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JOHANNA LINNANKOSKI

MATHEMATICAL MODELLING OF INTESTINAL DRUG ABSORPTION

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ABSTRACT

In the early 2000s, when the investigations of this thesis were started, new experimental methodologies were producing increasing numbers of pharmacologically interesting drug candidate molecules. Since poor pharmacokinetics of a compound had been a major contributor to expensive late-stage failures in drug development, methodologies for early prediction of the pharmacokinetics of a compound were needed more than ever.

The primary objective of this thesis was to explore methods for predicting passive molecule transport across the intestinal membrane. Novel Quantitative Structure-Property Relationship (QSPR) models to predict the kinetics of passive oral drug absorption in humans were developed using multivariate analysis. The predictivities of the developed models were compared to the predictivities of previously developed computational and experimental models. Literature data were used in the analyses. To better understand cell model predictions, the paracellular pathways of the three different cell models and human intestinal tissue were characterized. The pore sizes and porosities were ascertained by determining the permeabilities of polyethylene glycol (PEG) oligomers through the examined membranes.

The results of this work demonstrated that passive human intestinal absorption could be well predicted using a combination of two or three

simple computational lipophilicity and polarity descriptors. The developed QSPR models were shown to predict absorption equally well as the experimental Caco-2 and 2/4/A1 cell models, and, notably, one of the QSPR models predicted absorption nearly as well as the *in vivo* rat model. We suggest that previously reported tendency of the Caco-2 cell model to underestimate paracellular drug transport results from differences in the porosities of the Caco-2 monolayer and the human intestine. The porosity of the Caco-2 cell model was found to be about eight times lower than the porosity of the human intestinal epithelium. The pore sizes in the Caco-2 monolayer (6 and 10 Å) and the human jejunal membrane (7 and 10 Å) were similar. The human jejunal pore sizes determined in this thesis were in line with previously determined human and rat intestinal pore sizes. Our study was the first to experimentally determine the surface area of the paracellular pores relative to the total intestinal surface area in the human intestine and cell models. In summary, the research in this thesis has provided new insight into computational and experimental methods for assessing the permeability of drug candidates during the initial phases of drug discovery.

Keywords: Intestinal drug absorption; Epithelial permeability; Paracellular permeation; Pharmacokinetics; Oral administration; Human intestine; Caco-2; MDCKII; 2/4/A1; PAMPA; Computer simulation; QSPR modelling

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TIIVISTELMÄ

2000-luvun alussa, kun tämän väitöskirjan tutkimukset aloitettiin, sopimattomat farmakokineettiset ominaisuudet, kuten huono imeytyminen, olivat johtaneet monen molekyylin lääkekehityksen myöhäisvaiheen keskeytykseen. Siksi uusia menetelmiä yhdisteiden farmakokinetiikan varhaiseen ennustamiseen kaivattiin.

Tämän väitöskirjan aiheena on suun kautta annettavien lääkeaineiden imeytymisen ennustaminen. Ensimmäisen osatyön tavoitteena oli kehittää laskennallisia QSPR-malleja, joilla voidaan nopeasti ja helposti ennustaa molekyylin rakenteen perusteella sen imeytymistä ruuansulatuskanavasta. Toisen osatyön tarkoituksena oli verrata kehittämiemme laskennallisten mallien ennustekykyä aiemmin kehitettyjen laskennallisten ja kokeellisten mallien ennustekykyyn. Työssä käytettiin kirjallisuusdataa. Kolmannen osatyön tarkoituksena oli kokeellisesti määrittää ihmisen ohutsuolen ja kolmen eri solumallin huokoskoot ja huokoisuus ja siten lisätä ymmärrystä lääkeaineiden parasellulaarikulkeutumisesta ohutsuolessa ja solumalleissa.

Ensimmäisen osatyön tulokset osoittivat, että suun kautta annetun lääkeaineen passiivista imeytymistä ruuansulatuskanavasta voidaan ennustaa hyvällä tarkkuudella seuraavan yksinkertaisen yhtälön avulla: $\log K_a = 0.623 + 0.154 \log D_{6.0} - 0.007$ PSA, missä $\log K_a$ on molekyylin imeytymisnopeusvakio ruuansulatuskanavassa, $\log D_{6.0}$ on molekyylin oktanoli-vesi jakaantumiskerroin pH:ssa 6.0 ja PSA on molekyylin polaarinen

pinta-ala. Sekä $logD_{6.0}$ että PSA voidaan määrittää laskennallisesti. Toisessa osatyössä näytimme, että kehittämämme QSPR-malli ennusti lääkeaineiden imeytymistä yhtä hyvin kuin kokeelliset Caco-2- ja 2/4/A1-solumallit, ja lähes yhtä hyvin kuin *in vivo* imeytymiskokeet rotilla.

Kolmannen osatyön tulokset osoittivat, että ihmisen ohutsuolen sekä Caco-2, MDCKII ja 2/4/A1 solumallien huokoisuudet (huokosten pinta-ala suhteessa ohutsuolen koko pinta-alaan) ovat matalia; ihmisen ohutsuolen huokoisuus oli noin 0,000005. Yleisimmin käytetyn Caco-2-solumallin huokoisuus osoittautui noin kahdeksan kertaa pienemmäksi kuin ihmisen suolen huokoisuus. Huokosten koot olivat kuitenkin samaa luokkaa kuin ihmisen ohutsuolessa. Kolmannen osatyön tulosten perusteella Caco-2 solumallin aiemmin raportoitu taipumus aliarvioida lääkeaineiden parasellulaarista imeytymistä saattaisi selittyä Caco-2 solumallin ja ihmisen suolen huokoisuuksien eroista. Tutkimuksemme oli ensimmäinen, jossa kokeellisesti määritettiin ihmisen ohutsuolen huokoisuus.

Tämän väitöskirjan tutkimukset ovat tuottaneet uutta tietoa laskennallisista ja kokeellisista menetelmistä, joita käytetään lääkeainekandidaattien imeytymisen seulontaan lääkekehityksen varhaisessa vaiheessa.

Avainsanat: Lääkeaineen imeytyminen ohutsuolesta; Parasellulaarinen permeabiliteetti; Farmakokinetiikka; Caco-2; MDCKII; 2/4/A1; PAMPA; QSPR mallintaminen

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Linnankoski J, Mäkelä J, Ranta V-P, Urtti A, Yliperttula M: Computational Prediction of Oral Drug Absorption Based on Absortpion Rate Constants in Humans. Journal of Medicinal Chemistry. 2006, 49(12):3674-81.

II Linnankoski J, Ranta V-P, Urtti A, Yliperttula M: Passive oral drug absorption can be predicted more reliably by experimental than computational models – fact or myth. European Journal of Pharmaceutical Sciences. 2008, 34(2-3):129-39.

III Linnankoski J, Mäkelä J, Palmgren J, Mauriala T, Vedin C, Ungell AL, Lazorova L, Artursson P, Urtti A, Yliperttula M: Paracellular porosity and pore size of the human intestinal epithelium in tissue and cell culture models. Journal of Pharmaceutical Sciences. 2010, 99(4):2166-75.

CONTENTS

ABBREVIATIONS

$ACDlogD_x$	logarithm of the octanol-water distribution coefficient calculated by ACD/LogD software, pH defined with subscript x
ACDlogP	logarithm of the octanol-water partition coefficient calculated by ACD/LogP software
ClogP	calculated logarithm of the octanol-water partition coefficient
F	bioavailability
F_A	fraction absorbed
F_G	fraction avoiding intestinal metabolism
F_H	fraction avoiding liver extraction during a single pass
GI	gastrointestinal
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
K_a	absorption rate constant
K_{app}	apparent absorption rate constant describing the disappearance of the drug from the absorption site
$logD_x$	logarithm of the octanol-water distribution coefficient, pH defined with subscript x
logP	logarithm of the octanol-water partition coefficient
MW	molecular weight
PAMPA	parallel artificial membrane permeability assay

P_{app}	apparent permeability coefficient
P_{eff}	effective human jejunal permeability
PEG	polyethylene glycol
PCA	principal component analysis
PLS	partial least squares
PSA	dynamic polar surface area
TPSA	topologic polar surface area
Q^2	cross validated correlation coefficient
QSPR	Quantitative Structure-Property relationship
R^2	coefficient of determination
RMSE	root mean squared error
SD	standard deviation
TEER	transepithelial electrical resistance
Å	ångström

1 INTRODUCTION

Drug discovery is a complex process that involves many different steps. Typically, it costs hundreds of millions of euros and takes at least a decade to produce a single medicine (DiMasi et al., 2016, 2010; Wouters et al., 2020). Most drug candidates do not advance to the stage of human testing, and, unfortunately, 90% of those that do reach this stage are subsequently rejected for diverse reasons (Sun et al., 2022).

In the late 1990s and early 2000s, when the investigations of this thesis were started, new techniques were producing increasing numbers of pharmacologically interesting candidate compounds (Ferreira and Andricopulo, 2019). Therefore, reliable tools for identifying the molecules that were likely to be clinically successful were needed more than ever. Since poor pharmacokinetics had been a significant cause of costly late-stage failures, the pharmaceutical industry made considerable investments in developing methodologies for predicting the pharmacokinetics of a compound (Davies et al., 2020).

The interplay of absorption, distribution, excretion, and metabolism determines a drug's pharmacokinetics, influencing its therapeutic impact. This thesis explores oral drug absorption and specifically methods for predicting a key factor that influences it: transport across the intestinal membrane. Novel computational models for predicting intestinal drug absorption were presented and evaluated in articles 1 and 2 of this thesis. To better understand cell model predictions, the paracellular pathways of cell cultures were characterized and compared to the human paracellular pathway in article 3 of this thesis.

Structure-based computational models, developed and evaluated in this thesis, are especially useful during the early discovery phase. They enable the rapid screening of a substantial number of compounds even before the synthesis stage. The cell culture models, evaluated and characterized in this thesis, have the drawback of being more time-consuming. However, they offer the advantage of not only predicting the transport rate across the

intestine but also providing information about different drug transport mechanisms.

2 REVIEW OF THE LITERATURE

2.1 FUNDAMENTALS OF DRUG ABSORPTION AFTER ORAL DOSAGE

For an orally administered drug to be effective, it needs to be sufficiently absorbed from the gastrointestinal (GI) tract into the systemic circulation. Although the stomach has a relatively large surface area, its thick mucous layer and short transit time limit drug absorption. Consequently, the small intestine with its significant villous surface, is the primary location for drug absorption for the majority of drugs.

The fraction of the dose that arrives in the systemic blood circulation unchanged, bioavailability (F), is the result of a series of events in which the drug must (1) dissolve and stay intact in the GI tract, (2) cross the intestinal epithelium unchanged (3) pass through the liver unchanged. Bioavailability can be calculated according to the following equation:

$$F = F_A \times F_G \times F_H$$

where F_A is the fraction absorbed from the intestine to the enterocytes , F_G is the fraction avoiding intestinal metabolism, and F_H is the fraction avoiding liver extraction during a single pass.

The bioavailability of a drug is primarily influenced by its solubility, permeability and stability against metabolizing enzymes (Amidon et al., 1995). Approximately 85% of drugs on the market undergo varying degrees of first pass metabolism (Iversen et al., 2022). Metabolism in both the liver and gut wall is predominantly mediated by the cytochrome P450 enzyme superfamily (Thelen and Dressman, 2009).

The series of events involved in drug transport from the GI tract into the systemic blood circulation are illustrated in Figure 1. The rate-limiting step in drug absorption from the GI tract is primarily determined either by release and dissolution from the dosage form or permeability across the

intestinal membrane. This thesis focuses on passive permeability rate-limited intestinal membrane transport.

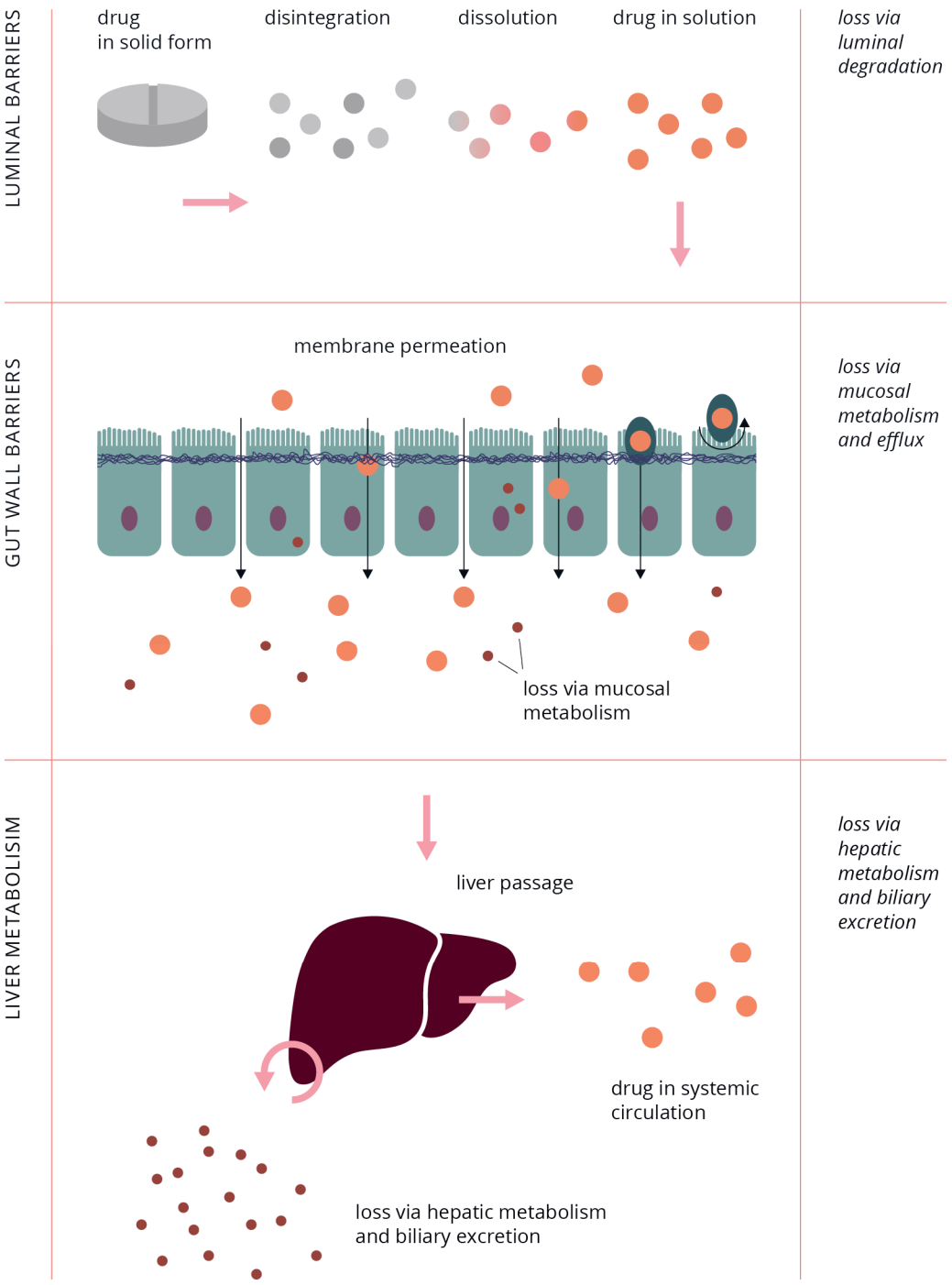

Figure 1. Schematic illustration of drug transport from the GI tract to the systemic circulation (adapted with permission from Pereira et al., 2016).

2.2 MECHANISMS OF INTESTINAL DRUG PERMEATION

The most common intestinal absorption mechanisms are (1) passive transcellular diffusion through the cells, (2) paracellular diffusion through the pores between the cells and (3) carrier-mediated active transport (Figure 2) (Sugano et al., 2010). Passive transcellular and paracellular diffusion involve the movement of molecules in the direction of the concentration gradient, while active transport may involve the passage of molecules against the concentration gradient. Different transport mechanisms can act in combination (Lennernäs, 2007; Matsson et al., 2005; Sugano et al., 2010) and it is not always easy to determine which process has the most significant role in transport (Lennernäs, 2007).

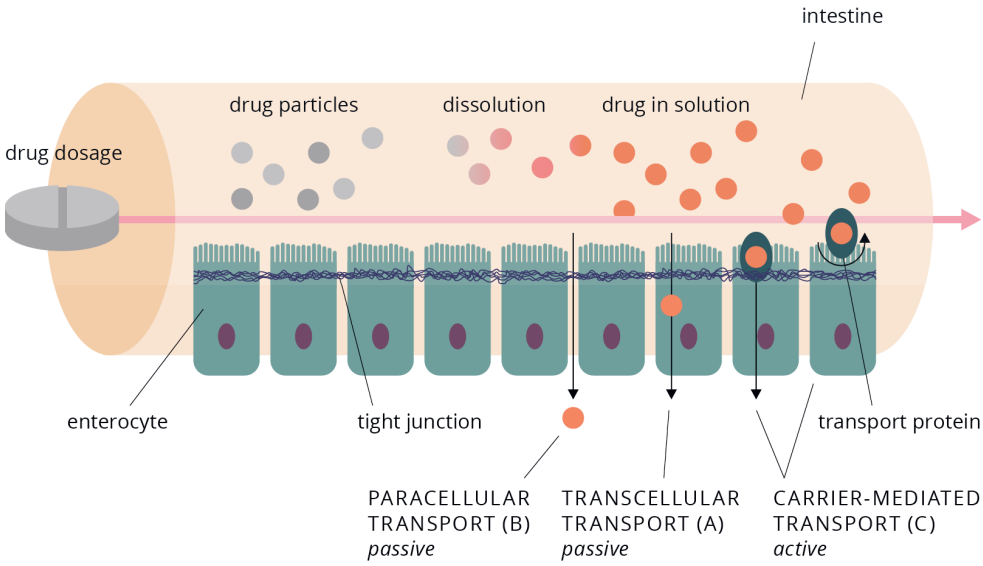

Figure 2. Schematic illustration of drug transport across the intestinal epithelium. Most traditional drugs are predominantly passively absorbed through the transcellular route (A). Hydrophilic compounds small enough to pass through the tight junction pores may favour the paracellular pathway (B). Actively transported drugs are recognized by a transporter protein. Active transport may occur in the absorptive or efflux direction (C).

2.2.1 Passive transcellular diffusion

Passive transcellular transport across intestinal epithelial cell membranes is regarded as the most common absorption mechanism for orally delivered drugs (Lennernäs, 2007; Matsson et al., 2005). Transcellularly diffusing compounds cross the intestinal enterocytes by passing first through the apical membrane, then the cytoplasm, and finally the basolateral membrane. The apical membrane is less fluid and permeable than the basolateral membrane (see section 2.3) and is therefore regarded as the rate-limiting barrier for transcellular diffusion (Lande et al., 1995, 1994). Transport across the cytoplasm usually occurs swiftly (Muranishi, 1990).

The lipid composition of the apical and basolateral enterocyte membranes limits the passage of hydrophilic compounds. However, if the drug is too hydrophobic, it may become trapped within the apical membrane and not traverse the aqueous cytoplasm (Wils et al., 1994). Thus, an appropriate balance between hydrophobicity and hydrophilicity is necessary. Moreover, passage across the non-polar interior of the phospholipid bilayer is restricted by ionization and increased polarity of a compound. Additionally, due to the ordered structure of the cell membrane, the smaller a molecule is, the easier it diffuses across the cell membrane. The molecular descriptors determining transcellular permeation are discussed in further detail in section 2.4.

2.2.2 Paracellular transport

Paracellular transport involves the movement of hydrophilic molecules through the aqueous pores between epithelial cells (Frömter and Diamond, 1972). It is generally accepted to be a passive process driven by transepithelial gradients. However, the negative charges of the paracellular space determine that cations pass through the paracellular pores more easily than neutral compounds, and in turn, neutral compounds are more permeable than anions (Adson et al., 1994; Karlsson et al., 1999; Pade and Stavchansky, 1997). Some researchers have proposed saturable transport mechanisms across the paracellular route (Lee and Thakker, 1999; Zhou et al., 1999).

The dimensions of the tight junction pores restrict paracellular diffusion to molecules of small to moderate size (Furuse et al., 1998; Madara, 1998). It has been suggested that hydrophilic compounds with molecular weights (MWs) below 200 may utilize the paracellular route (Lennernäs, 2007). However, the involvement of paracellular transport has also been proposed for slightly larger molecules, such as atenolol (MW 266), furosemide (MW 330) and a series of positively charged H2-antagonists (MWs 252-337) (Adson et al., 1994; Flanagan et al., 2002; Gan et al., 1998).

The intestinal absorption of compounds thought to be mainly transported through the paracellular route varies from low to high: e.g. foscarnet (MW 126, logP -1.8, negative charge) and erythritol (MW 122, logP -3, neutral charge) have F_A values of 0.17 and 0.9, respectively (Karlsson et al., 1999). The absorption of paracellularly transported compounds is, however, most often incomplete (Saitoh et al., 2004). Incomplete absorption has been proposed to result from the paracellular pores' low surface area relative to the intestine's total surface area. The dimensions of the paracellular space will be further discussed in section 2.3.

