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In vitro models of the human intestine for better prediction of drug absorption, pre-systemic metabolism, and bioavailability

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Abstract
Ceylan, M. 2025. *In vitro* models of the human intestine for better prediction of drug absorption, pre-systemic metabolism, and bioavailability. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 371. 55 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2400-5.

The human small intestine is the primary site for drug absorption, hence intestinal *in vitro* models can bridge the gap to *in vivo* studies ultimately improving the drug development processes. This PhD thesis explores the global proteome and functional characteristics of four *in vitro* models that recapitulate the epithelial barrier of the human small intestine.

In Project I, we analyzed the global proteome of 2D enteroid monolayer models, focusing on markers related to stem cells, differentiation, actin regulatory proteins, and ADME proteins. We assessed the maturation of these models to evaluate their potential for studying bacterial infections and drug disposition, thereby establishing their functional capacity to replicate *in vivo* conditions.

Project II built upon the findings from Project I by evaluating the differentiation level of human intestinal 3D enteroid models through global proteomics and functional assays. Our proteomics analysis revealed that the 3D models exhibit a higher level of differentiation compared to 2D models. We also demonstrated the functionality of some key clinically important proteins, including CYP3A4, Pgp/MDR1, and BCRP, within the enteroids. This project confirms that these advanced 3D models are well-suited for studying drug metabolism and transport.

While enteroids are derived from stem cells and differentiated under laboratory conditions, freshly isolated enterocytes come from human tissue and are already fully differentiated. This potentially makes them a more accurate representation of *in vivo* conditions. Leveraging this advantage, in Project III, we focus on improving the isolation method for enterocytes from fresh jejunum tissue specimens. We quantified important metabolic enzymes, performed drug metabolism assays and demonstrated the enterocytes functionality for intestinal drug metabolism studies.

In Project IV, we develop a co-culture model using two MDCK cell lines that overexpress human MDR1 and BCRP. Following global proteomics characterization, we performed drug transport studies with both specific and shared drug substrates in Transwell and the Enabling Absorption Device settings. Our findings demonstrate the potential of this co-culture model for studying the role of active efflux in the absorption of drugs that require advanced formulations.

Together, these projects enhance our understanding of intestinal *in vitro* models, supporting their application in drug development research.

Keywords: Human small intestine, enteroids, enterocytes, MDCK, proteomics, ADME, drug metabolism, drug transport, live-cell imaging

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dedicated to the memory of people who lost their lives
in the earthquake on February 6th, 2023 in
Adana,
Adıyaman,
Diyarbakır,
Elazığ,
Gaziantep,
Hatay,
Kahramanmaraş,
Kilis,
Malatya,
Osmaniye,
and Şanlıurfa.

“Eğer bir yerde bilime, demokrasiye, barışa ve aydınlığa aç bir çocuk senin ışığını bekliyorsa, sönmeye hakkın yoktur.”

Prof. Dr. Türkan Saylan

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. van Rijn, J. M., Lopes, A. C. C., **Ceylan, M.,** Eriksson, J., Florbrant, A., Ntokaki, A., