintain a constant applied concentration gradient. Also, in order to mimic the in vivo situation of drug transport, ‘sink’ conditions have to be maintained since, in vivo, the blood circulation represents a perfect sink, i.e. an open system.
In addition, differences in the cell culture conditions, such as seeding density, passage, degree of differentiation and expression level of the major proteins, can result in a lack of reproducibility between laboratories and need to be exposed for accurate comparison of results (Ungell, 2004; Ungell and Karlsson, 2003).

As long as there is a high rate of variability between assay conditions, a substantial variability in the results, e.g. drug transport rates, will be obtained. Hence, a profound understanding of the underlying mechanisms of drug transport is desirable and standardized assay conditions are necessary (Ungell, 2004).

1.4. The Caco-2 cell

The closer an in vitro model describes the in vivo bioavailability, the more complex it becomes. The models used in early drug development simulate as closely as possible the physiology of the intestinal epithelium in order to predict oral bioavailability, which is basically influenced by the lack of absorption from the GI tract, first-pass metabolism by enzymes in the gut wall, and first-pass metabolism by the liver.

Caco-2 cells are the most common cell models used to investigate intestinal absorption processes in vitro (Balimane and Chong, 2005; Ungell, 2004). The cell line was derived from a moderately differentiated colon adenocarcinoma in a 72-year-old patient (Fogh et al., 1977). It is well characterized with respect to its cyto- and biochemical properties (Lu et al., 1996; Wilson et al., 1990). Caco-2 cell line clones are highly standardizable; however, carriers for intestinal transport may be over- or under-expressed compared to the protein expression of the various parts of the human intestine (Sun et al., 2002).

The cells differentiate spontaneously under normal culture conditions and form tight monolayers of well-polarized absorptive cells (Artursson, 1990; Chantret et al., 1988; Hidalgo et al., 1989; Hilgers et al., 1990; Pinto et al., 1983). The cells show similarities in membrane function with human colon, but the carrier systems resemble those of the small intestine more closely (Ungell and Karlsson, 2003; Zweibaum et al., 1984). Their apical surface is equipped with the typical brush-border microvilli (Artursson, 1991; Artursson and Magnusson, 1990; Hidalgo et al., 1989; Wilson et al., 1990). Caco-2 membrane proteins include transport systems and enzymes typical of those in intestinal epithelial enterocytes in vivo (Sun et al., 2002) (Appendix, Figure 19). The transport of amino acids, bile acids, biotin, peptides and sugars has, for instance, been studied in this model (Bissonnette et al., 1996; Dantzig and Bergin, 1990; Hidalgo and Borchardt, 1990a; Hidalgo and Borchardt, 1990b; Ng and Borchardt, 1993). In addition, they express organic anion and cation transporters, mainly as uptake transporters and exchangers. They also express ATP-dependent efflux pumps such as P-gp, breast cancer resistance protein (BCRP) and several multidrug resistance-associated proteins (MRPs) (Taipalensuu et al., 2001). The activity and amount of the transporters can depend on the average age of the cells and the culture condition, as seen for P-gp and dipeptide transporters (Anderle et al., 1998; Herrera-Ruiz et al., 2001; Hosoya et al., 1996; Seithel et al., 2004).

Caco-2 cells also express various phase I (e.g., CYP1A1) and phase II (e.g., glutathione-S-transferases, and sulfotransferase) metabolizing enzymes. The culture
conditions are also important for the development (amount and expression) of these proteins. For instance, Caco-2 cells grown in the presence of 1-α-25-dihydroxy-vitamin D3 express enzymatically active CYP3A4 (Engman et al., 2003; Schmiedlin-Ren et al., 1997). The metabolic activity of Caco-2 cells is generally lower or between those obtained in the small intestine and colonic mucosae of animals or humans (Jumarie and Malo, 1991; Madara and Trier, 1994; Quaroni and Hochman, 1996; Sun et al., 2002). Caco-2 cells are representative of columnar absorptive cells, and, thus, they lack the complexity of the small intestinal wall, which contains several epithelial cell types, including mucin producing goblet cells. Thus, Caco-2 cells lack a mucus layer in culture and the cells do not maintain the acid microclimate. Instead, the selected buffer under standard experimental conditions maintains the pH.

In drug discovery and development settings, Caco-2 cells are widely used to:
- rapidly assess compound permeation across the intestinal epithelium of potential drug candidates and consequently determine suitable physicochemical properties for passive diffusion of drugs,
- study the function of intestinal epithelial cells (e.g. passive versus carrier-mediated pathways of drug transport),
- investigate formulation strategies in order to enhance membrane permeability,
- investigate potential toxic effects of compounds, pharmaceutical additives or formulations, and
- study presystemic drug metabolism.

1.5. pH and pH-gradients

The pH can affect the degree of dissociation and in turn the membrane permeability and, thus, the potency of a compound to interact with proteins in the membrane and behind the membrane barrier. Additionally, the pH varies in the different compartments of the body from the macroscopic to the microscopic level, i.e. in the body fluids and organs, as well as cell cytosol, membranes and transporter levels.

1.5.1. Henderson-Hasselbalch equation

Many drugs are either weakly acidic or weakly basic. When these drugs are in solution equilibria exist between undissociated molecules and their ions. Thus, in a solution of a weakly basic drug B (or a weakly acidic drug HA) the equilibrium can be described by the following equations:

Base: \[ B + H^+ \rightleftharpoons BH^+ \] Eq. 2a

Acid: \[ HA \rightleftharpoons H^+ + A^- \] Eq. 2b.

In solutions of most salts of strong basic or acidic compounds in water, such equilibria are shifted to the right, i.e. to the ionized species (A⁻ and BH⁺). The ionized constant (or dissociation constant) \( K_a \) of a weakly basic or acidic compound can be written as:
When calculating the negative logarithm of $K_a$ ($pK_a$) and of the proton concentration ($pH$), the Henderson-Hasselbalch equation can be obtained (Hasselbalch, 1916):

$$\text{Base: } pK_a = pH + \log \frac{[BH^+]}{[B]} \quad \text{Eq. 4a}$$

$$\text{Acid: } pK_a = pH + \log \frac{[HA]}{[A^-]} \quad \text{Eq. 4b}.$$

The degree of ionization of a weakly basic or acidic compound in a solution can be calculated from the Henderson-Hasselbalch equation, if the $pK_a$ values of the drug and the $pH$ of the solution are known.

1.5.2. $pH$ partitioning hypothesis

The $pH$-partitioning hypothesis states that only the uncharged species of a compound can permeate the membrane and that the permeation of a drug is therefore a function of the concentration of the uncharged species (Shore et al., 1957). The $pH$ of the solution at the site of absorption, i.e. close to the membrane on the luminal side, will affect the degree of ionization of weak acids and bases according to the $pH$ partition hypothesis. Acids will diffuse more slowly and bases more rapidly across the membrane as $pH$ increases. Deviations from the $pH$-partitioning theory for passively transported compounds have been attributed to the presence of an acid microclimate adjacent to the apical surface of the intestinal epithelium and to effects of the aqueous boundary layer (Lucas et al., 1978; Lucas et al., 1975; Tsuji et al., 1978).

1.5.3. $pH$ and $pH$-gradients in the GI tract

The intestine has to have the capacity to regulate the acidic compounds released by gastric secretion and by the activity of the intestinal microbes. Additionally, the $HCO_3^-$-ion secretions from the pancreas, the changes in $CO_2$ pressure and the $pH$ of the blood have to be accounted for.

The mean bulk $pH$ along the human intestine in vivo ranges between 5.5 and 7.5 (Fallingborg et al., 1989). The bulk $pH$ varies throughout the GI-tract in the fasted state from about 1.5-2.0 in the stomach, to 5.5-6.5 in the duodenum, 6.0-6.5 in the jejunum, 7.0-7.4 in the ileum and 5.5-6.5 in the colon (Dressman et al., 1990; Evans et al., 1988; Fallingborg et al., 1989). Following a meal, the gastric $pH$ is increased to a median peak value of 6.7, then declining gradually back to the fasted state value during less than 2 hours (Dressman et al., 1990). In contrast to the $pH$ changes observed in the stomach, the $pH$ in the duodenum decreases in the fed state to a median
value of 5.4. The pH in the GI tract can influence the absorption processes on several levels. Firstly, the pH of the GI fluids determines the degree of dissociation, as described by the Henderson-Hasselbalch equation. The degree of dissociation in turn can affect the solubility and, thus, the dissolution rate of a compound. Thus, the bulk pH affects solubility and release from the dosage form and the amount of compound interacting with the acid microclimate. Secondly, the pH of the acid microclimate can trigger the activity of active transport processes as described in the next chapter. Hence, the degree of dissociation can also affect the passive diffusion of the compound, according to the pH-partition hypothesis and, thus, the passive paracellular and transcellular pathways.

In 1959, Hogben and co-workers suggested that the metabolism of the epithelium may lead to a formation of an acid microclimate at the luminal surface and that this acid microclimate is responsible for deviations from the pH-partition hypothesis in observation on the intestinal transport of drugs (Hogben et al., 1959). In 1978, Lucas and co-workers measured an in vitro jejunal surface pH of 5.93 ± 0.05 in human proximal jejunum biopsy samples (Lucas et al., 1978). In another study, the surface pHs of five rectal biopsy specimens obtained from a normal human mucosa were between 6.23 and 6.80, while the buffer pH varied between 5.96 and 7.51 (McNeil et al., 1987; McNeil and Ling, 1984). Independently of the bulk pH, the acid microclimate on the apical surface of the intestinal epithelium (approximately 20 µm thick) is relatively constant, when measured, for example, at the tip of the villi of rat jejunal tissue (Figure 6). Thus, the acid microclimate offers a permanent natural pH gradient across the absorptive cell membrane in vivo.

Figure 6: Relationship between microclimate pH and bulk pH in the rat jejunum. Individual symbols represent results of separate publications, sometimes including several studies (Daniel et al., 1985; Lucas, 1983; Shiau et al., 1985). The measured microclimate pH was between 5.0 and 6.7, while the bulk pH ranged between pH 4.0 and 10.0. However, beyond this range, there was a steep drop and rise, respectively, in the microclimate pH. The alterations in microclimate pH were relatively dependent on the distance to the tissue and, thus, on the probe rather than on the bulk pH. The dashed line is the line of identity.

Further down the crypt-villus axis, the pH increases from approximately 6.0 to 8.0 (Figure 7) (Daniel et al., 1985; Hogerle and Winne, 1983). Thus, while the bulk pH
The acid microclimate exists because of the mucus, an ampholyte at the surface lining of the intestine. Several HCO₃⁻-ion exchangers (e.g. Cl⁻/HCO₃⁻-exchanger) in combination with sodium-proton exchangers (NHEs) and other transporters maintain the buffer system (Chang and Rao, 1994); however, the formation of the acid microclimate is not only dependent on HCO₃⁻-ion and hydrogen ion secretion. The expression and activity of the transporters along the GI tract, and also along the crypt-villus axis, will determine the buffer capacity at the particular site.

1.5.4. Impact on transporters

In order to distinguish between the possible pH-gradient systems, the concept of a 3-compartment model can be used (Jackson et al., 1974; Lucas and Whitehead, 1994) (Figure 8). A Caco-2 cell monolayer represents such a 3-compartment model. The pH of the buffer solutions on both sides of the monolayers can vary, while the cytosolic pH is assumed to be around 7.4. Caco-2 cells express at least three different sodium proton exchangers (NHE1, NHE2, and NHE3), which help to stabilize the intracellular pH (pHi) (Cavet et al., 2001; Janecki et al., 1999; Thwaites et al., 1999).
Transporters can be directly and indirectly affected by the bulk pH and the acid microclimate pH:

Antiporters or symporters can require a proton gradient in order to function. These transporters need a proton gradient either over one membrane (e.g. apical pH 6.0; intracellular pH 7.4) or over the entire cell (e.g. apical pH/basolateral pH: 6.0/7.4/7.4). Transporters which have been reported to be proton-dependent include OCTN1 (Yabuuchi et al., 1999), MCT1 (Tamai et al., 1999), PepT1 (Thwaites et al., 2002), and OATP-B (Kobayashi et al., 2003). Three of them are thought to be apically located in the GI tract: MCT1 (Cuff et al., 2002), PepT1 (Rubio-Aliaga and Daniel, 2002) and OATP-B (Kobayashi et al., 2003).

It has been suggested that PepT1 and NHE3 share a common location (Thwaites et al., 2002) since absorption via PepT1 is dependent on maintenance of a proton gradient over the apical membrane alone, which is, in part, established by NHE3.

In contrast, the MCT1 transporter may be counter-ion dependent. This means that either a proton is transported with the substrate (symport; MCT1 (Halestrap and Price, 1999)) into the cell or an \(\text{HCO}_3^-\) ion is transported out of the cell while the substrate is transported into the cell (antiport; SCFA/\(\text{HCO}_3^-\)-antiporter (Stein et al., 2000)). In both cases, the intracellular compartment gains one positive charge (proton) per transport cycle. Thus, the proton gradient over the membrane could influence the choice of counter-ion.

Transporters can favor one species of a compound, i.e. the charged or uncharged form. They can also favor one enantiomer over the other. For instance, L-lactic acid is a substrate of the MCT1 (Stein et al., 2000; Tamai et al., 1999; Tamai et al., 1995), while its enantiomer D-lactic acid is not. Flupentixol is an example for an allosteric inhibitor of P-gp. Both cis and trans isomers of the dopamine receptor antagonist flupentixol inhibit drug transport and reverse drug resistance mediated by P-gp with a stereoselective potency. A stimulatory effect of cis\((Z)\)-flupentixol and the inhibitory effect of trans\((E)\)-flupentixol on ATP hydrolysis and radioligand labeling were reported (Dey et al., 1999).

As indicated in Figure 4, the working direction and, thus, the location (apical or basolateral membrane) of the transporters relative to the concentration gradient of the compound, which determines the passive diffusion of the compound, is important. The expression of the same transporters in the various organs is also important in this context. It is expected that apical efflux transporters such as P-gp in the GI
tract can reduce the absorption of a compound before metabolism in the cell, while P-gp in the liver will affect transport after the metabolism step (Benet et al., 2003). The ease of use of relatively new methods such as RT-polymerase chain reaction (RT-PCR) technology has allowed determination of the transporters and their expression in relation to each other; however, this technique is quite expensive and comprehensive published data are currently limited (Seithel et al., 2004; Taipalensuu et al., 2001).

According to the pH-partitioning theory, the uncharged compound is more likely to permeate the membrane, and the extent of permeation, in turn, will determine the amount of compound available at the binding site of the efflux transporters. Thus, the impact of the transporter is indirectly affected by the bulk pH. For example, although efflux of drugs by P-gp is not directly affected by extracellular or intracellular pH (pHt) (Altenberg et al., 1993; Goda et al., 1996), an indirect connection between pHt regulation and the expression of functional P-gp has been suggested because of the acidification of the external medium observed in cells overexpressing P-gp. In addition, Johnstone and co-workers suggest, in an explanation of the regulation of apoptosis by P-gp, that expression of functional P-gp may increase the intracellular pH (2000). Thus, an active transporter can also change the intracellular and/or extracellular pH close to its location (Landwojtowicz et al., 2002).