2.2.3 Active transport

Transporter proteins within the apical membranes of the intestinal enterocytes actively move endogenous substances such as ions, vitamins, sugars, amino acids and peptides across the phospholipid bilayer. Active transport is an energy-dependent, substrate-specific and saturable process (Tsuji and Tamai, 1999). Due to substrate-specificity active transport is often constrained to drugs that share structural similarities to endogenous substances. In addition to uptake transporters acting in the absorptive direction, enterocytes have several efflux transporters that actively move compounds from the enterocyte into the GI tract.

The intestinal transporters can be categorised into two primary families: the uptake solute carrier (SLC) family and the efflux ATP binding cassette (ABC) family (Giacomini and Huang, 2013). The uptake SLC family includes e.g. the Human peptide 1 (PepT1), organic anion transporting polypeptide (OATP) and, organic cation (OCT) transporters, and the efflux ABC family

includes e.g. the P-glycoprotein (Pgp), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) transporters.

As transporter substrates also diffuse passively, active transport does not always have a substantial impact on the absorption of sufficiently lipophilic compounds that are swiftly absorbed across cell membranes (Lennernäs, 2007). Also, because the active transport mechanism is saturable, the contribution of passive transport increases with higher doses. Since this thesis describes passive drug permeation, the active transport mechanisms are not discussed in depth.

2.3 EPITHELIAL STRUCTURES DETERMINING PASSIVE INTESTINAL DRUG PERMEATION

The intestinal epithelium consists of a single layer of epithelial cells (Figure 3). It creates a barrier between the lumen of the intestine and the underlying connective tissue while also forming villous structures that increase the overall surface area of the intestine severalfold. The villi are separated by crypts where the regeneration of the intestinal epithelial cells takes place. Intestinal epithelial cells are connected by intercellular junctional complexes. In addition to sealing the intercellular space, the junctional complexes form pores that regulate the movement of ions and solutes through the epithelium. The tight junction, the most apical component of the junctional complex, is regarded as the primary factor controlling paracellular permeability (Madara, 1998). Since this thesis focuses on passive transcellular and paracellular drug transport, the epithelial structures determining these processes: (1) the enterocyte membrane and (2) the intercellular tight junctions are discussed below.

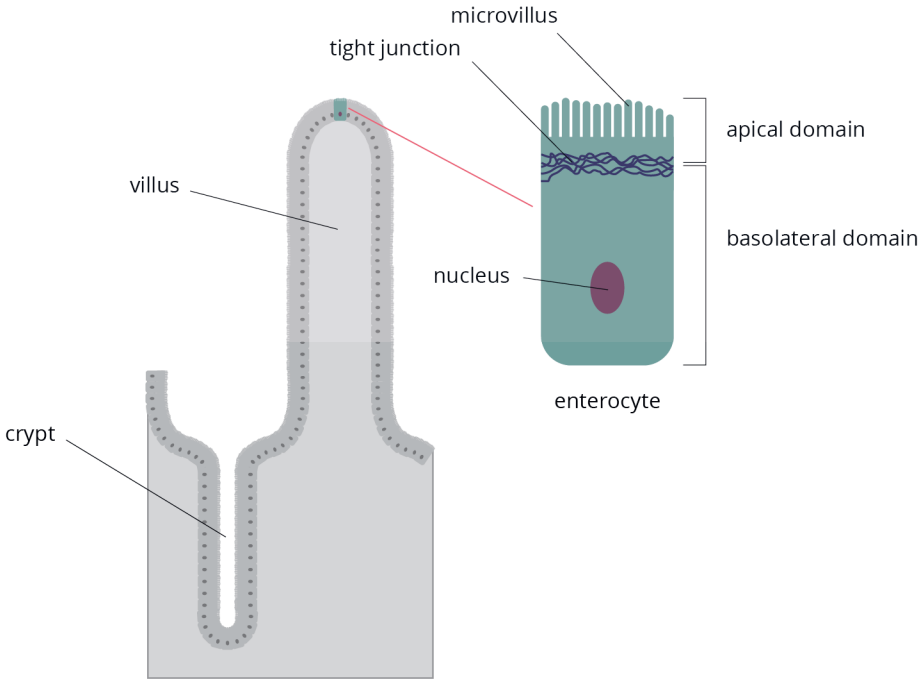

Figure 3. Schematic illustration of the intestinal lining and an enterocyte. The intestinal lining consists of a single layer of epithelial cells connected by intercellular junctional complexes, including tight junctions. The villi and microvilli enhance the absorptive surface area of the small intestine. The crypts contain stem cells responsible for the renewal of the intestinal epithelium.

2.3.1 Enterocyte membrane

Cell membranes are composed of a bilayer of phospholipids containing various proteins, cholesterol, and glycolipids (Figure 4). The membrane composition varies among different cell types and also in a single cell. Enterocytes have two distinct membrane domains: (1) the apical domain oriented towards the intestinal lumen and (2) the basolateral domain bordering the neighbouring cells and the underlying basement membrane (Figure 3). The apical membrane contains more cholesterol and glycolipids than the basolateral membrane (Fagerholm, 2007). This makes it thicker and less fluid and less permeable than the basolateral membrane (Fagerholm, 2007). The apical membrane surface is often called "the brush border" because it forms projections called microvilli (Figure 3). The microvilli are covered by a thin mucopolysaccharide coat called the glycocalyx. Since membrane lipids are amphiphilic molecules with hydrophilic headgroups and hydrophobic hydrocarbon chains, regional differences in membrane polarity and hydrophobicity make molecular movement across cell membranes a complex process (Martinez and Amidon, 2002).

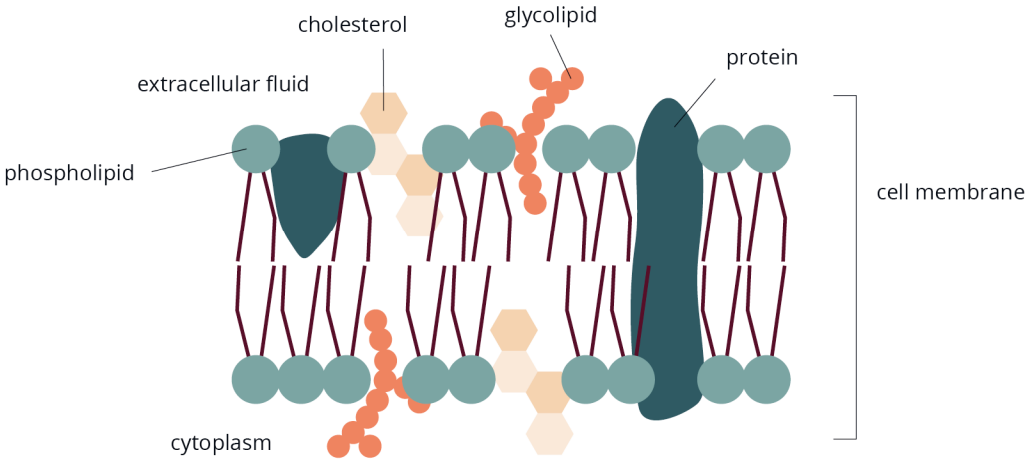

Figure 4. Schematic illustration of the cell membrane.

2.3.2 Tight junction pores

The paracellular pathway consists of small pores in the intercellular barrier (Figure 5). The most apical structures of the intercellular barrier, the tight junctions, are considered essential in regulating the flow of ions and solutes across the pores (Ballard et al., 1995). Tight junctions also prevent the diffusion of membrane molecules between the apical and basolateral cell domains and are connected to the intracellular machinery responsible for regulating the polarization of the enterocytes.

Tight junctions are composed of different types of (1) transmembrane proteins that form a linear barrier of strands between adjacent cells and (2) cytoplasmic scaffolding proteins that anchor the transmembrane proteins to the cytoskeleton. Of the numerous tight junction proteins, the transmembrane claudins are the most abundant and considered key components for tight junctions' structure and function (Van Itallie et al., 2008). Claudins polymerize within the cell membrane to form the strands that interact with each other between adjacent cells. The claudin-based strands also form the pores that mediate the movement of ions and solutes (Krug et al., 2012).

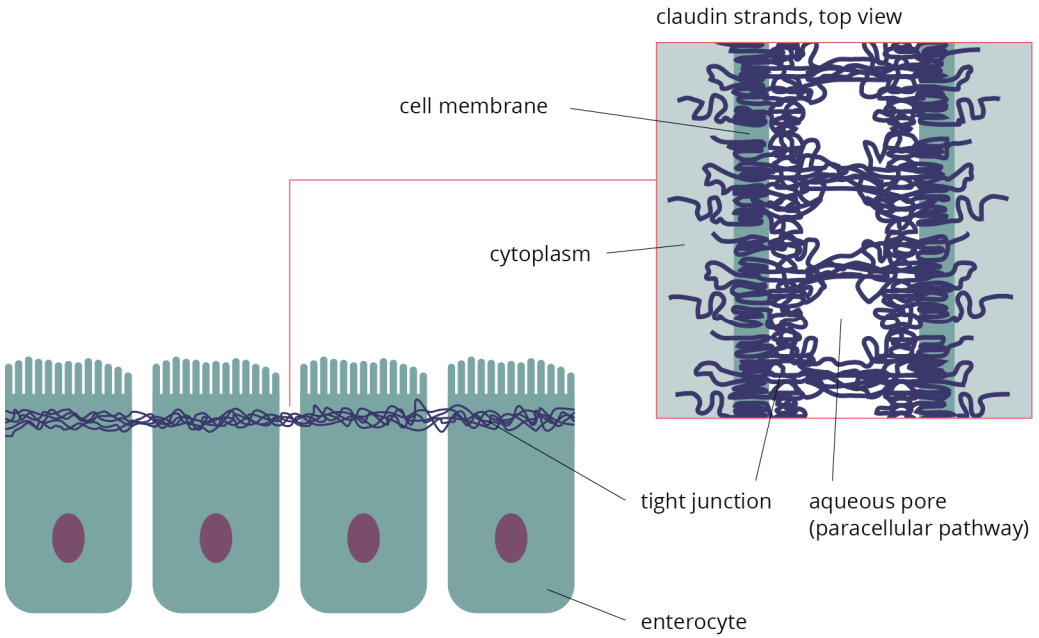

Figure 5. Schematic illustration of the tight junction pores formed by claudins (adapted with permission from Van Itallie and Anderson, 2004).

A perfusion study in humans suggests that that the villus tips are less permeable than the lower parts of the villus (Figure 3) and that the human intestine has pores with a radius between 8 and 13 Å (Fine et al., 1995). The average pore radii were determined by measuring the permeability ratio of L-xylose/urea during constant perfusion of the jejunum at varying flow rates. In a rat intestinal perfusion study conducted by Fihn et al. (2000), it was also suggested that the pores at the villus tips are smaller (radius ~6 Å) and more numerous compared to the pores at the lower part of the villus (radius 10-15 Å) and the crypt base (radius 50-60 Å). Fihn et al. (2000) examined the rat jejunal paracellular dimensions *in vivo* by utilizing radiolabeled probes of three varying sizes and employing tissue autoradiography. The hetero-porosity of the intestinal epithelium is supported by morphologic findings (Marcial et al., 1984). However, it should be noted that the largest pores at the crypt base might not be accessible to the luminal content and thus drug absorption (Fihn et al., 2000; Hollander, 1992). Paracellular permeability does not just differ across the crypt-villus axis but also along the intestine, with the small intestine exhibiting larger pores than the colon (Fihn and Jodal, 2001).

The pore-forming tight junctions are not only static structural elements but dynamically regulated by intracellular processes (Ballard et al., 1995). Pappenheimer and coworkers have proposed a controversial theory suggesting that glucose uptake serves as an intracellular signal to decrease tight junction integrity and, thus, increase tight junction permeability (Pappenheimer, 1993). The rat intestinal perfusion study of Fihn et al. (2000) supported the theory of the role of glucose in paracellular permeability: It was suggested that active glucose transport increases the number of small pores in the rat intestinal epithelium, while the size of the pores appears to remain constant.

A number of compounds such as ethylenediaminetetraacetic acid (EDTA), bile salts, chitosan, sodium caprate, and synthetic peptides have been shown to open tight junctions (Brunner et al., 2021). This has led to several studies exploring ways to enhance paracellular permeability.

2.4 MOLECULAR DESCRIPTORS DETERMINING PASSIVE INTESTINAL DRUG PERMEATION

Molecular properties that determine drug permeation across the intestinal epithelium have been studied extensively. Already in 1890s, Overton outlined that the membrane permeability of a molecule increases with its lipophilicity (Al-Awqati, 1999). In 1957 Brodie and coworkers presented the pH-partition theory, according to which the ionization state of a compound, influenced by its pK_a value and the pH of the surrounding environment, determines its ability to cross biological membranes. It was proposed that only the unionized form of a molecule can cross the intestinal cell membranes (Shore et al., 1957). Since then, it has been shown that polarity and size are, in addition to lipophilicity and charge, the key parameters determining passive diffusion across the intestinal epithelium. These descriptors are reviewed below.

Lipophilicity is the affinity a molecule has for a lipophilic or non-polar environment (Gold, 2019). In drug development, the lipophilicity of a compound is usually presented as logP or logD (octanol/water partition coefficient of the neutral form or octanol/water partition coefficient of all forms at a specific pH, respectively). The logD and logP descriptors correlate roughly with human intestinal permeability: sigmoid, linear, hyperbolic, bilinear and parabolic relationships between lipophilicity and permeability have been reported (Pham-The et al., 2018). Figure 7 illustrates the correlation between calculated $logD_{6.5}$ and human F_A values of 553 passively absorbed compounds (Hou et al., 2007). Hou and coworkers proposed that compounds with $logD_{6.5}$ values below -3.2 were more likely to have F_A values under 0.1. However, it has also been suggested that small hydrophilic compounds transported via the paracellular path may also be well absorbed (Karlsson et al., 1999).

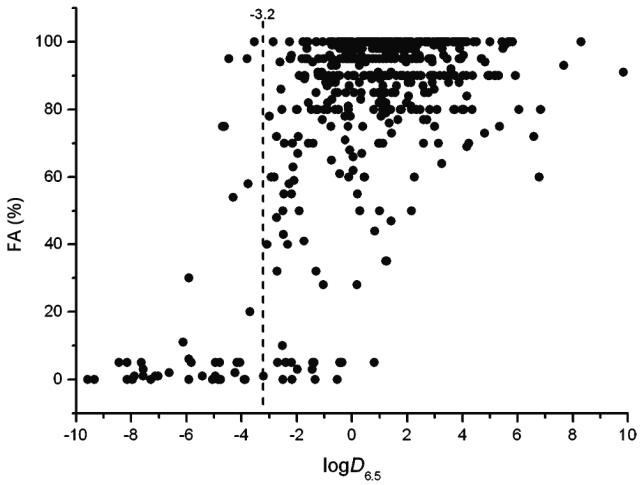

Figure 7. Correlation between $logD_{6.5}$ and F_A (reprinted with permission from Hou et al., 2007).

The inverse correlation of molecular polarity with intestinal absorption has been demonstrated in several studies. In the late 1990s it was shown that both dynamic and topologic polar surface areas (PSA and TPSA) have excellent correlations with human F_A (Clark, 1999; Palm et al., 1997). The analyses were performed with a set of 20 passively absorbed compounds and resulted in R^2 values of 0.94 and 0.92 respectively. A larger set of 553 compounds yielded a lower but still reasonable correlation between TPSA and F_A (R^2 = 0.49) (Hou and Wang, 2008). PSA and TPSA are defined as the molecular surface area occupied by nitrogen and oxygen atoms, including the polar hydrogens attached to them. While PSA correlates slightly better with human F_A than TPSA, the latter has the advantage of being rapidly calculated and not requiring determination of the 3D structure. Figure 8 illustrates the correlation between both PSA and TPSA and human F_A. In addition to PSA, molecular polarity can be quantified by the hydrogen bond donors and acceptors (HBD and HBA).

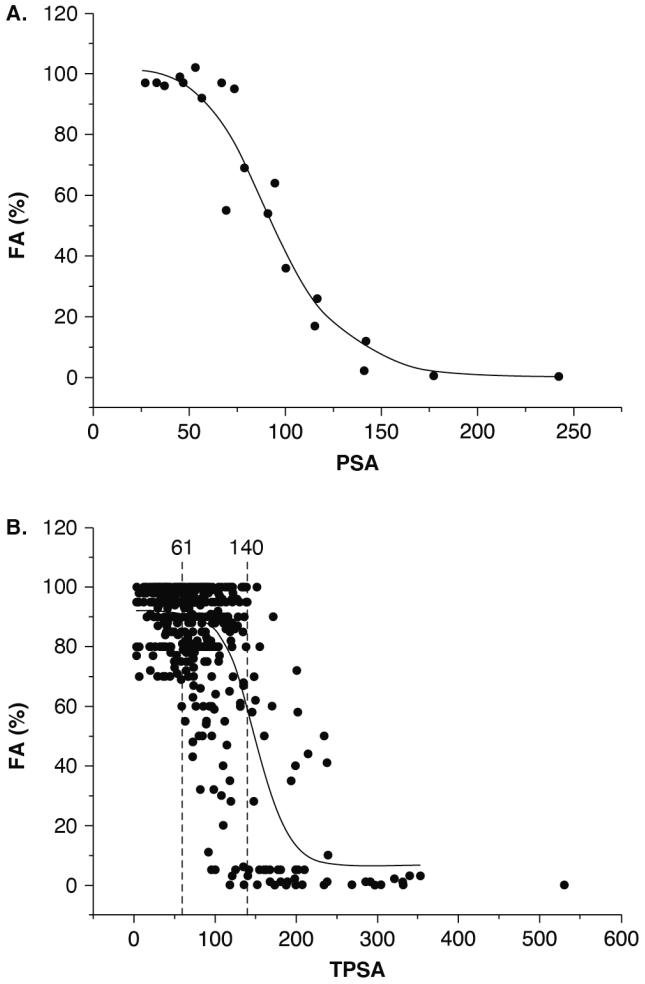

Figure 8. Correlation between PSA and F_A. (A) The correlation between PSA and human F_A for a set of 22 compounds resulted in an R^2 value of 0.94 (Palm et al., 1997). Compounds with a PSA < 61 $Å^2$ were shown to have F_A (%) values > 90, while compounds with a PSA > 140 $Å^2$ had F_A (%) values < 10. (B) The correlation between TPSA and human F_A for a set of 553 compounds resulted in an R^2 value of 0.49 (Hou and Wang, 2008). Reprinted with permission from Hou and Wang, 2008.

Molecular size, often represented by molecular weight (MW), is important in determining transport across the intestinal membrane. Small hydrophilic molecules may cross the intestinal epithelium via the aqueous paracellular route. However, since the paracellular space occupies only a minor portion of the intestinal membrane, the main route of absorption for most sufficiently lipophilic molecules is considered to be transcellular. Due to the ordered structure of the cell membrane, the smaller a molecule is, the easier it diffuses across the cell membrane. The inverse correlation of molecular size with transcellular transport has been demonstrated in several studies (Cohen and Bangham, 1972; Walter and Gutknecht, 1986; Xiang and Anderson, 2006, 1998). According to Lipinski's "Rule of 5" (see section 2.6), the MW of a compound should ideally be under 500, and the study of Doak and coworkers (2014) showed that orally delivered clinical compounds rarely have MWs over 1000.

2.5 EXPERIMENTAL METHODS FOR ASSESSING INTESTINAL DRUG PERMEATION

The rate and mechanisms of intestinal drug permeation can be assessed using experimental model systems. The complexity of experimental models varies greatly, from simple *in vitro* models to more complex *ex vivo* and *in vivo* models. The properties of the *in vitro*, *ex vivo* and *in vivo* methods relevant to this thesis are discussed below.

2.5.1 *In vitro* methods

Cell models, introduced in the 1980s (Artursson, 1990; Hidalgo et al., 1989), and artificial membrane models such as the parallel artificial membrane assay (PAMPA), developed in the late 1990s (Kansy et al., 1998), are popular *in vitro* tools for determining intestinal drug permeability. When cultured, the Caco-2, MDCKII and 2/4/A1 cells investigated in this thesis, form a monolayer of polarized cells with an apical brush border and tight junctions sealing the cells together. They also express varying levels of

transporter proteins and metabolizing enzymes. Caco-2 and transporter-transfected MDCKII cell models are commonly employed to study active transport. Artificial membranes completely lack transporters, enzymes and the paracellular pathway. Some key features of the Caco-2, MDCKII and 2/4/A1 cell models are shown in Table 1.

Table 1. Features of the Caco-2, MDCKII and 2/4/A1 cell models.

Membrane model	Origin	Culture time (d)	TEER* (Ωxcm^2)	Advantages
Caco-2	human colon carcinoma	21	250-4000	well characterized
MDCKII	dog kidney	3-5	100-640	short cultivation time
2/4/A1	rat fetal intestine	4-7	25-50	good prediction of paracellular permeation

*Transepithelial electrical resistance (TEER) reflects the tightness of a membrane. TEER values were obtained from Srinivasan et al. (2015); Thwaites et al. (1993); Dukes et al. (2011); Matsson et al. (2005).