If a drug is highly lipophilic and also has other physicochemical properties that favor a strong interaction with the cell membrane, it can become trapped in the apical membrane. After diffusion across the apical membrane, the intracellular pH will determine the fate of the compound. While weakly basic compounds can be trapped in the lysosomes (pHt: 4), acidic compounds can be trapped in the cytosol of epithelial cells (pHt: 7.2-7.4). All these processes lead to a loss of available compound at the site of action and result in poor or variable bioavailability. The intracellular drug concentration will also be increased, which could trigger a change in the transport direction of an exchanger.

1.6. Protein binding

1.6.1. Importance of protein binding in vivo

Protein binding of drugs to human plasma and tissue in vivo leads to several effects in the body. By affecting the effective concentration of the drug and the equilibrium levels between tissue and plasma, it can alter the storage (when acceptors are involved), the metabolic transformation (when enzymes are involved), the translocation (when transporters are involved) and the pharmacological effects (when receptors are involved) of the drug. When over 95% of the compound is plasma protein bound, a compound is regarded as a highly protein bound compound in vivo.

1.6.2. Protein composition

Albumin is the most abundant protein component of plasma, with a concentration of about 40 mg.ml⁻¹ (0.6 mM); it accounts for approximately 60% of the total protein content (Colmenarejo, 2003; Peters, 1996). The binding properties and the various
binding sites for drugs on BSA and HSA (human serum albumin) are similar but are not identical (Kosa et al., 1997). BSA is less expensive than HSA and is, therefore, commonly used as a replacement in in vitro studies (Saha and Kou, 2002; Taub et al., 2000). There are also additional proteins, e.g. α₁-acid glycoprotein, low-density lipoprotein (LDL) and high-density lipoprotein (HDL), in plasma (and intestinal fluid) which can affect the permeation of drugs (Shen et al., 2004). In humans, serum contains 56 g.l⁻¹ of total protein, 35-50 g.l⁻¹ (500-700 μM) of albumin, 0.4-1.0 g.l⁻¹ (9-23 μM) of α₁-acid-glycoprotein, about 2.4 g.l⁻¹ (10.4 μM) of HDL, and about 2.4 g.l⁻¹ (0.96 μM) of LDL (Bree and Tillement, 1986; Rowland and Tozer, 1989).

1.6.3. Protein binding in in vitro experiments

BSA can be applied basolaterally, i.e. on the serosal side, in the Caco-2 cell system in order to more closely approximate physiological conditions (Saha and Kou, 2002; Yamashita et al., 2000). This method of mimicking the extracellular tissue protein levels may improve the mass balance for lipophilic drugs, which will bind to the protein instead of to the plastic wells (Artursson, 1990; Wilson et al., 1990). The addition of extracellular protein will also help to maintain sink conditions during screening experiments (Krishna et al., 2001; Saha and Kou, 2002; Taub et al., 2002; Yamashita et al., 2000). In studies of the secretory (basolateral-to-apical; b-a) transport direction, however, applying BSA only to the basolateral side could reduce the concentration of unbound drug (Cᵢ) that is then available to permeate the membrane. Nonetheless, it would still reflect the in vivo situation more closely than the traditional Caco-2 set-up (without BSA).

Although also depending on the extent of protein binding and the degree to which the compound permeates the membrane passively, addition of BSA basolaterally will generally increase transport in the absorptive (apical-to-basolateral; a-b) direction (Saha and Kou, 2002) and reduce transport in the secretory direction (Walgren and Walle, 1999). This can result in false positive (apical influx or basolateral efflux) or false negative (apical efflux or basolateral influx) interpretations of the involvement of a carrier-mediated transport system. Since apical efflux systems such as P-gp, BCRP and MRP2 influence the transport of drugs from the site of administration to the site of action and thus influence the maintenance of adequate drug concentrations at the site(s) of action, it is necessary to develop predictive models for the transport of drugs in the presence of extracellular protein. Thus, it is important to understand the effect of protein binding on the efflux of model drugs. The validity of commonly used HTS methods in predicting efflux-related pharmacokinetic behaviour in vivo in humans must therefore be revisited under improved experimental conditions.

1.7. Surfactants

The complexity and likelihood of error in calculating transport rates across biological membranes are substantially increased for poorly soluble compounds. Aqueous media without any solubility enhancing surfactant are preferred in screening studies, and limits for solubility and recovery data often have to be set when conducting in vitro permeability studies, in order to ensure reasonably high throughput and an
acceptable (minimum) quality of the results. However, when screening for new
drugs, there are often few poorly soluble compounds that fulfil the minimum crite-
ria. For this reason, solubility enhancers have been investigated as additives for
adequate increase in solubility.

The common use of aqueous-organic solvent mixtures as a buffer medium is ques-
tionable, since organic solvents such as DMSO and ethanol are known to interact
with biological material. Thus, the concentration of these organic solvents in the
final buffer solution is restricted, which in turn limits their solubility enhancing
capacity.

Surfactant, amphiphile and tenside are all terms used to describe the same class of
compound. The word surfactant is an abridgement of the term surface-active agent,
i.e. a chemical species that is adsorbed at an interface. Common ionic and non-ionic
surfactants such as polyoxyethylene sorbitan monolaurate (Tween), polyoxyl castor
oil (Cremophor) and taurocholic acid can be used as solvent systems (Aungst, 2000;
Ingels and Augustijns, 2003; van Hoogdalem et al., 1989).

The concentration of surfactant needed to increase the solubility of the drug depends
on the critical micelle concentration (CMC) of the surfactant and the degree to which
the compound partitions into the micelle formed by the surfactant. The CMC is the
concentration (concentration range) which reflects the transition from a monomer
solution of surfactant to a colloidal solution of surfactant micelles. At very low con-
centrations of surfactant, no aggregates are formed. The surfactant molecules are
present in the solution as monomers or, at somewhat higher concentrations, as
dimers. When the concentration is raised to a certain value, specific for each surfac-
tant, the surfactant molecules form aggregates, which are called micelles. This spe-
cific concentration is called the CMC. Addition of surfactant will lead to a further
increase in micelles, while the concentration of the monomer will stay constant
(Figure 9). The CMC of the surfactant depends on the type of surfactant and the
buffer solution (pH, ionic strength, type of ions, co-solvents) and the temperature.
The partitioning of the compound into the micelle depends on factors such as the
degree of ionization of the compound and the size of the molecule (or particle). In
general, a linear relationship between solubility and surfactant concentration can be
assumed above the CMC.

![Figure 9: Concentration of micelles and monomers in relation to the total concentration of surfactant.](image)
1.7.1. Cremophor® EL

CEL is a non-ionic surfactant and a derivative (polyether) of Castor oil and ethylene oxide. The polarity of the head group is created by polyether or polyhydroxy moieties. The hydrophilic-lipophilic-balance value (Griffin, 1950) for CEL lies between 12 and 14 (BASF AG, July 1997); thus, CEL can be used as an oil-in-water emulsifier as well as as a wetting or solubilizing agent. CEL is used to emulsify and solubilize oils and water-insoluble substances. It is used as a vehicle for water-insoluble vitamins and drugs such as cyclosporine A. The CMC for CEL in HBSS at pH 7.4 and 25°C is 0.0095% (w/v) (0.0073-0.0091% (w/v) range), assuming a molecular weight for the monomer of 2515 (Nerurkar et al., 1997). In general, the CMC is lower for a non-ionic surfactant, than for an ionic surfactant because of the lack of repulsive electrostatic forces between the head groups.

1.7.2. Surfactants in in vitro assays

Surfactants used in pharmaceutical formulations can modulate drug absorption by many mechanisms. Of these only solubility enhancement is an acceptable application for in vitro screening of drug permeability. Other effects, such as membrane solubilization, tight junction modulation and interaction with transport proteins should be avoided (Aungst, 2000; van Hoogdalem et al., 1989). Experiments using surfactants in whole-tissue models offer complex information, which can be difficult to interpret (Florence, 1981). In contrast, in simpler models such as brush border membrane vesicle (BBMV) and cell monolayers the cell membrane, active transport systems and in the cell monolayers also the tight junctions can be studied under more controllable conditions (Anderberg and Artursson, 1993; Aungst, 2000; Nerurkar et al., 1996; Nerurkar et al., 1997).

While some apical additives seem to improve the experimental set-up, others should be used with caution, since surfactants can affect both the solubility and the permeation of a drug (Rege et al., 2002; Saha and Kou, 2000; Yu et al., 1999). At low concentrations, the surfactants are incorporated into the lipid bilayer and this can change the properties of the cell membranes. After the lipid bilayer is saturated, mixed micelles could be formed, which results in removal of phospholipids from the cell membranes and a solubilization of the membrane. This, in turn, can change the fluidity of the membrane, which could affect the functioning of membrane proteins, as has been suggested for P-gp (Dudeja et al., 1995; Woodcock et al., 1992). However, membrane fluidity modulators, such as cholesterol and benzylalcohol did not change the P-gp-associated drug transport of rhodamine 123 in Caco-2 cells (Rege et al., 2002). Controlled inhibition of P-gp or other apical efflux/basolateral uptake transporters by a surfactant (preferably one that is already used in pharmaceutical formulations, such as TPGS or CEL) might be useful for increasing the intestinal absorption (and bioavailability) of substrates for these transporters. This approach has been followed recently by several laboratories (Bogman et al., 2005; Yu et al., 1999).

Thus, the potential positive effects of inclusion of a surfactant (solubility enhancement; improved mass balance) should be compared with the potential negative effects (formation of mixed micelles; uncontrolled inhibition of active membrane transporters).
1.8. Clinical relevance of drug-drug interactions

In the clinic, a patient might obtain up to 21 different drugs concomitantly (Englund et al., 2004). All effective compounds have the potential to produce both benefits and risks associated with desired and undesired effects. Both types of effects are concentration dependent. Changes in the concentration of a drug can be triggered by a second effective compound (a drug (Lin and Lu, 2001), food (Deferme and Augustijns, 2003), etc.). Thus, one drug interaction can be considered as clinically significant when it is linked with a change in plasma drug concentration and when this change has the potential to alter the drug response.

DDIs can occur at a variety of sites, from the initial site of dissolution of the drug in the GI fluids to an effective site distant from the site of administration. Thus, a drug can affect the dissolution of another drug directly after oral administration, its plasma protein binding after both have reached the systemic circulation, or its metabolism (Lin and Lu, 2001). However, DDIs at the transporter level are still unresolved. It could be speculated that transporter-mediated DDIs are most likely to occur where variations in transporter genes have been observed e.g., in rheumatoid arthritis and Crohn’s disease (OCTN1, SLC22A4; (Grundemann et al., 2005)), cholestatic liver disease (BSEP, ABCB11; (Kullak-Ublick et al., 2004) and the human Dubin-Johnson syndrome (MRP2, ABCC2; (Keppeler and Konig, 1997)).

It is possible that transporter interactions have not yet been fully elucidated because of the difficulties in separating transport and metabolism in vivo, bearing in mind that 56% of a group of 315 marketed drugs were found to be primarily cleared by CYP (Bertz and Granneman, 1997) and that an estimated 30% of clinically marketed drugs are targeted to membrane transporters or channels (AAPS-Workshop, 2003). Not only might a compound have several different metabolic pathways but it might also interact with several different transporters. Thus, DDIs based solely on transporters can only be shown with two non-metabolizable compounds which share the same transporter. For example, digoxin and talinolol share P-gp (Westphal et al., 2000), and oral administration of talinolol increased the area under the concentration-time curve of co-administered oral digoxin significantly, increasing the maximum serum levels by 45% (Westphal et al., 2000). In this study the renal clearance and half-life of digoxin remained unchanged. However, infusion of talinolol concomitantly with oral digoxin had no significant effects on digoxin pharmacokinetics. Digoxin did not affect the disposition of talinolol after either oral or intravenous administration. Westphal and co-workers observed significant increases in the bioavailability of digoxin only with oral co-administration of talinolol, and they concluded that this is most likely caused by competition for intestinal P-gp (Westphal et al., 2000).

A negative effect of co-administration of multiple inhibitors has been described recently (Englund et al., 2004). Multiple DDIs at the same site, e.g. P-gp, may be the result of multiple drug prescriptions. For example, S-digoxin levels increased stepwise with an increased number of co-administered P-gp inhibitors, when S-digoxin and the P-gp inhibitors were taken at the same time (Englund et al., 2004). In unusual cases, intestinal transporter-based DDIs can be used to improve therapy, as suggested for the HIV-protease inhibitor and Vitamin E-TPGS (Yu et al., 1999). As
proof of concept, TPGS, which inhibits P-gp, was co-administered with talinolol. TPGS increased the area under the plasma concentration-time curve (0- infinity) of talinolol by 39% and the maximum plasma concentration of talinolol by 100% (Bogman et al., 2005). This study showed the intestinal relevance of the drug-formulation interaction; however, it remains to be shown that TPGS is also useful in the clinic.

An additional obstacle in determining the degree of a DDI is the fact that the extent and appearance of DDIs will be altered depending on the organ and, thus, the expect order of which the compound would be exposed to the proteins (e.g. GI tract: P-gp then CYP 3A; liver: CYP3A then P-gp) (Benet et al., 2003; Suzuki and Sugiyama, 2000; Wacher et al., 1998). The properties of the organ should also be considered. For instance, since the brain comprises less than 1% of the total body weight, changes in the overall distribution of a drug within the body will not be observed. However, inhibiting apically located efflux transporters in the blood-brain barrier (BBB) (e.g. P-gp, MRPs or BCRP) can result in significantly increased brain levels of compounds like cyclosporin A and asimadoline, without an observable change in total body distribution. Understanding this type of transporter-based DDI could be useful as a concept for new drug delivery systems, such as those involving cotransport of efflux inhibitor and drug by nanoparticles across the BBB (Gulyaev et al., 1999).

Thus, DDIs are a reflection of the interplay between two compounds and their effects upon each other. These effects are concentration dependent, i.e. dependent on the amount of ionized and un-ionized species of the compounds. Understanding the effect of various concentrations on active and passive transport processes might help to establish an experimental set-up that mimics some of the causes leading to DDIs, i.e. mimicking alterations in concentration at the site of an interaction.
2. AIMS OF THE THESIS

The objective of this thesis was to refine studies of the intestinal drug absorption of orally administered drugs using the *in vitro* Caco-2 cell model.

The specific aims were:

- To investigate the passive and active transport processes of weakly basic, weakly acidic, and neutral drugs under the influence of the physiological \( pH \) gradient of the intestine using inhibitors and concentration and temperature dependency.