Caco-2, MDCKII, and 2/4/A1 permeability values demonstrate a fairly good correlation with human F_A, for permeability rate-limited compounds and sigmoid relationships between cell model permeabilities and human F_A are observed (Figure 6). Especially studies performed with small datasets of passively transported compounds have yielded good correlations. Because of inter-laboratory variability, the predictive power of cell models should not be assessed by combining permeability values from different laboratories. Factors contributing to inter-laboratory variability include genetic drift and differences in experimental conditions and protocols (Hämäläinen and Frostell-Karlsson, 2004).

The Caco-2 cell model has been shown to underestimate the absorption of drugs that are considered to be mainly transported via the paracellular

route (Tavelin et al., 2003). The 2/4/A1 cell model, on the other hand, seems to predict the absorption of these compounds better (Matsson et al., 2005; Tavelin et al., 2003). The better predictivity of the 2/4/A1 cell model has been proposed to result from it forming a leakier paracellular pathway than the Caco-2 cell line (Tables 1 and 2).

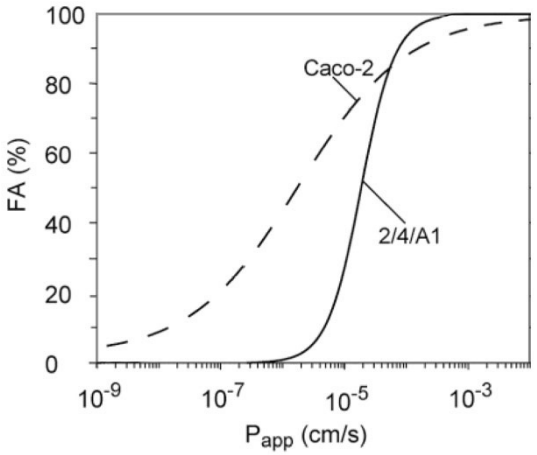

Figure 6. Permeability ($logP_{app}$) vs F_A curves of the Caco-2 and 2/4/A1 cell models. 2/4/A1 cell line has a steeper slope and higher P_{app} values than the Caco-2 cell line. Adapted with permission from Matsson et al., 2005.

The dimensions of the paracellular pathways of the cell models have been assessed by determining the permeability of inert hydrophilic probes of varying sizes. In line with *in vivo* studies cell line studies suggest the existence of paracellular pores of different sizes. Based on biphasic permeability profiles of PEG oligomers across cell monolayers, both Watson et al. (2001) and Van Itallie et al. (2008) determined that the Caco-2 and MDCKII cell lines have two distinct pore sizes. The radius of the smaller pores was shown to be ~4 Å, whereas the larger pores did not exhibit a size limit. It is, however, likely that the largest PEG examined (PEG25 ~7 Å) was too small to evaluate the size of the larger pores. Tavelin and coworkers (2003) also found that the Caco-2 cell line exhibits pores with a radius of ~4 Å. The same study suggested that the pore size of the 2/4/A1 cell line was 9 Å.

Table 2. Estimated pore size/sizes of cell models and the rat and human intestine.

Absorption model	Smaller pore size (Å)	Larger pore size (Å)	Reference
Caco-2	12	-	Adson et al., 1994
Caco-2	5	-	Knipp et al., 1997
Caco-2	4	>7	Watson et al., 2001
Caco-2	4	>7	Van Itallie et al., 2008
MDCKII	4	>7	
Caco-2	4	-	Tavelin et al., 2003
2/4/A1	9	-	
rat intestine	6	10-15 and 50-60	Fihn et al., 2000
human intestine	8	13	Fine et al., 1995

2.5.2 *Ex vivo* methods

Ex vivo transport studies use gut tissues removed from animals or humans to assess intestinal transport. These techniques provide a compromise between simple *in vitro* and more complex *in vivo* models. Diffusion chambers, such as the Ussing chamber (technique employed in article 3 of this thesis), provide physiological systems to determine drug permeation across excised intestinal tissues (Rozehnal et al., 2012). The Ussing chamber technique allows (1) intestinal permeability measurements under varying physiological conditions, (2) evaluation of transport mechanisms and (3) assessment of regional differences in intestinal permeability (Ungell et al., 1998). Limitations of the diffusion chamber technique include limited viability of the tissue and high inter- and intra-laboratory variability (Dahlgren and Lennernäs, 2019). A study by Sjöberg et al. (2013) demonstrated a good sigmoidal correlation (R^2 = 0.85) between human jejunal segments and experimental F_A for a set of 25 drugs.

2.5.3 *In vivo* methods

Pharmacokinetic studies in animals are routinely performed in preclinical drug discovery and development. The rat is the most frequently employed animal for assessing oral drug absorption, representing approximately 80% of all studies (Harloff-Helleberg et al., 2017). Rat F_A values have demonstrated a good correlation with human intestinal F_A values (Chiou and Barve, 1998; Zhao et al., 2003). Zhao and coworkers demonstrated a linear relationship between rat and human absorption: $F_A(\text{human}) = 0.997\ F_A(\text{rat})$; $R^2 = 0.88$, n = 98.

Despite the good correlation between rat and human absorption, human pharmacokinetic studies are always performed in drug development programs. Oral bioavailability is a critical parameter determined in these studies. The permeability of a compound is considered to be high when bioavailability (F) is ≥ 0.85 of the administered dose or when ≥ 0.85 of the dose is retrieved in urine as the parent drug or as the molar total of the parent drug and phase 1 and 2 metabolites (ICH, 2020).

2.6 COMPUTATIONAL METHODS FOR ESTIMATING INTESTINAL DRUG PERMEATION

Computational models are widely employed in the pharmaceutical industry to predict oral drug absorption. They are based on computationally calculated molecular descriptors and can be broadly classed into (1) simple qualitative approaches relating physicochemical properties to bioavailability and (2) Quantitative Structure-Property Relationship (QSPR) models (O'Shea et al., 2022). Computational models are generally designed to describe transcellular permeability because the transcellular route of absorption is predominant for the majority of sufficiently lipophilic molecules.

2.6.1 Drug-likeness rules

Drug-likeness is a concept that provides useful guidelines for predicting oral absorption in the early drug discovery stages. A molecule is considered drug-like if its physicochemical properties fall within the range of approved orally active drugs (Ajay et al., 1998). One of the best-known drug-likeness rules is the "Rule of 5", based on an analysis of 2245 approved drugs and clinical candidates (Lipinski et al., 2001). According to the "Rule of 5", poor oral absorption is most probable when: (1) calculated logP (ClogP) exceeds five, (2) MW is over 500, (3) there are over five hydrogen bond donors and (4) there are over ten hydrogen bond acceptors. Later other investigators suggested other versions of drug-likeness rules. Many of the later rules include the same, but also alternative descriptors, to the ones used in the "Rule of 5". Table 3 shows the Lipinski, Egan, Veber, Ghose and Muegge rules regarding the logP, MW, HBA, HBD and TPSA descriptors. Although drug-likeness rules have been successfully used in predicting oral absorption, not all molecules that comply with rules are well absorbed. Furthermore, it is not uncommon that compounds that are well absorbed deviate from the rules.

Table 3. The Lipinski, Egan, Veber, Ghose and Muegge rules regarding the logP, MW, HBA, HBD and PSA descriptors.

Rule	logP	MW	HBA	HBD	TPSA
Lipinski et al., 2001	≤ 5	≤ 500	≤ 10	≤ 5	-
Veber et al., 2002	-	-	-	-	≤ 140
Egan et al., 2000	≤ 5.88	-	-	-	≤ 131.6
Ghose et al., 1999	-0.4 – 5.6	160 – 480	-	-	-
Muegge et al., 2001	-2 – 5	200 – 600	≤ 10	≤ 5	≤ 150

2.6.2 QSPR models

Over the past two decades, numerous QSPR models have been developed to predict intestinal absorption (Table 4). Structural features have been related to (1) cell line permeability, (2) artificial membrane permeability, (3) human jejunal permeability and (4) F_A in humans. To establish the quantitative correlations between molecular descriptors and absorption, various statistical methods – from linear regressions to neural networks – have been utilized.

The building of a QSPR model typically consists of the following steps: (1) collection of a dataset (2) calculation and selection of molecular descriptors (3) establishing a quantitative correlation between the molecular descriptors and permeability/absorption using statistical methods (4) model validation (Figure 9). The model's validity relies on the dataset used, irrespective of the statistical tools used to develop it. It is common that the QSPR models in the literature are built with fewer than 50 compounds due to the lack of large public datasets with high quality data (Table 4) (Norinder and Bergström, 2006). This may result in models with limited predictive ability due to insufficient diversity of the compounds in the dataset (Norinder and Bergström, 2006).

Table 4 Quantitative computational models based on Caco-2, F_A, and P_{eff} data. Adapted with permission from Norinder and Bergström, (2006).

Caco-2	**R^2**	**n**	**Reference**
molecular surface areas	0.96	19	Stenberg et al., 1999
PSA, lipophilicity, size, flexibility	0.71	77	Hou et al., 2004
H bond capacity, lipophilicity, size	0.71	33	Marrero Ponce et al., 2004
H bond strength and electrostatics	0.85	9	Norinder et al., 1997
H bond capacity, lipophilicity, size, flexibility	0.80	16	Oprea and Gottfries, 1999
H bond capacity, lipophilicity	0.92	11	Osterberg and Norinder, 2000
size, surface tension, dielectric constant	0.90	16	Osterberg and Norinder, 2001
electrotopological indices	0.71	17	Stenberg et al., 2001
H bond strength, electrostatics	0.87	17	Stenberg et al., 2001
surface areas	0.93	17	Stenberg et al., 2001
electrotoplological indices	0.91	9	Norinder and Osterberg, 2001
surface areas	0.93	13	Bergström et al., 2003
H bond capacity, PSA, charge	0.83	20	Matsson et al., 2005
H bond capacity, charge, polarizability, dipole moment	0.62	87	Fujiwara et al., 2002
F_A			
structural fragments	0.79	417	Klopman et al., 2002
H bond capacity, lipophilicity, size, flexibility	0.5	85	Oprea and Gottfries, 1999
H bond capacity, lipophilicity	0.93	74	Osterberg and Norinder, 2000
electrotopological indices	0.83	13	Norinder and Osterberg, 2001
H bond capacity, size, flexibility	0.87	76	Wessel et al., 1998
H bond capacity, flexibility, hydrophobicity	0.86	76	Niwa, 2003
P_{eff}			
PSA. $logD_{5.5}$	0.93	13	Winiwarter et al., 1998
PSA, HBD	0.85	13	Winiwarter et al., 1998
PSA, ClogP, HBD	0.88	13	Winiwarter et al., 1998