- To investigate, using a mechanistic approach, DDIs at the level of a transport protein under the influence of the physiological \( pH \) gradient of the intestine.

- To investigate the mechanism behind the alterations in passive and active transport of drugs in the presence of drug binding to extracellular serum albumin.

- To investigate the effects of the solubilizing agent CEL on Caco-2 permeability to both actively and passively transported lipophilic, BCS class II (low solubility/high permeation) compounds.
3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Drugs and radiolabelled compounds

[\textsuperscript{3}H]-Digoxin (629 GBq/mmol), \textsuperscript{S}[-\textsuperscript{3}H]-propranolol (703 GBq/mmol), \textsuperscript{14}C]-indomethacin (740 MBq/mmol), \textsuperscript{14}C]-salicylic acid (2.1 GBq/mmol), and \textsuperscript{14}C]-mannitol (1.9 GBq/mmol) were purchased from PerkinElmer\textsuperscript{TM} (former NEN\textsuperscript{TM}) Life Science Products Inc. (Boston, MA, USA). Quinidine [9-\textsuperscript{3}H] (740 GBq/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, USA). \textsuperscript{14}C]-Doxorubicin (2.04 GBq/mmol) was purchased from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, UK). \textsuperscript{3}H]-Metoprolol (31.5 GBq/mmol), metoprolol, \textsuperscript{3}H]-felodipine (605 GBq/mmol), felodipine, \textsuperscript{3}H]-inogatran (58.5 GBq/mmol), inogatran, \textsuperscript{3}H]-asimadoline (1514 GBq/mmol), asimadoline, \textsuperscript{3}H]-atenolol (229 GBq/mmol), atenolol, \textsuperscript{3}H]-talinolol (639 GBq/mmol) and talinolol were synthesized at AstraZeneca (Mölndal, Sweden). L-[1-\textsuperscript{14}C]-Lactic acid (1.85 GBq/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), rifamycin SV, and sulfobromophthalein (BSP) were purchased from Sigma-Aldrich Co. (Stockholm, Sweden). Pravastatin sodium was obtained from LKT laboratories, Inc. (St. Paul, MN, USA).

3.1.2. Bovine serum albumin (BSA)

The quality of the BSA determines the binding capacity as well as the binding affinity to a drug. The BSA used in these studies was obtained from Sigma-Aldrich Co. (Stockholm, Sweden). The BSA was a Fraction V (Cat. No.: A-8022) of the following Lot No’s.: 78H1090; 107H0558, and 109H1051.

3.1.3. Other materials

The non-ionic surfactant CEL was obtained from Fluka Biochemika (Buchs, Switzerland). SOLUENE\textsuperscript{®}-350 tissue solubilizer solution and OptiPhase ‘Highsafe’ 3 were purchased from PerkinElmer\textsuperscript{TM} (Boston, MA, USA) and Wallac (Loughborough, UK), respectively, and 2-morpholino-ethanesulfonic acid monohydrate (MES) was obtained from Fluka (Gothenburg, Sweden). All other chemicals were obtained from Sigma-Aldrich Co. (Stockholm, Sweden).

3.2. Cell culture conditions

3.2.1. Caco-2 cell culture

Caco-2 cells, obtained from ATCC (Rockville, MD, USA) at passage 18, were maintained as described previously (Kamm et al., 2001) using heat-inactivated fetal calf serum. All Caco-2 cells used for this thesis were treated under the same conditions as those used for screening at AstraZeneca (Mölndal, Sweden): i.e. they were
used at passage 27-41 and routinely seeded at a density of 2 x 10^6 cells per 175 cm^2-flasks. The cells were grown to 90% confluence and harvested by regular trypsinization using a trypsin (0.05%) - EDTA (0.02%) solution.

The Caco-2 cells were cultivated at 37°C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 8% fetal calf serum (FCS), 1% Minimum Essential Medium of non essential amino acids and 2 mM l-glutamine. The medium was changed every second day. For transport experiments, 2.56 x 10^5 cells were seeded onto each 12 mm polycarbonate cell culture insert – 12 well plates with a filter area of 1.13 cm^2 and a pore size of 0.4 µm (Transwell®, Cat. No. 3401, Corning Costar® Corporation, Cambridge, MA, USA). The cells on the plates were maintained, using culture medium containing antibiotics (100 U ml^-1 penicillin, 100 µg ml^-1 streptomycin), in an atmosphere of 5% CO₂. The relative humidity was 90%. All tissue culture media were obtained from Gibco, Life Technologies (Paisley, Scotland).

3.2.2. MDCK cell culture

MDCK II wild type cells (MDCK-wt) and MDCK II cells transfected with the human MDR1 gene (MDCK-MDR1) were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands). All MDCK type II cells were cultured in DMEM supplemented with 8% FCS, 1% Minimum Essential Medium of non essential amino acids and 1.5% L-glutamine (200 mM) at 37°C, 90% humidity, and 5% CO₂. The final concentration in the medium was 7 mM L-glutamine.

Cells were trypsinized once weekly and seeded at a density of 1 million cells per 185 cm^2 flask. For transport studies, cells were seeded onto polycarbonate Transwell® filter supports (Cat. No. 3401) at a density of 150,000 cells per filter (filter area: 1.13 cm^2). The monolayers were ready for studies 3 days later. The transport experiments were performed as described for the Caco-2 cell monolayers (Transport studies – section 3.3).

3.3. Transport studies

Transport studies were performed at AstraZeneca (Mölndal, Sweden) as previously described (Palm et al., 1999) with slight modifications. Briefly, bidirectional transport rates (apical-to-basolateral: a-b; basolateral-to-apical: b-a) of the test compounds were measured using Caco-2 monolayer cultures grown in the Transwell® system for 23 ± 1 days (Paper I and Paper II) or for 17 ± 1 days (Paper III and Paper IV). Each experiment was performed at least in triplicate. Monolayer permeability to the paracellular marker mannitol was determined simultaneously.

The pH values and the osmolarity of the donor and receiver solutions were controlled at the beginning of the experiment as well as at the end of the experiments. The cell monolayers were preincubated for 25 to 30 minutes to stabilize the desired pH-gradient and BSA-gradient system, respectively.

The volumes of the appropriate incubation medium used were 0.5 or 1.5 ml on the apical and basolateral side, respectively. Samples were withdrawn from the donor side of the monolayers to determine the initial and final donor concentration of the
compound and for calculation of the recovery. The following sampling times were in general used for the receiver sample: 0, 5, 15, 25, 50, 80, and 120 minutes. For a-b transport, 1.5 ml of medium was removed from the basolateral side, whereas for b-a transport, 400 µl of the initial volume (500 µl) was removed from the apical side and replaced with fresh buffer solution.

3.3.1. pH Dependency in transport studies

The incubation medium was Hank’s balanced salt solution (HBSS) supplemented either with 10 mM MES (pH 5.0 – 6.5), or with 25 mM HEPES (pH 7.0 – 8.0). In general, the apically applied pH was altered between pH 5.0 and 8.0, while the basolateral pH was kept at pH 7.4. However, for some mechanistic studies, the pH was also altered (between pH 5.0 and 8.0) on the basolateral side of the monolayers. The pH values of the donor and receiver solutions were verified at the beginning of the experiment as well as at the end of the experiments.

3.3.2. Inhibition studies

Inhibition studies can be performed (i) as competitive inhibition, when inhibitor and test compound are simultaneously added, (ii) as ‘total’ inhibition, when the inhibitor is added in advance, i.e. independent from the test compound, (iii) as concentration-dependent inhibition, when the test compound is added at increasing concentrations, until the transport protein is saturated.

Competitive inhibition studies (Paper I)

For the DDI studies in Paper I, the culture medium was removed from both sides of the monolayers and they were washed with the desired HBSS-buffer. The cell monolayers were then preincubated for 30 min without the test compounds, and then incubated for 25 min at 37°C with the two test compounds for the DDI. The pH values used in the apical compartment were 5.0, 5.5, 6.0, 6.5, 7.0, 7.4, 7.7 or 8.0; while the pH used in the basolateral compartment was 7.4. The receiver medium was replaced after 5, 15, and 25 minutes with fresh buffer solution.

Inhibition of proton-dependent transporters (Paper II)

The aim of a ‘total’ inhibition study is to obtain a defined inhibition of one or several transport proteins. The inhibition can be the result of saturation of the protein or mechanism-based inhibition, e.g. allosteric inhibition. Different pH-gradient systems were applied in order to investigate the inhibition of proton-dependent transport. The cell monolayers were preincubated for 25 min at 37°C in the presence of the inhibitor on both sides without the test compound. The buffer was removed from both sides of the monolayers, which were then incubated for another 25 min in the presence of inhibitor at 37°C with fresh buffer containing the test compound.

Phloretin (nominal concentration: 1 mM), which is a nonspecific inhibitor of several active transport systems, was used as inhibitor. Phloretin inhibits glucose transport in Caco-2 cells (Bissonnette et al., 1996) and is thus a potent indirect but reversible inhibitor of the proton-dependent MCT1 (Stein et al., 2000). l-Lactic acid (10 mM) was included as a more specific and enantioselective competitive inhibitor of MCT1 (Stein et al., 2000; Tamai et al., 1995), and D-lactic acid (10 mM) was included as a
non-substrate of MCT1. In addition, pravastatin (5 mM), rifamycin SV (100 µM) and BSP (25 µM and 50 µM) were used as inhibitors of the proton-dependent OATP-B. Positive controls of transporter function, e.g. L-lactic acid for MCT1, were included in each experiment.

**Concentration dependency in transport studies (Paper II and Paper III)**

In Paper II, salicylic acid and indomethacin were investigated in the concentration range 0.025 to 33 mM and 0.025-0.1 mM, respectively.

In Paper III, each compound was investigated at two different donor concentrations. In order to cover a wide concentration range, the lowest concentrations to be investigated were determined by the specific activity of the radiolabeled compound, and the highest concentrations were in general determined by the drug solubility or toxicity to the monolayers. For quinidine, the highest concentration was that at which the bidirectional transport rates were not significantly different, i.e. 10 µM.

3.3.3. Temperature dependency in transport studies

To investigate the dependence of the permeability of the system on temperature, the transport rate for the compounds was measured at 4, 7, 17, 27 and/or 37°C in transport buffer after preincubation of the cell monolayers for 25 to 30 minutes at the desired temperature. The experiments at 4°C and 7°C were performed in a refrigerator room and a cold storage room, respectively. The experiments at 17°C were performed in a cooled incubator (Sanyo - Biomedical Gallenkamp, Loughborough, UK). The experiments at 27°C and 37°C were performed in a laboratory. In all cases, the plates with the monolayers were placed onto a calibrated plate shaker (BMG LabTechnologies GmbH, Offenburg, Germany), which was set at a high stirring rate (450 rpm) throughout the experiment in order to minimize the influence of the aqueous boundary layer, and was adjusted for heat if necessary, i.e. during the experiments at 27°C and 37°C.

3.3.4. Transport studies using extracellular additives

**Transport studies in the presence of BSA**

Transport studies for Paper III were performed as described above (chapter 3.3) using the following sampling times: 0, 5, 15, 25, 50, 80, and 120 minutes. The incubation medium was HBSS-HEPES supplemented with 0 to 4% BSA (w/v). All experiments were performed at a basolateral pH of 7.4, in order to take the possible effect of pH in the blood on the binding rate into account. To avoid the impact of a pH gradient on drug transport, the apical pH was also set to 7.4. Influence of basolaterally applied BSA (1, 2, and 4%) on the permeability of the monolayer to the drug was determined bidirectionally.

In the initial experiments in Paper IV, BSA was applied apically, basolaterally or on both sides of the monolayers. The receiver medium was replaced after 5, 15, and 25 minutes.
Transport studies in the presence of CEL and BSA

The cell monolayers were preincubated for 30 min without the test compound, and thereafter incubated for 25 min at 37°C with the test compound. The CEL concentrations used in the apical compartment were between 0.00001 – 10% (w/v), whereas no CEL was added to the basolateral compartment. Four percent BSA was added to the basolateral compartment.

3.4. Cell monolayer integrity

3.4.1. Electrophysiological measurements

The initial and final values of transepithelial electrical resistance (TER) of the Caco-2 cell monolayers grown on permeable supports were measured with an epithelial volt-ohmmeter (EVOM), equipped with an SX-2 electrode (World Precision Instruments Inc., Sarasota, Florida, USA). The monolayers were equilibrated for 25-35 minutes before TER measurements. Only cell monolayers with TER values over 220 Ω cm² were used. When inhibitors were used, the effect of the inhibitors on TER was measured after preincubation with the inhibitor.

3.4.2. Mannitol transport

The lower threshold for the permeability of the monolayer to the paracellular marker, mannitol, was set at < 0.5 x 10⁻⁶ cm.s⁻¹.

3.5. Drug concentration in monolayer and filter support

After the final receiver and donor samples were collected, the filters containing the cell monolayers were washed twice with ice-cold HBSS-HEPES buffer (pH 7.4). The filter supports were then cut out with a scalpel and transferred into 20 ml glass vials. The cell monolayers were dissolued with the filter support in 2 ml SOLUENE®-350 tissue solubilizer solution and, thereafter, 15 ml OptiPhase ‘Highsafe’ 3 solution was added to each vial. The radioactivity content of the well mixed samples was measured using a liquid scintillation counter (Wallac, Turku, Finland) and the amount of drug in the sample was then calculated. The radioactivity associated with the cells or adsorbed onto the filter was determined.

The binding of compound to the filter supports was determined in control wells without cells. The filter supports were preincubated at 37°C for 10 minutes in cell culture medium, washed twice with transport buffer and then incubated for 25 minutes in transport buffer including the compound. Afterwards, the filter supports were washed twice with ice-cold HBSS-HEPES buffer (pH 7.4) and treated like the filters containing the cell monolayers.

3.7. Sample analysis

The amount of drug transported was determined from the radioactivity content in the samples using a scintillation counter (Wallac, Turku, Finland), with a quench curve for BSA when necessary.
3.8. Calculations of permeability coefficients and efflux ratios

The transport rate is described by the following equation obtained from Fick’s law

\[ \frac{dQ}{dt} = dC_r(t)\frac{dt}{V_r} = P_{app} \cdot A \cdot (C_d(t) - C_r(t)) \]  

Eq. 5

where \( Q \) [mg] is the amount transported over the cell monolayer, \( C_r(t) \) [mg/ml] the receiver concentration, \( C_d(t) \) [mg.ml\(^{-1}\)] the donor concentration, \( V_r \) [ml] the receiver volume, \( P_{app} \) [cm.s\(^{-1}\)] the apparent permeability coefficient, \( A \) [cm\(^2\)] the area of the filter and \( t \) [s] the time.

For all experiments that were performed under 'sink' conditions, the apparent permeability coefficients were calculated according to the following equation:

\[ P_{app} = \frac{\frac{dQ}{dt}}{A \cdot C_d(0)} \]  

Eq. 6

where the transport rate (\( dQ/dt \)) was determined as the slope obtained by linear regression of cumulative fraction absorbed-time profiles. Equation 6 is obtained from Equation 5, where \( C_d(t) \) have been approximated constant and \( C_r(t) \) on the left side of Equation 5 has been approximated as zero.

When the sink conditions could not be maintained, \( P_{app} \) was determined using the following equation:

\[ C_r(t) = \frac{M}{V_d + V_r} + \left( C_r(0) - \frac{M}{V_d + V_r} \right) \cdot e^{-P_{app}A(V_r + V_d)t} \]  

Eq. 7 a.

Where \( C_r(0) \) is the initial receiver concentration \( V_d \) [ml], the donor volume and \( M \) the total amount compound in the system. Equation 7 a is the exact solution of the differential equation (Equation 5), i.e. \( C_d(t) \) and \( C_r(t) \) is no longer considered constant and \( C_d(t) \) has been recalculated in terms of \( M \) and \( C_r(t) \).

The removing of compound in sampling procedure was considered by the recalculation of the total amount of compound in the system and initial receiver concentration for each sample interval. With these considerations the theoretical value for the receiver concentration \( C_r(t) \) for a certain \( P_{app} \) at each sampling time point, \( t_s \), is expressed by:

\[ C_r(t_s) = \frac{M_i}{V_d + V_r} + \left( C_r(t_{s-1}) \cdot f - \frac{M_i}{V_d + V_r} \right) \cdot e^{-P_{app}A(V_r + V_d)(t_s - t_{s-1})} \]  

Eq. 7 b.

Where \( M_i \) is the amount substance in the system at the sampling interval and \( f \) is the dilution factor caused by the sampling and replacement procedure. The \( P_{app} \) was determined by curve fitting, using the Levenberg-Marquardt algorithm when mini-
minizing the sum of squared residuals \[ \Sigma(C_r(t)_{obs} - C_r(t))^2 \], where \( C_r(t)_{obs} \) is the observed receiver concentration and \( C_r(t) \) is the corresponding calculated concentration at the end of the interval \((i)\).

The ratios of the transport in the b-a direction to that in the a-b direction, and vice versa, were calculated in order to obtain information regarding any asymmetry in the transport of the drug. Thus, (apical) drug efflux ratios and uptake ratios were calculated using the following equations:

\[
\text{Efflux Ratio} = \frac{P_{app(b-a)}}{P_{app(a-b)}} \quad \text{Eq. 8 a}
\]

\[
\text{Uptake Ratio} = \frac{P_{app(a-b)}}{P_{app(b-a)}} \quad \text{Eq. 8 b.}
\]

The relative contribution of the P-gp transport to the overall transport in MDCK-MDR1 monolayers were determined by calculating the efflux ratio obtained in MDR1 transfected cells and dividing this value by the calculated efflux ratio obtained from the transport rates across MDCK-wt cells.

### 3.9. Accounting for low recovery

The recovery \( R [\%] \) is the percentage of original drug mass accounted for at the end of the experiment (the sum of the amounts on the apical and basolateral sides). The recovery was calculated using the following equation:

\[
R = \left[ \frac{(C_a(t) \cdot V_a) + (C_b(t) \cdot V_b)}{C_d(0) \cdot V_d} \right] \cdot 100 \quad \text{Eq. 9}
\]

where \( C_a(t) \) and \( C_b(t) \) are the drug concentrations on the apical and basolateral sides of the monolayer at time \( t \), \( C_d(0) \) is the concentration of the donor at time zero \((t = 0)\), \( V_a \) and \( V_b \) are the volumes of the apical and basolateral compartments, and \( V_d \) is the volume of the donor solution added to the appropriate side of the monolayer.

It was assumed that reductions in recovery were due to a process that occurred immediately, thus affecting the initial concentration on the donor side, and the following equation was used to account for low recovery:

\[
P_{app\ (recovery\ -\ corrected)} = \frac{P_{app} \cdot 100}{R} \quad \text{Eq. 10.}
\]

Note, that this equation cannot be used when time-dependent processes, e.g. metabolism, are the reason for the low recovery.
3.10. Arrhenius equation and the activation energy

Transporters are temperature sensitive. The movement of the transporters within the lipid bilayer, the movement of counterions in the buffer solution and, thus, the activity of the transporters are all decreased, when the temperature is decreased because of the reduced Brownian molecular movement with temperature. The diffusion of the compound which might interact with the transporter in the membrane, is also affected.

Arrhenius plots of the logarithmic permeability values at the same donor concentration versus the reciprocal of the absolute temperature within one pH-gradient system were prepared. Values for the activation energy $E_a$ were determined from the slope of the linear regression lines of the Arrhenius plots, where the rate constant $k$ is correlated with the temperature $T$ [°K] and were calculated in units of [kJ.mol$^{-1}$] according to the Arrhenius equation:

\[
\frac{-E_a}{2.303 \cdot R} = -\frac{E_a}{2.303 \cdot R} \cdot \frac{1}{T}
\]

were $R$ is the gas constant with a value of 8.31J.mol$^{-1}$.K$^{-1}$ and $A$ is the frequency factor for the process.

3.11. Protein binding determination

To determine protein binding an ultrafiltration method was used. Standardized conditions for binding experiments were used to mimic physiological conditions. The pH was 7.4, and the maximal albumin concentration was 4% (w/v), i.e. 40 g.l$^{-1}$. Centrifree® micropartition devices from Millipore (Bredford, MA, USA) were pre-warmed to 37°C and a 400 µl sample volume was loaded into the reservoir. Each protein binding estimation was determined twice using two micropartition devices. After centrifuging in a Hettich Rotixa/KS centrifuge (Tutlingen, Germany) with a fixed-angle bucket at 3,080 rpm (2,000 g) for 20 minutes, radioactivities in 100-µl ultrafiltrate were determined using a liquid scintillation counter (Wallac, Turku, Finland). The initial total concentration ($C_{initial}$) was also determined from the radioactivity content in the initial samples. The fraction of the compound that was unbound ($f_u$) was then determined according to the following equation:

\[
f_u = \frac{C_u}{C_{initial}}
\]

where $C_u$ is the concentration of unbound drug available for permeation. An assumption was made that only the unbound drug is able to move across membranes. Low recovery from either protein-free ultrafiltrate or buffer could indicate adsorptive losses and/or membrane rejection of ligand. Thus, comparable concentrations of
drug in HBSS-HEPES buffer (pH 7.4) without protein were analyzed to ensure that binding to the filter material did not occur.

3.12. Accounting for protein binding

The values for $C_i$ were used in the appropriate equation (Equations 5 to 7) to replace the values of the measured initial concentration on the donor side.

3.13. Theoretical solubility calculation

Prediction models for aqueous solubility are still under development; however, rough estimations are often possible based on the existing models and the Henderson-Hasselbalch equation. The following three linear correlations by Hansch and co-workers (1968), Yalkowsky and Valvani (1980) and Meylan and co-workers (1996) were used to estimate the solubility of the compounds.

The equation by Hansch and co-workers is a linear correlation between $\log P$ and solubility ($r^2 = 0.87$) based on organic liquids (1968).

$$\log \left( \frac{1}{s_0} \right) = 1.34 \cdot c \log P - 0.98 \quad \text{Eq. 13}$$

The estimated solubilities for asimadoline and felodipine based on Equation 17 are 4.6 µM and 2.4 µM, respectively.

Yalkowsky and Valvani combined the lipophilicity term ($\log P$) with the melting point, a solid state characteristic, in order to predict the solubility of solids.

$$\log s_0 = 0.5 - [0.01 \cdot (T_m - 25)] - c \log P \quad \text{Eq. 14}$$

The estimated solubilities for asimadoline and felodipine based on Equation 18 are 3.6 µM and 2.6 µM, respectively.

The predicted solubility of un-ionized asimadoline at 25°C was estimated as 0.5 µM, using the following equation according to Meylan and co-workers (1996):

$$\log s_0 = 0.978 - 0.935 \cdot c \log P - 0.0082 \cdot (T_m - 25) - 0.00468 \cdot MW \quad \text{Eq. 15}$$

where $s_0$ is the water solubility in mol.l$^{-1}$ of the un-ionized species, $T_m$ is the melting temperature of 222°C (van Osdol and Watanabe, 1999) and $MW$ is the molecular weight of 414.5 g.l$^{-1}$. Values for $c\log P$ ($ACD\log P$) and $pK_a$ ($ACDpK_a$) were calculated using the ACDlabs databases version 4.56 (Advanced Chemistry Development Inc., Toronto, Canada). The predicted solubility at a given buffer $pH_d$ ($s_{solid}$, $pH_d = pH$ in the donor compartment) can then be estimated using the Henderson-Hasselbalch equation:
For asimadoline the predicted solubility increased by a factor of 54 (from 0.54 µM to 29.4 µM) when the pH was reduced from pH 8.0 to 5.0. In contrast, felodipine does not ionize over the pH range in the GI tract; therefore, pH-dependent variations in its solubility are not likely. The corresponding predicted solubility of felodipine was 0.4 µM (25°C, pH 7.4, Tm = 142°C, pKa = 3.97). This value is in reasonably good agreement with the measured aqueous solubility of felodipine, which is 2.6 µM at 37°C (Scholz et al., 2002).

In order to ensure that the compounds were in solution in HBSS (pH 7.4, 37°C), an initial donor concentrations below the (predicted) solubility limit, i.e. asimadoline at 25 nM and felodipine at 60 nM, was used in Paper IV.

3.14. Critical micelle concentration determination

For the approximation of the CMC of CEL the following method was used. 1,6-Diphenyl-1,3,5-hexatriene (DPHT) is a fluorescence dye; the intensity of fluorescence increases greatly above the CMC of CEL because of incorporation of the compound into the hydrophobic interior of the micelles. The abrupt change in intensity represents the CMC value (Zhang et al., 1996). A DPHT stock solution was added to various concentrations of CEL solution prepared in HBSS-HEPES buffer (pH 7.4) in a 96-well plate. The final DPHT concentration was ¼ of a saturated solution (pH 7.4) in all wells and the final volume per well was 200 µl. These samples were equilibrated for 24 h in the dark at 37°C. A SPECTRAmax® GeminiXS spectrofluorophotometer from Molecular Devices (Sunnyvale, California, U.S.A.) was used to measure DPHT fluorescence. Each surfactant concentration was measured in quadruplicate. The wavelengths of excitation and emission were 355 nm and 428 nm, respectively. The temperature was controlled at 37°C.

3.15. Statistical analysis

Values are expressed as the means ± standard deviation (SD). All cell culture experiments were performed at least in triplicate. The protein binding was determined in duplicate.

The statistical difference between the permeabilities of the cells to the drugs at different pH gradients or in the absence or presence of 4% BSA was calculated using an unpaired t-test with a two-tailed distribution for comparison of two mean values.

ANOVA was used when more than two mean values were compared; i.e. when the statistical difference between the permeabilities of the cells to the drug at different BSA concentrations was calculated (0, 1, 2, and 4%). A p-value of less then 0.05 was considered statistically significant.
4. RESULTS and DISCUSSION

4.1. pH gradient systems across the Caco-2 monolayers
To investigate the passive and active transport processes under the influence of the physiological pH gradient of the intestine, the pH-dependent transport rates of weakly basic (Paper I), weakly acidic (Paper II) and neutral drugs (Paper I) were studied bidirectionally across Caco-2 monolayers (pH 5.0-8.0).

4.1.1. Effect on weakly basic drugs
Weakly basic compounds, which were chemically and metabolically stable under the in experimental conditions, were selected for Paper I. The compounds were classified as either poorly permeable or highly permeable using calculated physicochemical properties such as polar surface area and lipophilicity (Palm et al., 1996). Atenolol, a poorly permeable compound that is predominantly passively transported across the cell membrane, and metoprolol, a compound that is highly permeable, were selected. Two more compounds that are actively effluxed from cells via P-gp were also selected: talinolol, which is poorly permeable, and quinidine, which is highly permeable (Table I).

<table>
<thead>
<tr>
<th>Permeability</th>
<th>Efflux</th>
<th>Compound</th>
<th>pKa(^{(a)})</th>
<th>(f_a)(^{(b)}) [%]</th>
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<tbody>
<tr>
<td>Low</td>
<td>No</td>
<td>Atenolol</td>
<td>9.6</td>
<td>51 ± 22</td>
</tr>
<tr>
<td>High</td>
<td>No</td>
<td>Metoprol</td>
<td>9.7</td>
<td>98 ± 41</td>
</tr>
<tr>
<td>Low</td>
<td>Yes</td>
<td>Talinolol</td>
<td>9.17(^{(c)})</td>
<td>52 ± 14</td>
</tr>
<tr>
<td>High</td>
<td>Yes</td>
<td>Quinidine</td>
<td>4.2; 7.9</td>
<td>78 ± 12</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Experimentally determined pKa values (Craig, 1990).
\(^{(b)}\) S-atenolol (Mehvar et al., 1990); metoprolol (Feliciano et al., 1990; Ungell and Karlsson, 2003); S-talinolol (Bogman et al., 2005; Zschiesche et al., 2002); quinidine (Rakhit et al., 1984; Ungell and Karlsson, 2003)
\(^{(c)}\) Value for pKa was calculated using the ACDlabs databases version 4.56 (Advanced Chemistry Development Inc. Toronto, Canada).

At equal buffer pH values of 6.5 or 7.4 on both sides of the monolayers, the bidirectional permeabilities for metoprolol were not significantly different (Figure 10). In contrast, the transport rates in the absorptive direction were lower, and those in the secretory direction were higher in the presence of a pH gradient (apical pH < basolateral pH, Figure 10). This behavior was also observed for the poorly permeating compound atenolol (Paper I) and is in accordance with the pH partition hypothesis. In Paper I, the efflux ratio (apical pH 6.5, basolateral pH 7.4) of metoprolol was determined as 4.5 ± 0.4. A comparable efflux ratio (4.8 ± 0.2), displaying one log unit in difference between the two buffer pH values, was seen with an apical pH 5.5 and basolateral pH 6.5 (Figure 10). These efflux ratios, obtained under different pH conditions, but with the same relative change in the ratios of uncharged to charged amounts of compound in both compartments, were in good agreement and, thus, could be taken to reflect passive efflux. These results illustrated that passive (pseudo-) efflux can occur across Caco-2 cell monolayers under certain experimental
conditions. Thus, the asymmetric transport was indeed the result of the pH-dependent concentration of un-ionized species of the drug in the apical compartment.

The transport rates in the absorptive direction were in general higher at a basolateral pH of 6.5 (Figure 10A) than those at pH 7.4 (Figure 10B; data published in Paper I), when values at the same apical pH were compared. In contrast, the transport rates in the secretory direction were consistently lower at a basolateral pH of 6.5 than at 7.4. These results were in agreement with the pH-partitioning hypothesis. The fraction of a basic compound that was ionized decreased as the pH changed from 6.5 to 7.4, and the concentration of un-ionized compound was lower at basolateral pH 6.5, thus offering an increased sink for the absorptive transport direction. In the secretory transport direction, however, the un-ionized species, which drove the passive transmembrane transport of the compound, was lower at pH 6.5 than at 7.4 for a weakly basic drug, leading consequently to lower transport rates at basolateral pH 6.5.