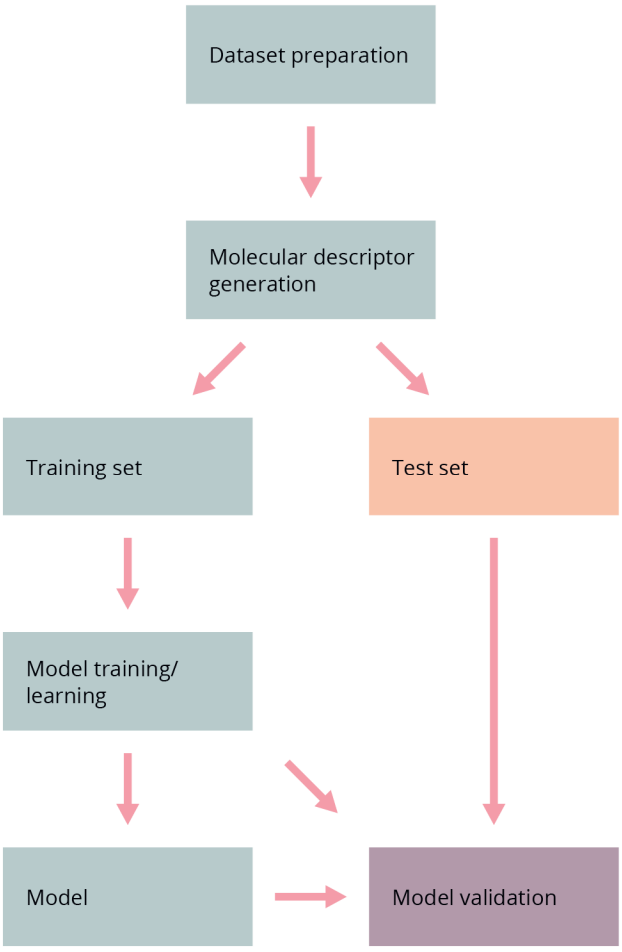

Figure 9 Typical workflow for building a QSPR model. Adapted with permission from Sahlgren et al., 2017.

3 AIMS OF THE STUDY

The general aims of the studies conducted in this thesis were to (1) develop and evaluate computational and experimental methods for predicting intestinal drug absorption that is rate-limited by passive permeability and (2) characterize the paracellular pathway of drug absorption.

The specific aims were to:

1. Develop QSPR models for predicting passive drug permeation across the human intestinal wall. (article 1)
2. Evaluate the predictive capability of QSPR models and preclinical experimental models. (article 2)
3. Characterize the pore sizes and porosity of the paracellular drug permeation pathway in the human intestine *ex vivo*. (article 3)
4. Characterize the pore sizes and porosity of the paracellular drug permeation pathway in the Caco-2, 2/4/A1, and MDCKII cell models. (article 3)
5. Enhance our understanding of cell model predictions by comparing the paracellular pathways of the Caco-2, 2/4/A1 and MDCKII cell models to the human paracellular pathway. (article 3)

4 MATERIALS AND METHODS

The following sections give an overview of the materials and methods used in this thesis. For a more comprehensive description of the materials and methods, please refer to the original articles.

4.1 QSPR MODELS FOR PREDICTING INTESTINAL ABSORPTION

The following strategy was applied to develop and validate the QSPR models for predicting human intestinal absorption rate constants (K_a):

1) Selection of compounds for datasets.
2) Determination of K_a values.
3) Determination of molecular descriptor values.
4) Investigation of structural diversity of compounds with principal component analysis (PCA).
5) Establishment of the relationship between molecular descriptors and K_a with partial least squares (PLS) analysis.
6) Validation of models.

4.1.1 Compound datasets

Two main compound datasets were used in the study. Dataset 2 consisted of 169 permeability rate-limited compounds with known F_A values, as reported by Zhao et al. (2001). From this dataset, 23 compounds were selected for dataset 1 based on the following criteria: (1) iv and po data available (various literature sources), and (2) passive diffusion is the predominant absorption mechanism according to the Ozawa transporter database (Ozawa et al., 2004). The final models were constructed using the 22 compounds from dataset 1. The compounds in dataset 2 were employed for external validation of the developed models.

4.1.2 Human intestinal absorption rate constants

The K_a values of dataset 1 compounds were calculated from their literature iv, po and F_A data according to the following equation:

$$K_a = K_{app} \times F_A$$

where K_{app} is the apparent absorption rate constant (1/h) describing the disappearance of the drug from the absorption site. K_{app} was calculated from the fraction input profiles obtained by deconvolution analysis of the iv and po data. WinNonlin software was used in the deconvolution analysis.

4.1.3 Molecular descriptors

Molecular descriptors relevant to intestinal absorption were selected. The values of MW, ACDlogP, and ACDlogD were calculated using ACD/Labs software. The values of PSA, HBA, HBD, ClogP were obtained from the study of Zhao et al. (2001).

4.1.4 Multivariate data analyses

The structural diversity of dataset 1 was compared to the structural diversity of dataset 2 by PCA analysis. The analysis was based on the following descriptors: MW, $ACDlogD_{6.0}$, HBD, HBA, and PSA.

The relationship between the molecular descriptors and K_a values of the molecules in dataset 1 was established using the multivariate PLS method. Cross-validation was conducted to assess the predictive ability of the developed models. The predictive capability of the models was further assessed by partitioning the compounds into a training and a test set. All multivariate analyses were carried out with SIMCA-P 10.5 software by UMETRICS.

4.1.5 External validation of developed models

The developed models were externally validated by employing them to predict the log K_a values of 169 drugs with known F_A values (dataset 2). A sigmoidal curve was fitted to the predicted log K_a versus experimental human F_A data.

$$F_A\% = 100 / 1 + e^{-((x - x_0) / y)}$$

where x is log K_a, x_0 is log K_a at 50% F_A and y is a slope factor. The curve was fitted to the data by minimizing the sum of squared residuals, and the fit was assessed using R^2, the coefficient of determination.

4.2 COMPARISON OF THE PREDICTIVE PERFORMANCES OF QSPR AND EXPERIMENTAL MODELS

The following strategy was applied to compare the predictive capabilities of experimental and computational models:

1) Selection of compounds for datasets
2) Calculation of molecular descriptors
3) Employment of the QSPR models to predict intestinal absorption
4) Correlation of model predictions with human F_A and assessment of the fit using the coefficient of determination

4.2.1 Models included in evaluation

The evaluated models included seven QSPR models based on human absorption values (Table 5), two cell models (Caco-2, 2/4/A1), three parallel artificial membrane models (Double-Sink PAMPA, HDM-PAMPA, Hydrophilic Filter Membrane PAMPA) and, the *in vivo* rat model.

Table 5. Equations of QSPR models included in the evaluation.

Model	Equation	Study
(1)	$\log K_a = 0.623 + 0.154 \log D_{6.0} - 0.007$ PSA	Linnankoski et al., 2006
(2)	$\log K_a = 0.424 + 0.143 \log D_{6.0} - 0.129$ HBD	Linnankoski et al., 2006
(3)	$\log K_a = 0.636 + 0.098 \log D_{6.0} - 0.004$ PSA – 0.088 HBD	Linnankoski et al., 2006
(4)	$\log P_{eff} = -2.883 - 0.01$ PSA + 0.192 $\log D_{5.5}$ – 0.239 HBD	Winiwarter et al., 1998
(5)	$\log P_{eff} = -2.546 - 0.011$ PSA – 0.287 HBD	Winiwarter et al., 1998
(6)	$\log P_{eff} = -3.067 + 0.162$ ClogP – 0.01 PSA – 0.235 HBD	Winiwarter et al., 1998
(7)	$Abs\% = 100 \times [1 - \exp(-10^{0.435+0.0848E+0.0405S-0.348A-0.403B+0.232V})]$*	Zhao et al., 2002

*E is an excess molar refraction, S the dipolarity/polarizability, A and B the hydrogen-bonding acceptors and donors, respectively, and V is the McGowan characteristic volume.

4.2.2 Compound datasets

Compound selection was based on the following requirements: (1) a reliable F_A value found in the literature; (2) diffusion limits the absorption rate. The selected compounds are shown in Table 6. The size of the datasets used in the evaluations ranged from 17 to 178. To ensure unbiased comparisons, datasets with *in vitro* permeability values determined within a single laboratory were employed.

Table 6. Datasets used to evaluate the models.

Study	Absorption data	n
Zhao et al., 2002	Computational	178
Zhao et al., 2003	Rat	66
Yazdanian et al., 1998	Caco-2	33
Matsson et al., 2005	Caco-2, 2/4/A1, HDM-PAMPA	17
Stenberg et al., 2001	Caco-2	20
Yee, 1997	Caco-2	19
Avdeef, 2003	Double-Sink PAMPA	17
Zhu et al., 2002	AMP	57

4.2.3 Structural diversity of the drugs

PCA analyses were conducted to assess the molecular diversities of the datasets (Table 6). The analyses were based on the dataset of 178 compounds and the following computational molecular descriptors: MW, ClogP, HBD, HBA, and PSA. The range of the physicochemical variable values of the 178 compounds was: MW 102–873, ClogP –10.62–6.35, $ClogD_{6.0}$ –7.31–5.48, HBD 0–19, HBA 1–19, PSA 5–354. The PCA analyses were carried out with SIMCA-P 10.5 software by UMETRICS.

4.2.4 Employing the computational models to predict intestinal absorption

Each computational model was utilized to predict the intestinal absorption of the 178 selected drugs. The molecular descriptors employed in the modelling process were calculated as in the original studies. However, there were a couple of exceptions regarding the PSA and logD values in the models predicting P_{eff}. In the case of PSA values, we used calculations performed with SAVOL software (Tripos Inc., St Louis, MO, USA), whereas the original study employed PSA values determined using SYBYL software (Tripos Inc., St Louis, MO, USA). Similarly, for logD values, we utilized computational logD values, calculated with the ACDlabs 6.0 software (Advanced Chemistry

Development, Toronto, Canada), whereas the original study used experimental values.

4.2.5 Correlating model predictions with human F_A

The predictive abilities of QSPR models 1-6 (Table 5) and the artificial membrane and cell models were evaluated by curve fitting the predicted absorption rate constant versus human F_A data using the equation below:

$$F_A\% = 100 / 1 + e^{-((x - x_0) / \gamma)}$$

where x is the logarithm of the absorption rate constant and x_0 is the logarithm of the absorption rate constant at 50% F_A, and γ is a slope factor. The equation was fitted to the experimental data by minimizing the sum of squared residuals, and the fit was assessed using R^2, the coefficient of determination.

The predictive ability of QSPR model 7 (Table 5) and the rat absorption data were assessed by applying linear least squares regression to the human F_A vs predicted F_A data.

4.3 PARACELLULAR PATHWAY OF THE HUMAN JEJUNUM AND CELL CULTURES

The following strategy was employed to determine the pore sizes and porosities of the human jejunum and the Caco-2, MDCKII and 2/4/A1 cell culture monolayers:

1) The permeabilities of polyethylene glycol (PEG) oligomers across the membranes under investigation were measured.
2) An effusion theory-based equation was applied to the permeability data to calculate the pore sizes and porosities of the investigated membranes.

4.3.1 Human jejunal tissue

The human jejunal tissue was collected during gastric bypass surgery from a 62-year-old female patient. A detailed description of the procedure performed at the Sahlgrenska University Hospital in Sweden can be found in the original article (Linnankoski et al., 2010).

4.3.2 Cell culture

Caco-2 cells obtained from the American Type Culture Collection were seeded at a density of 0.8 x 10^5 cells/cm^2 onto uncoated permeable polycarbonate filters and cultured for 21-23 days. Cells at passage number 46 through 54 were utilized.

MDCKII cells obtained from the Netherlands Cancer Institute were seeded at a density of 0.43 x 10^6 cells/cm^2 onto uncoated permeable polycarbonate filters and cultured for four days. Cells at passage number 32 through 38 were employed.

2/4/A1 cells obtained from the University of Uppsala were seeded at a density of 0.18 x 10^6 cells/cm^2 onto uncoated permeable polycarbonate filters and cultured for four days. Cells at passage number 37 were used.

A detailed description of the cultivation procedures can be found in the original article.

4.3.3 Polyethylene glycol permeability studies

A solution of polyethylene glycol (PEG) oligomers (Table 7) was used in the permeability experiments. All experiments were conducted at 37 °C in the apical to basolateral direction. The initial concentration of each PEG oligomer at the apical side was 100 µmol/L. Samples were collected from both acceptor and donor chambers at regular intervals, up to 240 minutes in the cell culture experiments and up to 210 minutes in the diffusion chamber experiment (jejunum). The collected sample volumes were exchanged with fresh buffer in the case of Caco-2, MDCKII, and jejunum experiments, while the filters were transferred to new wells containing buffer in the case of the 2/4/A1 experiment. Integrity of the membranes was

investigated by measuring (1) permeability of radiolabeled mannitol and (2) TEER values before and after the experiments.

Table 7. Properties of investigated PEG oligomers

MW (g/mol)	Radius* (Å)
238	4.51
282	4.87
326	5.15
370	5.47
414	5.78
458	6.03
502	6.27
546	6.55
590	6.77
634	7.03
678	7.24
722	7.45
766	7.69
810	8.89
854	8.09
898	8.27
942	8.51

*Calculated based on radius of gyration as in Linnankoski et al., (2010)

4.3.4 Calculating pore sizes and porosities

Apparent permeability coefficients (P_{app}) of the PEG oligomers were calculated according to the following equation:

$$P_{app}\ (cm/s) = dQ/dt \times 1/(A \times C_o)$$

where dQ/dt represents the PEG flux across the membrane at steady state (mol/s), A denotes the membrane surface area (cm^2) and C_o is the concentration in the donor chamber at the beginning of the experiment (mol/ml).

The porosities (ε) and pore sizes of the membranes were determined based on PEG oligomer permeabilities using an effusion theory based equation (Hämäläinen et al., 1997):

$$\frac{J_h}{c} = \frac{RT\varepsilon}{12\pi\eta N_A \lambda}\frac{1}{r_s} = [slope]\frac{1}{r_s}$$

where, (J_h/C) represents the permeability of a PEG oligomer, r_s is the radius of a PEG oligomer, ε is the membrane porosity, R is the gas constant, T is the temperature, η is the water viscosity, N_A is Avogadro's constant, λ is the jump length of PEGs in water.

The pore sizes were estimated by extrapolating f($1/r_s$) to a permeability of zero and thereby determining the largest PEG oligomer capable of penetrating through the paracellular pores.

5 RESULTS AND DISCUSSION

An overview of the main results of this thesis will be provided and discussed below. Detailed results can be found in the original articles.

5.1 QSPR MODELS FOR PREDICTING INTESTINAL ABSORPTION

The QSPR model equations resulting from our analyses are presented in Table 8. Consistent with previous studies we found that the lipophilicity and polarity of a compound play a significant role in passive intestinal permeation. The developed models combine $\log D_{6.0}$ with either PSA, HBD, or both.

The models were the first to predict absorption rate constants (K_a) in humans. Prior QSPR models had been developed to predict (1) *in vitro* membrane permeability, (2) human jejunal permeability and (3) human F_A. Predicting the rate of absorption instead of the extent of absorption was a logical choice, considering that completely absorbed compounds can exhibit varying absorption rates. In our study, the range of K_a values for drugs with F_A values of 1 was found to be between 0.15 and 6.4 h^{-1}. Furthermore, building models based on human *in vivo* absorption rate data was motivated by the fact that *in vitro* permeability studies can only simulate the human absorption process.

Table 8. Equations of the developed QSPR models. The models were developed using 22 structurally diverse compounds.

Model	Equation
1a	$\log K_a = 0.623 + 0.154 \log D_{6.0} - 0.007$ PSA
2a	$\log K_a = 0.424 + 0.143 \log D_{6.0} - 0.129$ HBD
3a	$\log K_a = 0.636 + 0.098 \log D_{6.0} - 0.004$ PSA $- 0.088$ HBD

The developed models showed good statistics (Table 9). External validation of the developed models also demonstrated a good correlation between predicted K_a and experimental F_A for a set of 169 compounds with F_A values ranging from 0.03 to 1 (Figure 10). The predictivity of the models may have been limited by the data range ($F_A > 0.43$) within the training set used to build the models. However, accurate predictions were shown not only for compounds with moderate to high absorption but also for compounds with poorer absorption (Figure 10). Obviously, we would have also used compounds with low F_A values in model development if it had been possible. Experimental human iv and po data required for the calculation of K_a were, however, not available in the literature.

Table 9. Statistics of the developed QSPR models.

Model	Q^2	R^2	RMSE
1a	0.75	0.76	0.25
2a	0.74	0.75	0.26
3a	0.69	0.71	0.28

Q^2 cross validated correlation coefficient;
R^2 coefficient of determination RMSE, root mean squared error

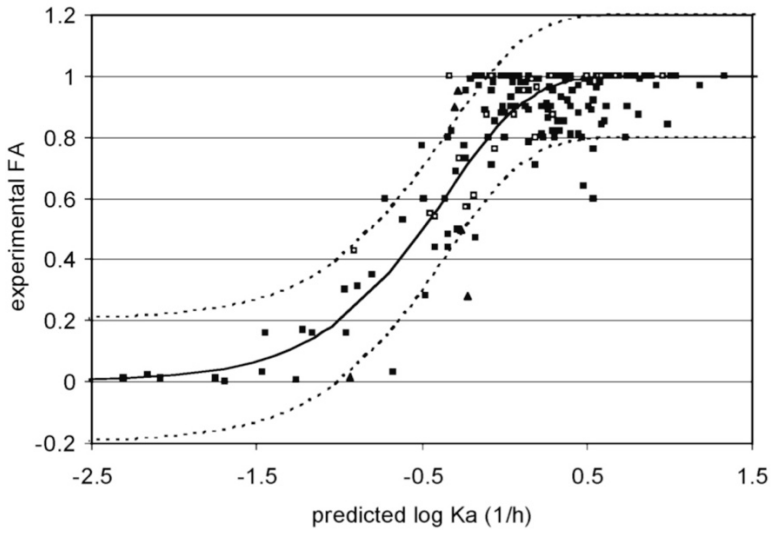

Figure 10. Correlation between predicted log K_a and experimental F_A values for a set of 169 compounds. Reprinted with permission from Linnankoski et al., 2006.

Although the training set used to develop the final models lacked poorly absorbed compounds, PCA analysis showed that it was structurally fairly diverse regarding the MW (range 144-384), PSA (range 24–182), $logD_{6.0}$ (range –2.87–4.82), and HBD (range 0–8) descriptors. The narrowish MW range might explain why MW was not found to be important in describing K_a. Obviously, the absorption of molecules not conforming to the defined property space should not be predicted using the developed models. Also, the models should only be applied to predict the absorption of permeabilty rate-limited passively absorbed compounds.

It is worth emphasizing that calculated logD values were used to develop the models. Due to the potential inaccuracies of calculated values, computational ACDlogD predictions were compared to experimental logD values. The correlation between the computational and experimental $logD_{7.4}$ values of 22 drugs used to build the models was excellent (R^2 = 0.96, RMSE 0.31). A similar high correlation was observed for a larger set of 88 drugs (R^2 = 0.86, RMSE 0.59). $LogD_{7.4}$ was used instead of $logD_{6.0}$ due to the availability of experimental values in the literature. Hou and coworkers (2007) later performed a similar analysis for 68 compounds: 48 compounds had

$ACDlogD_{7.4}$ prediction errors less than 1.0 log units, and seven had prediction errors exceeding 2.0 log units. They concluded that while the ACDLABS software satisfactorily predicts the logD values of most compounds, there is room for improvement in both logP and pK_a prediction accuracy.

5.2 PREDICTIVE POWER OF QSPR AND EXPERIMENTAL MODELS

The comparison of the computational models indicated that the simple models for predicting K_a and P_{eff} have similar predictivities as the QSPR model based on Abraham descriptors (Table 10). The K_a-based computational model combining $logD_{6.0}$ with PSA predicted human passive permeability rate-limited intestinal absorption (1) better than the artificial membrane models, (2) at least as well as the Caco-2 and 2/4/A1 cell models and (3) nearly as well as the *in vivo* rat model. Table 11 presents the comparison of the predictive capabilities of the K_a-based QSPR model and the established experimental intestinal absorption models. The most surprising result of the analysis was that the K_a-based QSPR model developed by us predicted the absorption of the set of 65 compounds almost as well as the *in vivo* rat model. Previous studies suggested that the *in vivo* rat model predicts human intestinal absorption better than *in vitro* models (Zhao et al., 2003). Among the *in vitro* models, the 2/4/A1 and Caco-2 cell models had been shown to have better predictive power than the artificial membrane models (Matsson et al., 2005). Our analyses supported these findings.

Table 10. Comparison of the QSPR models.

Prediction target	Descriptors	R^2
K_a	$logD_{6.0}$, PSA	0.79
P_{eff}	logD, PSA, HBD	0.80
F_A%	Abraham	0.78

Coefficients of determination (R^2) were obtained by correlating model predictions and human F_A% values of a set of 178 compounds.

Table 11. Comparison of the statistics of a QSPR model developed by us and experimental models. R^2 values were obtained by correlating model predictions and human F_A% values.