For the actively effluxed weakly basic compounds talinolol and quinidine, the transport rates in the absorptive direction increased and those in the secretory direction decreased as the apical pH increased. However, at equal buffer pH values on both sides of the monolayers, the impact of the apical efflux transporter P-gp was still reflected by the significant efflux ratios of 6.0 and 4.0 at concentrations of 58 nM (talinolol) and 50 nM (quinidine), respectively (Paper I).

Several approaches for improving the predictive power of the Caco-2 cell assay have been investigated. Firstly, as suggested by Yamashita and co-workers (Yamashita et al., 2000) and Yee (Yee, 1997), an optimized permeability model for predicting \( f_a \) should include transport rates obtained in a pH-gradient system using an apical pH of 6.0 or 6.5, respectively, and a basolateral pH of 7.4. These pH-gradient systems mimic the acid microclimate in vivo. Secondly, a smaller pH gradient of 6.8/7.4 (apical pH/basolateral pH), as suggested by Volpe (Volpe, 2004), might be not efficient enough to establish a good functioning of proton-dependent transporters (Figures 14 and 16). Finally, extreme pH gradients (e.g. 5.0/7.4 and 5.5/7.4) can be a
challenge for the cells and the investigator. (The proportion of cell monolayers retaining good integrity (TER, Mannitol) is generally lower than at apical pH 6.0 or 6.5.) However, the data presented here and in Paper I show that a system with different pH values on the apical and basolateral sides will lead to different concentrations of uncharged drug species, which in turn will lead to asymmetry in bidirectional transport. This asymmetry in the transport rate over the membrane can occur independently of active transport. Therefore, it is not advisable to use, as suggested in the literature (Volpe, 2004), an efflux ratio obtained in a pH-gradient system for predictions of active transport involvement.

While drug efflux ratios obtained from a pH-gradient system can falsely indicate active apical efflux for weakly basic drugs in in vitro systems, these pH-gradient effects may influence drug absorption in vivo. Therefore, a refined screening model for the prediction of $f_{m}$, assuming that the jejunum is the major site of absorption for the drug, passive permeation of a compound should be measured at pH 6.0/7.4 in the absorptive transport direction only. This pH gradient mimics the acid microclimate and supplies a proton gradient over the entire monolayer as well as over the apical membrane.

4.1.2. Effect on weakly acidic drugs

The model acidic compounds indomethacin and salicylic acid were selected for Paper II since they are sufficiently soluble for the purpose of this study (Yazdanian et al., 2004), chemically and metabolically stable and ionizable within the physiological pH range of the GI tract. While indomethacin is predominantly passively transported across the cell membrane, salicylic acid is both passively and actively transported (Table II).

<table>
<thead>
<tr>
<th>Permeability</th>
<th>Main transport</th>
<th>Compound</th>
<th>$pK_{a}$ (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Passive</td>
<td>Indomethacin</td>
<td>4.5</td>
</tr>
<tr>
<td>High</td>
<td>Active</td>
<td>Salicylic acid</td>
<td>2.97; 13.4</td>
</tr>
</tbody>
</table>

(a) Experimentally determined $pK_{a}$ values (Craig, 1990).

In contrast to the results obtained for weakly basic compounds, transport of the acidic compounds was higher in the absorptive direction than in the secretory direction when the apical pH was lower than the basolateral pH. Figure 12 shows the bidirectional pH-dependent transport of indomethacin when the apical pH was varied between 5.0 and 8.0, while the basolateral pH was kept at 6.5 or at 7.4. The values for Figure 11B are published in Paper II. The transport rates of indomethacin decreased 22-fold and 14-fold for the basolateral pH of 6.5 and 7.4, respectively, in the absorptive direction with increasing apical pH, while transport rates in the secretory direction increased 10-fold and 20-fold for the basolateral pH of 6.5 and 7.4, respectively (Figure 11).

* Indomethacin is practically insoluble in water. The predicted solubility of the un-ionized species at 25°C is, according to Equation 13, estimated as 1.8 µM, Equation 14 as 8.5 µM, and Equation 15 as 2.3 µM. The dissolved radioligand was supplied in ethanol. The final concentration of ethanol in the donor solution was no more than 1%.
Bidirectional pH-dependent transport of the rapidly transported weakly acidic drug, indomethacin, across Caco-2 monolayers. When the basolateral pH was kept at 6.5 (A) or 7.4 (B) and the apical pH was increased, the apparent permeability coefficient ($P_{app}$) in the absorptive direction (black bars) decreased and the $P_{app}$ in the secretory direction (white bars) increased. Each bar indicates mean ± SD (n ≥ 3). The values used in Figure B are published in Paper II.

This was expected, according to the pH-partitioning hypothesis, since the amount of uncharged species that drove the passive permeation of the drug, in the apical compartments decreased with increased buffer pH from 5.0 to 8.0.

Using data presented in Paper II, the efflux ratio (apical pH 6.5, basolateral pH 7.4) for indomethacin was determined as $7.4 ± 0.5$ by calculating the permeability in absorptive direction in the non-sink equation (Equation 7; $P_{app (a-b)} = 257 ± 8 \times 10^{-6}$ cm.s$^{-1}$ and $P_{app (b-a)} = 34.6 ± 2 \times 10^{-6}$ cm.s$^{-1}$). A comparable efflux ratio for indomethacin (apical pH 5.5, basolateral pH 6.5) of $7.7 ± 0.4$ was obtained using the transport rates shown in Figure 11. These values were one indication of the mainly passive transport of indomethacin across Caco-2 cell monolayers.

In addition, indomethacin transport was not inhibited by MCT1-, OATP-B-, or glucose-transport inhibitors (L-Lactic acid, pravastatin, phloretin), or by salicylic acid (Paper II). Most importantly, there was no concentration dependency of indomethacin observed in any pH-gradient system, i.e. in a system where the apical pH was lower than the basolateral pH and in a system where a pH of 7.4 was applied in both compartments. Hence, the asymmetric transport of indomethacin was not caused by real carrier-mediated uptake in the Caco-2 cell system. As with weakly basic compounds in Paper I, it was shown in Paper II that asymmetric transport of weakly acidic compounds, observed using a pH-gradient system, can falsely indicate active transport in in vitro systems. However, physiological pH-gradient effects of at least one pH unit may influence drug absorption in vivo as they do for weakly basic compounds (Paper II).

Salicylic acid was the second compound investigated in Paper II. The results for salicylic acid indicated interplay of both pH-dependent active transport and passive diffusion. General inhibitors were used to inhibit the active transport of salicylic

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* It should be noted that indomethacin is actively transported in vivo by OATs. However, these transporters are probably not expressed in the Caco-2 cell clone used (Seithel et al., 2004).
acid. The data indicated that OATP-B and MCT1, two proton-dependent transporters in human intestine, which are also expressed in Caco-2 cells (see appendix), are involved in the uptake of salicylic acid. These data also left room for the speculation that a third transporter is involved in the transport process. This transporter can be either an apical uptake or a basolateral efflux transporter, which seems to respond only to a pH gradient over the whole monolayer. Since no accumulation of salicylic acid in the cell monolayer was observed, a basolateral efflux transporter that transports salicylic acid actively out of the cell was suggested. A possible candidate is the heterodimeric organic solute transporter (OST)α/β for which mRNA was recently detected in kidney and intestine (Dawson et al., 2005; Hubbert et al., 2004). In Paper II, an extreme pH gradient (apical pH < 6.0) was used, which can be helpful for mechanistic studies, investigating proton-dependent transport, to mimic a high proton concentration located close to the transporter or to inhibit the NHE3 (Thwaites et al., 2002). Therefore it can further be concluded that the proposed additional transporter for salicylic acid should be directly or indirectly pH-gradient dependent but should be independently working of the NHE3, since the NHE3 is inhibited under the investigated pH-gradient conditions (5.0/7.4/7.4).

The results of Papers II indicated that a pH-gradient over the monolayer, rather than over the apical membrane alone, is important for the function of some transporters. As a refinement it can be suggested that weakly acidic compounds should be studied in both a pH-gradient system across cell monolayers (apical pH 5.0-6.0 and basolateral pH 7.4) and without a pH gradient, when investigating active uptake (basolateral efflux) systems.

4.1.3. Effect on concentration dependency

The transport of the neutral P-gp substrate, digoxin, was unaffected by changes in buffer pH, but was affected by changes in donor concentration (Papers I and III). Constant efflux ratios (8.9 ± 0.8; n = 90) were obtained over the physiological pH range, reflecting the efflux of the neutral drug digoxin by a transporter, with an activity that is independent of the pH gradient. However, the efflux ratios were reduced when the donor concentration was increased. This was mainly a consequence of a decrease in the transport rates in secretory direction, caused by saturation of the transporter. Thus, the function of P-gp seems not to depend on pH, but the efficiency of the transport depends on the amount of compound available at the binding site. Thus, any pH change along the GI tract would not affect digoxin directly. This result is in agreement with the decreased permeation of digoxin further down the intestine, which is consistent and can be correlated with an increase in P-gp expression further down the GI tract (Stephens et al., 2001; Zimmermann et al., 2005).

Concentration-dependent transport and inhibition studies of compounds are normally investigated with the same buffer pH on both sides of the monolayers, e.g. taurocolic acid (Wilson et al., 1990), vinblastine (Hunter et al., 1993), biotin (Ng and Borchartd, 1993) and fexofenadine (Petri et al., 2004). However, proton-dependent transporters have also been investigated in pH-gradient systems (apical pH < basolateral pH), e.g. salicylic acid (Takanaga et al., 1994), benzoic acid (Tsuji et al., 1994) and Gly-Sar (Bravo et al., 2004).
For weakly basic compounds, the pH gradient used has to be accounted for (Paper I). This can be illustrated by the change in the efflux ratios at different concentrations and in different pH-gradient systems. Figure 12 shows the calculated pH-dependent efflux ratios for quinidine at three different donor concentrations.

**Figure 12:** Efflux ratios calculated from the bidirectional pH-dependent transport rates of the weakly basic drug, quinidine, across Caco-2 monolayers at three concentrations. When the basolateral pH was kept at 7.4 and the apical pH was increased, the apparent permeability coefficient ($P_{app}$) in the absorptive direction increased ($n \geq 3$) and the $P_{app}$ in the secretory direction decreased ($n \geq 3$). Hence, with increased apical pH the efflux ratios decreased. Within each pH-gradient system, an increased concentration of quinidine gave a reduction in the efflux ratio. Each bar indicates mean ± SD.

The dramatic change in efflux ratios between the various pH-gradient systems reflected the combined alterations in the transport rates of the various active and passive transport processes that were involved. The alterations within a pH-gradient system can be assigned to the concentration-dependent part of the transport, which is generally assumed to be the active part. For quinidine, the most important transporter is probably P-gp (Emi et al., 1998). Since quinidine is a weakly basic compound, only the concentration-dependency observed, when an equal pH of 7.4 was applied on both sides of the monolayer, reflects the active efflux. The concentration-dependency in all other pH-gradient systems is a mixture of active and passive efflux. The functioning of P-gp is independent of intracellular and extracellular pH (Altenberg et al., 1993) and, if P-gp would be the only transporter involved in the transport of quinidine, comparable $K_m$ values within the different pH-gradient systems should be obtained, when calculating only with the un-ionized amount of the drug.

As with the weakly basic compounds, the concentration-dependency relationship within one particular pH-gradient system does not necessarily contain the information for distinguishing between active and passive transport of a weakly acidic compound. Figure 13 shows the concentration-dependent and pH-gradient dependent bidirectional transport rates for salicylic acid.

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* The efflux ratios of the lowest concentration (50 nM) are calculated from data of the pH-dependent bidirectional transport rates for quinidine published in Paper I.
Figure 13: Bidirectional pH-dependent transport of the rapidly transported weakly acidic drug, salicylic acid, across Caco-2 monolayers at three concentrations. When the basolateral pH was kept at 7.4 and the apical pH was increased, the apparent permeability coefficient (P_{app}) in the absorptive direction (black bars) decreased and the P_{app} in the secretory direction (white bars) increased. The extent of the change in permeability was concentration and pH dependent. Each bar indicates mean ± SD (n ≥ 3).

No alterations in the transport rates for salicylic acid were observed when the apical and basolateral pHs were equal at 7.4. However, transport rates in the absorptive direction increased and those in the secretory direction decreased with decreased apical pH and within a pH-gradient system with decreased concentration of salicylic acid.

When the pH-gradient system 5.0/7.4/7.4 (apical pH/intercellular pH/basolateral pH) was used, a K_m value of 5.4 ± 0.7 mM was calculated (Paper II). Takanaga and co-workers reported a K_m value, probably measured at apical pH 6.0 and basolateral pH 7.4 across Caco-2 monolayers, of 5.28 ± 0.72 mM (Takanaga et al., 1994). However, at pH 7.4 on both sides of the monolayers, a K_m value cannot be obtained. The apparent K_m values calculated for salicylic acid will decrease with increased apical pH as indicated by these results (Figure 13). It can therefore be argued that the use of apparent K_m values obtained from a pH-dependent process can be questioned especially when several transporters are working simultaneously.

* The pH-dependent bidirectional transport rates for salicylic acid at 25 µM are published in Paper II.
4.1.4. Effect on temperature dependency

It has been suggested that alterations in drug transport across tissue and monolayers due to temperature changes can be used to identify active transport processes (Hidalgo and Borchardt, 1990a). Hidalgo and Borchardt state that "the normal range for \( E_a \) values associated with enzymatic reactions or carrier-mediated processes lies between 7 and 25 kcal.mol\(^{-1}\) (30-107 kJ.mol\(^{-1}\)), while \( E_a \) values associated with simple diffusion processes are less than 4 kcal.mol\(^{-1}\) (17 kJ.mol\(^{-1}\))." In Paper II, the effect of temperature change on the passive and active transport of two acidic compounds, indomethacin and salicylic acid, was investigated. It was found that the transport rates of both compounds were highly affected by temperature and \( pH \) as illustrated for salicylic acid in Figure 14A. The \( E_a \) values were obtained from the slope of the corresponding Arrhenius plot (Figure 14B, Table III).

The results presented here and in Paper II do not support the hypothesis that passive and active transport of weakly acidic or weakly basic compounds across a lipid membrane are distinguishable by change in temperature. In contrast, \( E_a \) values for passively and actively transported compounds significantly overlap (Table III); precluding their use as a reliable criterion for the identification of active transport.

\[^{*}\text{permeability values were obtained at the same initial donor concentration}\]

Figure 14: (A) Temperature dependence of the pH-dependent transport of salicylic acid (25 µM) across Caco-2 monolayers. (B) Arrhenius-plot. The apparent permeability coefficients (\( P_{app} \)) in the absorptive direction were measured at 4, 7, 17, 27 and 37°C, using the non-gradient systems (\( pH 7.4 \)) on both sides of the monolayer, or in the presence of a \( pH \)-gradient (apical \( pH 5.0, 5.5 \) or 6.5 and basolateral \( pH 7.4 \)), or in the presence of a double \( pH \)-gradient system (\( pH 5.0, 5.5 \) or 6.5 on both sides of the monolayer, while the \( pH \) was always assumed to be around 7.3). Each point indicates mean ± SD, (\( n \geq 4 \)).
Table III: Activation energies

<table>
<thead>
<tr>
<th>Compound</th>
<th>Main transport</th>
<th>pH on the apical side</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Passive</td>
<td>35(a)</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>Active</td>
<td>50</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>Passive</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. not determined. The basolateral pH was always kept at 7.4.
(a) Activation energies were expressed in units of [kJ.mol⁻¹].

4.1.5. pH Dependency of drug-drug interactions

Apart from digoxin, several compounds that are clinically often co-administered with digoxin also use P-gp as a transporter, thus, there is potential for DDIs (Englund et al., 2004). In the screening phase, the final dose of the drug is often not known; however, data presented here and in Paper I indicate the importance of a good estimate for this parameter in designing a relevant DDI study in vitro.

In Paper I, the dependency of the clinically relevant digoxin-quinidine interaction on pH was investigated. The concentrations used were estimated from (i) the given dose, (ii) the relative relationship of the doses of each drug in vivo, (iii) the concentration causing toxicity to the cells and (iv) the C_max of the compound from in vivo studies. The transport rates of the two compounds both, in combination and separately, were measured across Caco-2 cell monolayers. The digoxin-quinidine interaction was clearly dependent on pH, although digoxin transport itself is not pH dependent (Paper I), indicating that the interaction is dependent on the amount of charged and uncharged species of quinidine available at the binding site of P-gp.

By using different ratios of the dose of quinidine and digoxin, the relevance of this interaction becomes even more apparent (Figure 15). If the quinidine concentration is low, the interaction in vitro is not significant (Figure 15A) and from this result, the relevance in vivo would be predicted to be insignificant; however, if the quinidine concentration is high in comparison to the relative dose ratio, then the interaction in vitro is dramatic (Figure 15B). Thus, conclusions relevant to the in vivo situation could be drawn from these data.

Figure 15: pH-Dependent permeability values for bidirectional transport of the neutral P-gp substrate, digoxin (2 nM), in the presence of (A) 10 µM quinidine or (B) 100 µM quinidine. Values are means ± SD (n ≥ 3).
It should be borne in mind that the *in vitro* experiments should help to predict and explain *in vivo* observations. The concentration-dependency relationship of the competitive inhibition studies shown in Figure 15, indicate that the relative dose ratio will determine the extent of the DDI. Thus, the concentration of free drug in its un-ionized form will determine the amount of drug that enters the cell via the apical membrane. This amount has to be high enough to reach a concentration that affects the transporter (above the apparent $K_m$ of the drug on P-gp) and thus inhibit the transport of the competing drug. Thus, the dependence on $pH$ in combination with the relative concentration ratio has clinically relevant repercussions for P-gp-dependent DDI and should be considered in the further design of *in vitro* models for DDIs.

Independently of whether the inhibition is competitive or non-competitive, the $pH$-dependency of the inhibitor has to be taken into account, since the $pK_a$ of the inhibitor will determine the amount of inhibitor available at the inhibitor-binding site of the transporters. If the $pK_a$ value of the compound is known, the appropriate conditions can be applied in the permeability assay. If the $pK_a$ value is not known, the concentration-dependency and inhibition studies might have to be performed twice, with and without a $pH$ gradient.

4.1.6. The paracellular pathway

*In vivo* pharmacokinetics indicate that atenolol is slowly and incompletely absorbed (Mehvar et al., 1990). This is in agreement with *in vitro* data that suggest that atenolol slowly permeates the GI membrane, thus resulting in a low fraction absorbed. Consistent with the $pH$-partition hypothesis, basic molecules are less ionized in the ileum ($pH = 7.3$) than in the jejunum ($pH = 6.5$) and are therefore more quickly absorbed in the lower parts of the GI tract (Taylor et al., 1985). This $pH$-dependent transport of bases was confirmed in the *in vitro* experiments published in Paper I using Caco-2 cell monolayers. However, the hydrophilic and poorly permeating basic β-blocker atenolol shows slightly faster transport in the upper GI tract (Taylor et al., 1985), indicating a more significant role of the paracellular route in the jejunum.

The paracellular pathway cannot be ignored in the Caco-2 cell model; in fact, paracellular diffusion has been investigated previously in this model (Adson et al., 1995; Adson et al., 1994; Karlsson et al., 1999; Nagahara et al., 2004; Pade and Stavchansky, 1997; Tavelin, 2003). The impact of the paracellular pathway can be seen in the different efflux ratios obtained for atenolol and metoprolol in the presence of a $pH$ gradient (apical $pH$ 6.5; basolateral $pH$ 7.4) (Paper I). Although atenolol and metoprolol have approximately the same $pK_a$ values and should, according to $pH$-partitioning theory, be equally affected by the changes in the buffer $pH$, the efflux ratio for atenolol is lower (1.7 ± 0.2) than for metoprolol (4.5 ± 0.4). The contribution of the paracellular pathway affects the ratio more for the poorly permeating drug, atenolol, than for the rapidly permeating drug, metoprolol. Adaptation of the calculations in the publication by Palm et al. (Palm et al., 1999) reveal that the
paracellular route contributes less than 20% to the overall transport of atenolol, compared with around 0% for metoprolol.\(^*\)

At low donor concentrations (25 \(\mu\text{M}\)) and in a \(pH\) gradient of 5.0/7.4/7.4 (apical \(pH\)/intracellular \(pH\)/basolateral \(pH\)), over 80% of the salicylic acid transport is via active carrier-mediated transport, 17% is via pure passive diffusion and less than 3% is via the paracellular pathway as estimated from the data presented in Paper II. In the non-gradient system, only passive transcellular diffusion and the paracellular pathway are involved. Pade and Stavchansky estimated the contribution of the paracellular pathway to the overall transport of salicylic acid as 1% at apical \(pH\) 7.4 and 0% at apical \(pH\) 5.4 (Pade and Stavchansky, 1997). Thus, the extent to which the various active and passive transport processes are involved in drug transport is affected by the fraction of ionized and un-ionized species according to the \(pH\)-partitioning theory.

4.2. BSA-gradient systems across Caco-2 monolayers

Two groups of drugs were investigated in Paper III. Group 1 included compounds transported by passive diffusion only and group 2 comprised compounds whose transport is influenced by active apical efflux systems such as P-gp and/or BCRP (Table IV). Each group contained compounds with either high or low protein binding and high or low \(f_a\), as ascertained from the literature (Table IV). In order to cover a wide variety of compounds and to fulfill the above criteria for the \(in vitro\) experiments, the ‘low’ and ‘high’ limits for both protein binding and \(f_a\) were set as 0-30% and 70-100%, respectively (Table IV). This was despite the general view that a compound is highly protein bound \(in vivo\) when over 95% of the compound is plasma protein bound and that a compound is considered to be easily absorbed when the extent of absorption in humans is 90% or more of an administered dose.

Table IV: Oral fraction absorbed \((f_a)\) and protein binding \((PB)\) of the investigated drugs.

<table>
<thead>
<tr>
<th>(PB)</th>
<th>Permeability</th>
<th>Efflux</th>
<th>Compound</th>
<th>(PB) [%]</th>
<th>(f_a) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Low</td>
<td>No</td>
<td>Mannitol</td>
<td>-</td>
<td>16-26</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
<td>No</td>
<td>Metoprolol</td>
<td>11</td>
<td>80-100</td>
</tr>
<tr>
<td>High</td>
<td>High</td>
<td>No</td>
<td>Propranolol</td>
<td>87</td>
<td>90-100</td>
</tr>
<tr>
<td>High</td>
<td>Low</td>
<td>No</td>
<td>n. a.</td>
<td>n. a.</td>
<td>n. a.</td>
</tr>
<tr>
<td>Low</td>
<td>Low</td>
<td>Yes</td>
<td>Inogatran</td>
<td>10-20</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
<td>Yes</td>
<td>Digoxin</td>
<td>25</td>
<td>81</td>
</tr>
<tr>
<td>High</td>
<td>High</td>
<td>Yes</td>
<td>Quinidine</td>
<td>87</td>
<td>66-90</td>
</tr>
<tr>
<td>High</td>
<td>Low</td>
<td>Yes</td>
<td>Doxorubicin</td>
<td>76</td>
<td>&gt; 5</td>
</tr>
</tbody>
</table>

n. a. = not available.


\(^*\) For the purpose of the study described in Paper I atenolol was selected as a poorly permeating drug that is predominantly passively transported across the cell membrane, since the paracellular contribution (< 20%) does not affect the general interpretation of the data. However, a discussion of the paracellular transport in Paper I was avoided, since the aim was to emphasize the impact of a \(pH\)-gradient on the transcellular pathway.
The results presented in Paper III show that basolaterally applied BSA, with the aim of mimicking \textit{in vivo} protein binding, modified both passive and active drug transport in the Caco-2 cell model. The inclusion of BSA helped to maintain sink conditions during drug transport in the Caco-2 cell model. Basolateral BSA increased transport in the absorptive direction and decreased transport in the secretory direction. Thus, the inclusion of BSA in the receiver compartment increased the transport rates, while inclusion of BSA in the donor compartment decreased the rates. This was also observed in Paper IV, where BSA was applied either on one side or on both sides of the monolayers. In order to account for the unbound substance in the presence of BSA, the unbound fraction \( f_u \) was determined and the unbound concentration \( C_u \) of each of the compounds was calculated, according to Equation 16. Determination of \( C_u \) provides an opportunity to elucidate the different mechanisms behind the alterations.

4.2.1. Accounting for the maximal effect of protein binding

If the recovery of the compound is around 100\% in the absence of BSA and the compound is highly protein bound (99.9\%; \( f_u = 0.1\% \)), then according to Equation 17, the expected maximal increase in permeation due to extracellular BSA would be approximately twice that obtained without BSA, assuming that the BSA represents a perfect sink which reduces the back flux of the compound across the basolateral membrane to zero. Thereby also reducing the intracellular concentration to such a low level that no compound passively effluxed over the apical membrane.

\[
P_{\text{app}}(a-b) (0\% \text{BSA}) \cdot \frac{100 + (100 - f_u(4\% \text{BSA}))}{100} \approx P_{\text{app}}(a-b)(4\% \text{BSA})
\]

Eq. 17

where \( P_{\text{app}}(a-b)(0\% \text{BSA}) \) is the apparent permeability in the absorptive direction in the absence of BSA, \( P_{\text{app}}(a-b)(4\% \text{BSA}) \) is the apparent permeability in the absorptive direction in the presence of 4\% BSA, and \( f_u(4\% \text{BSA}) \) is the percentage of unbound compound in the presence of 4\% BSA.

This equation can be used for compounds that have poor recovery in traditional studies of \textit{in vitro} permeability (without BSA), often seen for lipophilic BCS class II compounds, which are per definition poorly soluble and highly permeating. For these compounds a maximal theoretical transport rate in the absorptive direction can be estimated with Equation 17. Felodipine and asimadoline are two highly lipophilic BCS class II compounds which can be easily trapped in the lipid membrane (Paper IV). Inclusion of BSA reduced the amount of felodipine and asimadoline associated with the cell monolayer, the filter support and to the plastic of the equipment (Figure 16). These results explain the observed increase in the recovery of these two compounds at the end of the experiments in the presence of BSA compared to experiments without BSA.
Figure 16: Effect of increased concentrations of basolaterally applied bovine serum albumin (BSA, w/v) on the intracellular concentration of asimadoline at an initial donor concentration of 25 nM. Values are means ± SD (n = 3).

Using Equation 17, a theoretical maximum transport rate for the passively transported felodipine in the absorptive direction would be $75 \times 10^{-6} \text{ cm.s}^{-1}$. For the actively transported asimadoline, the corresponding theoretical maximum transport rate in absorptive direction would be $62 \times 10^{-6} \text{ cm.s}^{-1}$ at the investigated initial donor concentration (20 nM). This theoretical transport rate (for 100% recovery of the compound) can then be compared to permeation values obtained in the traditional Caco-2 cell model (without BSA), for instance, for the prediction of $f_a$. This conversion facilitates the use of BSA in the Caco-2 cell model as a secondary screening tool.

4.2.2. Impact of basolateral BSA on transport in the absorptive direction

For passively transported compounds, the relative increase in the permeation of the compound across the Caco-2 cell monolayers in the absorptive direction was directly proportional to the extent of protein binding for each compound (Figure 17A). Mannitol showed no change in permeability as expected for a compound without PB (Figure 17A). The slope of 0.78 can be used to estimate the maximal expected impact of 78% change if a protein binding of 100% is assumed using the experimental set-up with basolaterally applied BSA and the limited set of compounds (mannitol, metoprolol and propranolol) (Figure 17A). The difference between the theoretical maximal change of 100% and the experimentally obtained maximal change of 78% can probably be explained with the amount bound to the monolayer (as representative of the intracellular concentration, Figure 16) that was only reduced in the presence of BSA but not totally abolished. Thus, the sink was improved in the presence of BSA, but not perfect.
The relative change in the apparent permeability coefficients ($P_{app}$) in the absorptive direction caused by the sink conditions created by basolaterally applied 1, 2 or 4% BSA is correlated to the fraction of compound bound to BSA. A good correlation for the passively transported compounds (mannitol, metoprolol, propranolol) was obtained ($r^2 = 0.9$) (A), while a very weak correlation was observed for the actively effluxed compounds (inogatran, doxorubicin, digoxin, quinidine) ($r^2 = 0.4$) (B), probably due to the dose dependency of the active transport component. Values are for each compound from two initial donor concentrations. The trendlines for the passively (solid line) and actively (dashed line) transported compounds are shown.

Figure 17B shows that the correlation coefficient for the actively effluxed compounds was $r^2 = 0.4$ and the slope was slightly lower (0.74) than the slope of the correlation for the passively transported compounds (Figure 17A). For the actively transported compounds, binding to BSA led to a scattered and compound-specific deviation from the trendline obtained from the passively transported compounds. One reason for this could be that the relative change in permeation of actively effluxed compounds in the absorptive direction was not only increased proportionally to the extent of protein binding for each compound, but at the same time additionally decreased by active transport in secretory direction. The total effect was then observed in the transport rates. The impact of active transport in the secretory direction on the overall transport in the absorptive direction was concentration dependent and more efficient at initial donor concentrations around the $K_m$ of the compound for the active transporter. The concentration at the transporter binding site was reduced because of the presence of BSA which, at least in the case of doxorubicin, reduced the amount of compound in the intracellular compartment (Paper III).