Dataset	QSPR*	Caco-2	2/4/A1	HDM-PAMPA	DS-PAMPA**	Rat
Matsson et al., 2005 (n=17)	0.87	0.72	0.88	0.37		
Yazdanian et al., 1998 (n=33)	0.86	0.67				
Yee, 1997 (n=19)	0.70	0.78				
Stenberg et al., 2001 (n=20)	0.89	0.85				
Avdeef, 2003 (n=17)	0.85				0.60	
Zhao et al., 2003 (n=66)	0.73					0.77

Each comparison has experimental permeability values determined in a single laboratory. The absorption of all compounds was assumed to be permeabilty rate-limited.

* $\log K_a = 0.623 + 0.154 \log D_{6.0} - 0.007$ PSA

** Double-Sink PAMPA

The differences in the ability of *in vitro* models to predict oral drug absorption have been suggested to result, at least partly, from varying capabilities in predicting paracellular permeation. Artificial membrane models entirely lack the paracellular pathway. By theoretically including paracellular transport into artificial membrane permeability data, it has been possible to improve their predictions to some extent (Matsson et al., 2005). The differences in predictions based on 2/4/A1 and Caco-2 cell model data may be attributed to the differences in the dimensions of their paracellular pathways. These dimensions were assessed in article 3 of this thesis and will be discussed below.

5.3 PARACELLULAR PATHWAY OF THE HUMAN JEJUNUM AND CELL CULTURES

The porosities and pore sizes of the paracellular drug permeation pathways of the human intestinal epithelium and the Caco-2, 2/4/A1 and MDCKII cell membranes investigated are presented in Figure 11 and Table 12. The porosities represent the surface area of the paracellular pores relative to the total surface area.

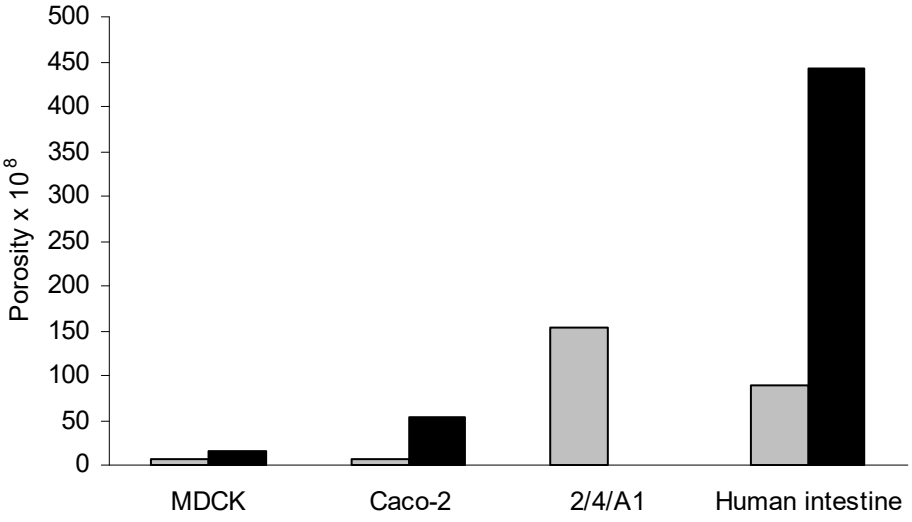

Figure 11. Porosities created by large pores (grey) and small pores (black) in MDCKII, Caco-2, 2/4/A1 cell models and human intestine. The total porosities in all membranes is low: 24 x 10^{-8}, 61 x 10^{-8}, 154 x 10^{-8} and 531 x10^{-8} in the MDCKII, Caco-2, 2/4/A1, and human jejunum, respectively. Reprinted with permission from Linnankoski et al., 2010.

Table 12. Tight junction pore dimensions in the investigated membranes.

Membrane	Small pores (Å)	Large pores (Å)
MDCKII	6	31
Caco-2	6	10
2/4/A1		15
Human intestine	7	10

Based on the biphasic permeability profiles of the PEG oligomers, two distinct pore sizes (7 and 10 Å) were identified in the human jejunal membrane. Our estimates of the human intestinal pore sizes are in line with the values obtained in human and rat intestinal perfusion studies (Table 13). The perfusion studies suggest that the smaller pores are located at the villus tips while the larger pores are located at the lower part of the villus. Hetero-porosity of the intestinal epithelium is supported by morphologic findings (Marcial et al., 1984). In line with the rat and human perfusion studies, we found that the smaller pores are more numerous than the larger pores (Figure 11).

Table 13. Tight junction pore dimensions in the human and rat intestine.

Study	Small pores (Å)	Large pores (Å)	Reference
Human intestine ex vivo	7	10	Our study
Rat intestine in vivo perfusion	6	10-15 and 50-60	Fihn et al., 2000
Human intestine in vivo perfusion	8	13	Fine et al., 1995

To better understand cell model predictions, the porosities and pore sizes of the Caco-2, 2/4/A1, and MDCKII cell models were compared to the porosity and pore size values of the human paracellular pathway. Among the investigated cell models, the porosity of the 2/4/A1 model was closest to the porosity of the human jejunum. The porosities of the MDCKII and Caco-2 cell models were found to be eight times lower than the porosity of the human intestinal epithelium. This might explain the tendency of the Caco-2 cell line to underestimate the absorption of compounds that are considered to be mainly transported via the paracellular route (Tavelin et al., 2003). Previously the better predictivity of the 2/4/A1 cell model has been explained by it forming larger pores than the Caco-2 cell line (Tavelin et al., 2003). We also found that the 2/4/A1 cell lines form larger pores than the Caco-2 cell model. However, the pore sizes of the Caco-2 cell model most closely resembled the pore sizes of the human intestine.

Our study was the first one to experimentally determine the surface area of the paracellular pores relative to the total surface area in the human intestine and cell models. Nellans (1991) proposed that the porosity of the human intestine may approach 0.1%. However, this estimate was not based on quantitative experimental research. The total porosities in all investigated membranes were low: 24×10^{-8}, 61×10^{-8}, 154×10^{-8} and 531×10^{-8} in the MDCKII, Caco-2, 2/4/A1 and human jejunum respectively. These values were approximately 100 fold lower than the eye porosity values determined using the same method (Hämäläinen et al., 1997).

5.4 FUTURE PROSPECTS

At the time when the investigations of this thesis were carried out, Matsson and coworkers (2005) wrote: "Diametrically opposed views regarding the paracellular pathway are presented in the literature, ranging from complete disregard of the influence of paracellular drug permeability to proposals that this pathway is a significant contributor to total drug permeability for many incompletely absorbed, low permeability compounds." Almost twenty years later, researchers agree that more research is still needed on the mechanisms of paracellular absorption (Dahlgren and Lennernäs, 2019;

Markovic et al., 2022). Larregieu and Benet (2013) emphasised the necessity for larger single-source *in vitro* permeability datasets to analyse the contribution of paracellular permeability.

The lack of extensive and reliable *in vivo* absorption and *in vitro* permeability data available publicly to the scientific community is well known to have hindered the development and evaluation of models for predicting human intestinal absorption. If it had been possible, we would have used larger datasets in developing the computational models for predicting human K_a (article 1) and evaluating the computational and experimental models for predicting intestinal absorption (article 2). Obviously, different results might have been obtained if larger compound datasets had been available for the analyses. Encouragingly, in recent years larger datasets, at least regarding F_A values, have been published (Price et al., 2021).

In the future, it would be interesting to analyse in further detail how the QSPR models developed in this thesis predict the absorption of small hydrophilic compounds. Because the paracellular and transcellular pathways are distinctly different, a single QSPR model obviously cannot predict drug transport across both routes. The predominant absorption mechanism of the drugs used to build our QSPR models was assumed to be transcellular, at least for the majority of the compounds. All except one compound had MWs > 200. Thus, the models should underpredict the absorption of compounds with significant paracellular transport. Also, it would be interesting to see alternative methods applied to determine the porosities of the human intestine and cell culture models.

6 CONCLUSIONS

The investigations of this thesis have provided new insight into computational and experimental techniques for screening the permeability of drug candidates in the early drug discovery stages. Novel QSPR models for predicting passive permeability rate-limited intestinal absorption were developed (article 1). The predictive powers of QSPR models, including the one presented in article 1, were compared to the predictivities of experimental models (article 2). To better understand cell model predictions, the paracellular pathways of the Caco-2, 2/4/A1 and MDCKII cell lines were characterized and compared to the human paracellular pathway (article 3). Our study was the first to experimentally determine the surface area of the paracellular pores relative to the total intestinal surface area in the human intestine and cell models.

The specific conclusions of this thesis are:

Passive intestinal permeability rate-limited drug absorption in humans can be successfully explained by a combination of two or three simple polarity and lipophilicity descriptors. The equation of the statistically best QSPR model developed in this thesis is: $\log K_a = 0.623 + 0.154 \log D_{6.0} - 0.007$ PSA.

The developed QSPR models were shown to predict absorption equally well as the Caco-2 and 2/4/A1 cell models, and furthermore, one of the models predicted absorption nearly as well as the *in vivo* rat model.

The porosities in the human intestine and the cell models are low. The porosity of the Caco-2 cell model was found to be eight times lower than the porosity of the human intestinal epithelium. The pore sizes in the Caco-2 monolayer (6 and 10 Å) and the human jejunal membrane (7 and 10 Å) were similar. The human jejunal pore sizes determined in this thesis aligned with previously determined human and rat intestinal pore sizes.

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ORIGINAL PUBLICATIONS (I-III)

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I

Computational prediction of oral drug absorption based on absorption rate constants in humans

Linnankoski J, Mäkelä J, Ranta V-P, Urtti A, Yliperttula M

Journal of Medicinal Chemistry 49(12): 3674-3681, 2006

II

Passive oral drug absorption can be predicted more reliably by experimental than computational models – fact or myth

Linnankoski J, Ranta V-P, Urtti A, Yliperttula M

European Journal of Pharmaceutical Sciences 34(2-3): 129-139, 2008

III

Paracellular porosity and pore size of the human intestinal epithelium in tissue and cell culture models

Linnankoski J, Mäkelä J, Palmgren J, Mauriala T, Vedin C, Ungell AL, Lazorova L, Artursson P, Urtti A, Yliperttula M

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Integrating pharmacokinetic considerations into the drug development process is crucial for developing effective medications. Models predicting the pharmacokinetics of a drug candidate are routinely used in early drug discovery phases, significantly contributing to the success of drug development programmes. This thesis explores oral drug absorption, focusing on intestinal permeability. The studies conducted have provided new insights into computational and experimental techniques for screening the intestinal permeability of drug candidates in the early drug discovery stages.

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