When Saha and Kou investigated transport in the absorptive direction, they concluded that the inclusion of 4% BSA in the receiver chamber during transport studies can dramatically affect the estimated permeation across Caco-2 monolayers and the
BCS permeability ranking of highly lipophilic compounds. This is presumably due to improved sink conditions and/or a reduction in non-specific drug adsorption to plastic wells (Saha and Kou, 2002) and the lipid bilayer as shown for asimadoline (Paper IV). They also suggested that the permeability classification of a compound with a log P less than 2.4 will not be affected by the presence of receiver BSA (Saha and Kou, 2002). However, as shown for doxorubicin ($clogP = -1.42$; BioByte MacLogP V. 2.0 Software, measured $logP = 0.1$), the impact of BSA is strictly related to the extent of protein binding and not to the lipophilicity of the compound (Figure 17B).

Inclusion of BSA can increase the recovery, and will affect all compounds in proportion to the extent of their protein binding under the experimental conditions. In an ideal case, it would be possible to account for the unbound concentration, e.g. by determining the free concentration via an ultracentrifugation method (Paper III). However, even when $C_u$ cannot be measured, an estimate for purely passively transported compounds can be arrived at, as discussed for felodipine (Paper IV). Inclusion of basolateral BSA without correction for protein binding probably gives a more physiologically sound model of absorption, since it mimics both protein binding in the blood and the sink effect better than the aqueous buffer solution normally used in in vitro systems. However, this hypothesis remains to be shown on an increased number of compounds.

4.2.3. Impact of basolateral BSA on the efflux/uptake ratios

Efflux and uptake ratios are used to identify any asymmetry in the bidirectional transport of a compound, which might be due to active transport. Ratios from comparable transport rates, i.e. good recovery (< 95%) calculated for the same cut-off sink, at the same initial donor concentration and without a pH gradient (Chapter 4.1.), can be used to estimate the in vivo relevance of asymmetrical transport.

An uptake ratio is observed for passively transported compounds in the presence of basolateral BSA. Similarly, transport rates for the actively transported compounds increased in the absorptive direction and decreased in the secretory direction in the presence of basolateral BSA. Thus, the efflux ratios of the actively transported compounds were reduced in the presence of basolaterally applied BSA. This change suggests that active secretory transport for highly protein bound compounds might have less effect on in vivo absorption than predicted from traditional Caco-2 cell models (without BSA).

Under controlled experimental conditions, the transport rates in the absorptive and secretory directions are affected to the same degree by BSA. Since extracellular BSA can reduce the amount of compound at the binding site of active transporters, as observed for doxorubicin in Paper III and asimadoline in Paper IV, their impact on the observed overall transport will be altered in a concentration dependent manner. However, the presence of BSA does not directly affect the functionality of active transport systems (such as P-gp) as shown in Paper III.

Scheme 1 illustrates this deviation. In the presence of BSA a lower $P_{app}$ is obtained in secretory direction (step 1), but this $P_{app}$ actually represents a values for a lower
concentration of drug, $C_u$, (step 2). Calculating a new $P_{app}$ for this lower donor concentration, $C_u$, will lead for a passively transported compound to a $P_{app}$, which is not different from the original $P_{app}$ (Scheme 1A), but for an actively transported compound the $P_{app}$ can increase (Scheme 1B).

**Scheme 1:** Basolaterally applied BSA reduces the unbound concentration of the compound in proportion to the protein binding of the compound. Thus, all the apparent permeability coefficients ($P_{app}$) in the secretory direction obtained in the presence of BSA are lower than those obtained without BSA. Due to the different dose dependency of the passive (A) and active (B) transport component (dotted lines), $P_{app}$ calculated with the unbound concentration as donor concentration can be higher than the $P_{app}$ observed in the absence of BSA for actively transported compounds.

Thus, the involvement of active transport processes can be estimated, when the efflux ratios re-calculated from permeabilities considering the $C_u$ for a compound differ from the efflux ratios obtained from the traditional model (without BSA) at the same initial donor concentration (Scheme 1). The greatest increase should be observed around the concentration of the $K_m$ value. This principle can be used to distinguish after re-calculation for $f_u$ between actively and passively transported compounds as shown in Paper III.

It has been argued that the role of apical efflux transporters as an intestinal barrier to oral bioavailability may have been overemphasized, particularly when evaluated in in vitro systems such as the Caco-2 cell model (Lin and Yamazaki, 2003). Significant transport in the secretory direction across cell monolayers does not necessarily correspond to unsatisfactory oral bioavailability in vivo, as seen for compounds like digoxin and cyclosporin A. Walgren and Walle observed in their study that the efflux of the investigated compounds in the presence of basolateral BSA was significantly reduced, mainly due to a reduction in the transport rates in the secretory direction. They concluded that, if the effect of plasma binding is not considered, overestimation of secretory transport could result in a misleading net flux calculation (Walgren and Walle, 1999). The inclusion of 4% BSA basolaterally significantly
reduced the efflux ratio for digoxin (30 nM) from $7.73 \pm 0.49$ to $5.86 \pm 0.46$, as reported in Paper III, indicating that lower amounts of the compound interacting with the apical efflux pump. Thus, in the presence of BSA the efflux will be estimated to be lower than in the absence of BSA.

Thus, efflux or uptake ratios calculated from transport rates obtained in the presence of BSA applied on the basolateral side of the monolayers can be used to determine the degree of overestimation of the apical efflux component in comparison with data obtained without BSA. This approach can be used as a selection tool in a small set of compounds, since the compounds can be ranked according to the change in their efflux or uptake ratios. Additionally, by accounting for $C_{p}$ actively and passively transported compounds can be distinguished (Paper III), as illustrated in Scheme 1.

Table V lists the advantages and disadvantages of the possible models that can be studied by including BSA in the Caco-2 monolayer assays and the 'traditional system' without BSA. In Paper III the ‘GI-mimicking model’ (basolateral application of BSA) was investigated. In Paper IV, BSA was applied either on both sides of the monolayer (‘protein model’) or only on the receiver side (‘receiver-side model’).

The data presented in Paper IV indicate that a compound with a high intrinsic (passive) permeability like asimadoline can show a small, but ‘relevant’ asymmetry (efflux ratio) across in-vitro cell monolayers. While in the GI-mimicking model an uptake ratio was obtained, in the receiver-side model a small but significant efflux ratio was calculated (in agreement with calculated efflux ratios obtained from MDCK-MDR1 experiments), indicating the possible relevance of efflux transporter for asimadoline in the brain, but not in the GI tract. This interpretation is in agreement with in vivo studies of asimadoline in the rat (Jonker et al., 1999). However, the binding to BSA should in a blood-brain barrier (BBB) model be considered also on the apical side (blood side of a BBB-model), the sink will be provided by BSA in the blood, i.e. at the ‘donor’ side, and for a protein-bound drug, such as asimadoline, an additional effect of P-gp efflux and the BSA sink will result. Thus as a suggestion, the apical addition of 4% BSA and an addition of 1% BSA basolaterally, to mimic the protein content in the brain, should be considered in in vitro models of the BBB. This may improve predictions of in vivo efflux transport (‘BBB-mimicking-model’).
Table V. Advantages and disadvantages of the absence or presence of bovine serum albumin (BSA) on either or both sides of the cell monolayers for lipophilic compounds.

<table>
<thead>
<tr>
<th>System</th>
<th>(+) Advantages;</th>
<th>(-) Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional system</td>
<td>(-) poor recovery</td>
<td>(+) equal initial donor concentrations</td>
</tr>
<tr>
<td></td>
<td>(+) high adsorption to plastic, high</td>
<td>(-) poor data quality for permeability a-b and b-a</td>
</tr>
<tr>
<td></td>
<td>cellular binding</td>
<td>(+) requires no determination of protein binding</td>
</tr>
<tr>
<td>a-b (0/0); b-a (0/0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘GI-mimicking model’</td>
<td>(+) low adsorption to plastic, low</td>
<td>(+) good recovery</td>
</tr>
<tr>
<td></td>
<td>cellular binding</td>
<td>(+) good data quality for permeability a-b</td>
</tr>
<tr>
<td>a-b (0/4); b-a (0/4)</td>
<td>(-) poor data quality for permeability</td>
<td>(-) free drug concentration has to be measured; compound-specific</td>
</tr>
<tr>
<td></td>
<td>a-b and b-a</td>
<td></td>
</tr>
<tr>
<td>‘Protein model’</td>
<td>(+) low adsorption to plastic, low</td>
<td>(+) equal initial donor concentrations on both sides</td>
</tr>
<tr>
<td></td>
<td>cellular binding</td>
<td>(-) free drug concentration has to be measured; compound-specific</td>
</tr>
<tr>
<td>a-b (4/4); b-a (4/4)</td>
<td>(+) good recovery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(+) good data quality for efflux</td>
<td></td>
</tr>
<tr>
<td>‘Receiver-side model’</td>
<td>(-) poor data quality for permeability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a-b and b-a</td>
<td></td>
</tr>
<tr>
<td>a-b (0/4); b-a (4/0)</td>
<td>(+) equal initial donor concentrations</td>
<td></td>
</tr>
<tr>
<td>‘BBB-mimicking model’</td>
<td>(+) low adsorption to plastic, low</td>
<td>(+) equal initial total donor concentrations</td>
</tr>
<tr>
<td></td>
<td>cellular binding</td>
<td></td>
</tr>
<tr>
<td>a-b (4/1); b-a (4/1)</td>
<td>(+) good recovery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-) poor quality permeability data a-b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b-a, since the initial donor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>concentration is affected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-) free drug concentration has to be</td>
<td></td>
</tr>
<tr>
<td></td>
<td>measured at different protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>concentrations</td>
<td></td>
</tr>
</tbody>
</table>

4.3. Cremophor® EL and Caco-2 cells

Two compounds were chosen as representatives of BCS class II drugs (low solubility/high permeation, (Amidon et al., 1995)). The model drugs, felodipine and asimadoline, were selected because of their known (felodipine; (Scholz et al., 2002)) and predicted (asimadoline) low solubility within the physiological pH range (5.0-8.0) of the GI tract. In order to study the effect of CEL on passive transport only,
felodipine was chosen, since felodipine is permeating highly and passively across the lipid membrane. To study the effect of CEL also on active transport, asimadoline was selected, since it is known that asimadoline is effectively transported via the apically located efflux transporter P-gp (Bender and Dasenbrock, 1998). Asimadoline is a compound which is also completely absorbed over the intestinal membranes.

Table VI: Oral Fraction Absorbed ($f_a$), Solubility and Protein Binding ($PB$) of the Investigated BCS class II Drugs.

<table>
<thead>
<tr>
<th>PB</th>
<th>Permeability</th>
<th>Solubility</th>
<th>Efflux</th>
<th>Compound</th>
<th>$PB$</th>
<th>$f_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>No</td>
<td>Felodipine</td>
<td>99.6(a)</td>
<td>100(a)</td>
</tr>
<tr>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Yes</td>
<td>Asimadoline</td>
<td>97(b)</td>
<td>100(c)</td>
</tr>
</tbody>
</table>

(a) Data for oral absorption and protein binding are taken from (Edgar et al., 1985) and (Artursson and Karlsson, 1991).
(b) Data for plasma protein binding in rats are taken from (Bender and Dasenbrock, 1998) and (Jonker et al., 1999).
(c) Data for oral absorption was predicted from permeation data across Caco-2 cell monolayers.

BSA was applied on the basolateral side and CEL on the apical side. This approach mimicked the in vivo situation in which BSA is in the blood/plasma of the capillaries and CEL is delivered with the drug. In addition, this design separated the two additives, thus avoiding direct interactions between them (Vidanovic et al., 2003). The CMC for CEL was approximated in the concentration range of 0.0050 to 0.0075% (w/v) in HBSS-HEPES buffer. This estimated CMC range is comparable to that reported for CEL by Nerurkar and co-workers (0.0070-0.0088%, w/v) (Nerurkar et al., 1997).

Some surfactants are capable of incorporating themselves into the lipid bilayer. This can alter the membrane permeability. Mannitol and TER measurements were followed to monitor the monolayer integrity (Anderberg et al., 1992). No significant alterations in TER values or mannitol permeation were observed for concentrations up to 10% (w/v) CEL on the apical side and 4% BSA on the basolateral side in the study presented in Paper IV. Thus, the paracellular pathway and the electrical parameters were not affected by CEL.

In contrast to the result of the paracellular marker, the transport rates for felodipine, a transcellularly transported compound, were affected above the estimated CMC of CEL (Figure 18, Paper IV). For instance, in the presence of 0.1% (w/v) CEL, a 3-fold reduction in felodipine permeation in the absorptive direction was observed, suggesting that micelle solubilization of felodipine decreased the fraction of free drug in the donor chamber. This, in turn, reduces the available amount of compound for transport across the monolayers. As a consequence, apparent permeability values based on the initial total felodipine concentration were substantially lower than without CEL.
Figure 18: Transport of the transcellularly transported compound, felodipine, across Caco-2 monolayers in the absorptive (a-b) (black bars) and secretory (b-a) (white bars) directions in the presence of various Cremophor® EL concentrations (0 – 0.1%; w/v) on the apical side and bovine serum albumin (4%; w/v) on the basolateral side. Values are means ± SD (n ≥ 3).

If the free concentration of felodipine, thus the solubility in the aqueous solution in the donor compartment, could be determined, then it should be possible to correct the measured transport rates for felodipine in a similar way, as shown for BSA (Page 50). Since the micellar interaction is a fragile system the determination of the free concentration of felodipine is not made easily. However, the use of CEL would give a possibility to include compounds for which the dissolution rate is the limiting step to absorption, since the micelles are serving as a reservoir that is easily accessible.

Non-ionic surfactants such as polysorbate 80 and CEL are among the substances reported to inhibit the activity of apically polarized efflux systems such as P-gp (Nerurkar et al., 1996). However, the bidirectional transport rates of asimadoline were unaffected by the presence of CEL (Paper IV). Thus, it can be speculated that any increase in asimadoline transport as a result of the inhibition of P-gp efflux by monomers of the CEL or decrease in transport due to the effect of surfactant micelles was insignificant beside the much larger effect of the high capacity for binding to BSA in the basolateral compartment. This efficient BSA binding is supported by the high lipophilicity of the compound. Thus, for an efflux compound with high lipophilicity, inclusion of CEL will be difficult to evaluate since several effects are present concurrently.

Inclusion of BSA on either or both sides of the monolayers is generally sufficient to increase the recovery of lipophilic BCS class II compounds. The overall effect of CEL on the permeation of a drug is more compound specific and less reliable and, therefore, less predictable for these drugs. Thus, the inclusion of BSA is recommended for BCS class II drugs, and the apical addition of a solubility enhancer such as CEL cannot be recommended.
Taub and co-worker suggested a pH gradient (apical pH 6.0, basolateral pH 7.4), together with basolateral BSA (0.25%) and apically applied sodium taurocholate (5 mM) as an optimized system for MDCK permeability determinations (Taub et al., 2002). This approach combines the use of pH-gradient and the use of additives. As shown in this thesis, each parameter changed in the Caco-2 cell system had its own impact on the results. In several cases the effects were working in opposing directions. A combination of all parameters can lead to unpredictable changes as observed for CEL in Paper IV and may obscure the interpretation of the data.
5. SUMMARY AND CONCLUSIONS

The results of the first part of this thesis (Papers I and II) show that the \( p\text{H} \)-dependent bidirectional transport of weakly basic and weakly acidic drugs may be biased by a false (passive) efflux and influx component, respectively. The false efflux can often be eliminated when the fraction of un-ionized drug species is equal in all compartments. In contrast, investigations on proton-dependent influx should be undertaken in both the presence and absence of a \( p\text{H} \) gradient. Concentration dependency and inhibition studies can be used to distinguish between active and passive transport processes, while temperature dependency failed to differentiate between these processes. The \( pK_a \) of a compound can be used to choose the \( p\text{H} \)-gradient system for concentration-dependency and inhibition studies and to interpret the obtained permeability data. For predictive models of \( f_u \) for the jejunum, ileum and colon transport a \( p\text{H} \) gradient of 6.0/7.4, 7.4/7.4 and 7.4/7.4 (apical \( p\text{H} \) basolateral \( p\text{H} \)) should be applied, respectively. Furthermore, the possibility of \( p\text{H} \) dependence should be considered in studies of DDIs involving efflux transporters such as P-gp, even when the functionalities of the transporters do not depend on the extracellular \( p\text{H} \).

The results of the second part of this thesis (Papers III and IV) show that BSA alters both passive and active drug transport in the Caco-2 cell model. Using the unbound concentration in the calculations of the transport rate allowed elucidation of the mechanisms behind these alterations. Firstly, the inclusion of basolateral BSA will improve the mass balance and in combination with \( C_u \) allows the permeability of the system to the compound to be predicted (for 100% recovery of the compound) and, thus, prediction of \( f_u \) values for poorly recovered compounds in the traditional Caco-2 cell model (without BSA). Secondly, BSA does not affect the functionality of active transport systems such as P-gp. However, the amount of compound available at the binding sites of the efflux transporters may be reduced in the presence of BSA, which, in turn, will alter the transport rates of actively transported compounds. This observation may also be used to distinguish between actively and passively transported compounds, after re-calculating with \( C_u \). Finally, the data suggest that active secretory transport for highly protein-bound compounds might have less effect \textit{in vivo} than predicted from traditional Caco-2 cell models. BSA in the Caco-2 cell model can be used as a secondary screening tool. Ranking of a limited subset of compounds based on efflux (or uptake) ratios obtained by inclusion of basolateral BSA might be used as a model in the CD (Candidate Drug) selection process. Thus, the inclusion of BSA in Caco-2 experiments results in a physiologically sound improvement of the model and can be recommended; however, the inclusion of CEL affects both passive and active drug transport in the Caco-2 cell model and, since the results are unpredictable, CEL cannot be recommended as an additive in permeability assays, as long as a determination and accounting of the free drug concentration is not possible.
Table VII is a summary of the refined experimental systems derived from Papers I-IV.

**Table VII: Refined models discussed in Papers I-IV**

<table>
<thead>
<tr>
<th>Paper</th>
<th>Model for...</th>
<th>Refinement</th>
<th>(+)/(-) Advantages/Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>apical efflux</td>
<td>a) pH 7.4</td>
<td>(+) study of active efflux, e.g., P-gp mimics physiological conditions in the ileum and colon, (-) proton-gradient dependent transport is inactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) pH 7.4</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>apical uptake</td>
<td>a) pH 6.0</td>
<td>(+) study of active uptake, e.g., MCT1, OATP-B, PepT1 mimics physiological conditions in the jejunum, (-) false efflux and uptake components</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(basolateral efflux) b) pH 7.4</td>
<td></td>
</tr>
<tr>
<td>III +IV</td>
<td>low recovery</td>
<td>a) pH 7.4</td>
<td>(+) increased recovery mimics physiological conditions, (-) free drug concentration has to be measured compound-specific</td>
</tr>
<tr>
<td></td>
<td>compounds</td>
<td>b) pH 7.4, 4% BSA</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>sparingly soluble compounds</td>
<td>a) pH 7.4; CEL</td>
<td>(-) compound-specific micellar incorporation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) pH 7.4, 4% BSA</td>
<td></td>
</tr>
</tbody>
</table>

a) = apical; b) = basolateral
6. REMAINING ISSUES

- The fraction absorbed in vivo is the sum of the permeation of the compound through the cell membranes along the GI tract. This permeation is affected by the changes in the pH gradients in vivo. The effect of these various pH changes on predictions of $f_a$ is an issue that remains to be resolved.

- The relative contribution of active and passive transport processes in the various pH-gradient systems of the GI tract is variable and complex. A remaining issue is to understand these alterations further, and additional mechanistic studies of the effect of pH on the function of transporters are needed, e.g. of the interplay between NHEs and H⁺-cotransporters.

- Efflux ratios are concentration dependent for actively transported compounds and, therefore, the efflux ratios should be determined at relevant drug concentrations. Approaches of estimating relevant in vivo concentrations for an in vitro assay are warranted.

- Another issue is the standardization of methods of accounting for reduced compound recovery. In order to do this, the reasons for poor recovery must first be identified. For instance, a compound may be trapped in the cell cytosol because of changes in the pH (in this compartment) and this may be interpreted as a loss of recovery. It is necessary to account for this loss in order to interpret the transport data and evaluate transport mechanisms.

- It remains to be shown, on a subset of compounds, covering a wide range of protein binding and ease of permeation, that basolaterally applied BSA will improve the prediction for $f_a$. 
7. APPENDIX

**Figure 19:** Schematic illustration of some intestinal epithelial transporters verified to be expressed in the Caco-2 cell line. (A) Transporters shown as a square demonstrate active or facilitated transport. Ion-exchangers are shown as oval shapes. The name of the corresponding transporter for the process is shown within the square or oval shape. For active transporters, the black triangles represent an efflux transporter, the white triangles an uptake transporter and the grey triangles represent symport or antiport of the substrate and the driving force. Grey marked squares represent an unverified location for the transporter. (B) The corresponding gene names of the transporters is shown in the right illustration. SLC: solute carrier, ABC: adenosine triphosphate binding cassette. The illustration is based on information collected from references listed in Table VIII.
**Table VIII:** Some transporters that have been identified in Caco-2 cells and that are relevant for drug transport

Transporter (Gene)*  Protein or Gene identified (References)

<table>
<thead>
<tr>
<th>Efflux transporter:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1 (ABCB1)</td>
<td>by immunoblotting with P-gp C219 antibody (Wils et al., 1994); by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>MDR3 (ABCB3)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>MRP1 (ABCC1)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>MRP2 (ABCC2)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>MRP3 (ABCC3)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>MRP4 (ABCC4)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>MRP5 (ABCC5)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>MRP6 (ABCC6)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
<tr>
<td>BCRP (ABCG2)</td>
<td>by RT-PCR (Taipalensuu et al., 2001).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Uptake transporter:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT1 (SLC22A1)</td>
<td>by RT-PCR (Hayer-Zillgen et al., 2002; Martel et al., 2001; Zhang et al., 1999).</td>
</tr>
<tr>
<td>OCT2 (SLC22A2)</td>
<td>by RT-PCR (Hayer-Zillgen et al., 2002).</td>
</tr>
<tr>
<td>OCT3 (SLC22A3)</td>
<td>by RT-PCR (Hayer-Zillgen et al., 2002).</td>
</tr>
<tr>
<td>OCTN2 (SLC22A5)</td>
<td>by Western blot analysis and by immunofluorescence (Elimrani et al., 2003); by RT-PCR (Seithel et al., 2004).</td>
</tr>
<tr>
<td>OATP-B (SLC02B1)</td>
<td>by RT-PCR (Seithel et al., 2004).</td>
</tr>
<tr>
<td>PepT1 (SLC15A1)</td>
<td>by RT-PCR and Northern blot analysis (PepT2 / SLC15A2 was negative)(Ganapathy et al., 1995); by RT-PCR and Western blot analysis (Sun et al., 2002).</td>
</tr>
<tr>
<td>HPT1</td>
<td>by monoclonal antibody inhibition, identification and subsequent cloning of the gene (Dantzig et al., 1994).</td>
</tr>
<tr>
<td>MCT1 (SLC16A1)</td>
<td>by RT-PCR (Seithel et al., 2004). MCT1 protein and RNA expression profile (Lambert et al., 2002).</td>
</tr>
<tr>
<td>PAT1 (SLC36A1)</td>
<td>by immunofluorescence (Chen et al., 2003).</td>
</tr>
</tbody>
</table>

* Supporting list of references for Figure 19. Name within parentheses or single name corresponds to the *Homo sapiens* Official Gene Symbol, according to the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/).
Table VIII (continues): Transporters that have been identified in Caco-2 cells.

**Glucose transporters and ion-exchangers:**

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Identification Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1 (SLC2A1)</td>
<td>by immunoelectron microscopy and by immunofluorescence (Harris et al., 1992); by Nothern blot analysis (Mahraoui et al., 1994).</td>
</tr>
<tr>
<td>GLUT2 (SLC2A2)</td>
<td>by Nothern blot analysis (Mahraoui et al., 1994).</td>
</tr>
<tr>
<td>GLUT3 (SLC2A3)</td>
<td>by immunoelectron microscopy and by immunofluorescence (Harris et al., 1992); by Nothern blot analysis (Mahraoui et al., 1994).</td>
</tr>
<tr>
<td>GLUT5 (SLC2A5)</td>
<td>by immunoelectron microscopy and by immunofluorescence (Harris et al., 1992); by Nothern blot analysis (Mahraoui et al., 1994).</td>
</tr>
<tr>
<td>SGLT1 (SLC5A1)</td>
<td>by Nothern blot analysis (Mahraoui et al., 1994).</td>
</tr>
<tr>
<td>NHE1 (SLC9A1)</td>
<td>by RT-PCR (Thwaites et al., 1999); by Western blot analysis and immunofluorescence (Janecki et al., 1999).</td>
</tr>
<tr>
<td>NHE2 (SLC9A2)</td>
<td>by RT-PCR (Thwaites et al., 1999).</td>
</tr>
<tr>
<td>NHE3 (SLC9A3)</td>
<td>by RT-PCR on poly(A)+RNA using primers specific for NHERF1 and NHERF2 (Thwaites et al., 2002); by Western blot and immunofluorescence (Janecki et al., 1999).</td>
</tr>
</tbody>
</table>

**Other important proteins identified in Caco-2 cells (a), or possibly available (b):**

(a) CYP1A1 (Boulenc et al., 1992); CYP3A (Carriere et al., 1994; Engman et al., 2001); villin (Chantret et al., 1988);
(b) OCTL1 (SLC22A13) (Nishiwaki et al., 1998), OCTL2 (SLC22A14) (Nishiwaki et al., 1998), OSTα/β (Dawson et al., 2005), OATP-E (Tamai et al., 2000); OATP-D (Tamai et al., 2000)
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9. REFERENCES


Artursson, P. and J. Karlsson, 1991, Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells, Biochem Biophys Res Commun 175, 880.


Boulenc, X., M. Bourrie, I. Fabre, C. Roque, H. Joyeux, Y. Berger and G. Fabre, 1992, Regulation of cytochrome P450IA1 gene expression in a human intestinal cell line, Caco-2, J Pharmacol Exp Ther 263, 1471.


Dantzig, A.H. and L. Bergin, 1990, Uptake of the cephalosporin, cephalaxin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2, Biochim Biophys Acta 1027, 211.


Dey, S., P. Hafkemeyer, I. Pastan and M.M. Gottesman, 1999, A single amino acid residue contributes to distinct mechanisms of inhibition of the human multidrug transporter by stereoisomers of the dopamine receptor antagonist flupentixol, Biochem 38, 6630.


Hidalgo, I.J. and R.T. Borchardt, 1990a, Transport of a large neutral amino acid (phenylalanine) in a human intestinal epithelial cell line: Caco-2, Biochim Biophys Acta 1028, 25.


Hogerle, M.L. and D. Winne, 1983, Drug absorption by the rat jejunum perfused in situ. Dissociation from the pH-partition theory and role of microclimate-pH and unstirred layer, Naunyn Schmiedebergs Arch Pharmacol 322, 249.


Ingels, F.M. and P.F. Augustijns, 2003, Biological, pharmaceutical, and analytical considerations with respect to the transport media used in the absorption screening system, Caco-2, J Pharm Sci 92, 1545.


Keppler, D. and J. Konig, 1997, Expression and localization of the conjugate export pump encoded by the MRP2 (cMRP/cMOAT) gene in liver, FASEB Journal 11, 509.


Lee, K., C. Ng, K.L. Brouwer and D.R. Thakker, 2002, Secretory transport of ranitidine and famotidine across Caco-2 cell monolayers, J Pharmacol Exp Ther 303, 574.
Lentz, K.A., J. Hayashi, L.J. Lucisano and J.E. Polli, 2000, Development of a more rapid, reduced serum culture system for Caco-2 monolayers and application to the biopharmaceutics classification system, Int J Pharm 200, 41.
Li, A.P., 2001, Screening for human ADME/Tox drug properties in drug discovery., DDT 6, 357.
Lipinski, C.A., 2003, Chris Lipinski discusses life and chemistry after the Rule of Five, DDT 8, 12.


Pade, V. and S. Stavchansky, 1997, Estimation of the relative contribution of the transcellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 cell culture model, Pharm Res 14, 1210.


Pinto, M., S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh and A. Zweibaum, 1983, En-
terocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture, Biol Cell 47, 323.
Stein, J., M. Zores and O. Schroder, 2000, Short-chain fatty acid (SCFA) uptake into Caco-2 cells by a pH-dependent and carrier mediated transport mechanism, Eur J Nutr 39, 121.


Tavelin, S., 2003, New approaches to studies of paracellular drug transport in intestinal epithelial cell monolayers, in: Department of Pharmacy (Uppsala University, Uppsala) p. 66.


Ungell, A.L., 2004, Caco-2 replace or refine?, DDT 1, 423.
rectional, and pH-dependent transport of organic cations, J Pharmacol Exp Ther 289, 768.


Yee, S., 1997, In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestine) absorption in man - Fact or myth, Pharm Res 14, 763.


Zimmermann, C., H. Gutmann, P. Hruz, J.-P. Gutzwiller, C. Beglinger and J. Drewe, 2005, Mapping of multidrug resistance gene 1 and multidrug resistance-associated protein isoform 1 to 5 mRNA expression along the human intestinal tract, Drug Metab Dispos 33, 219.


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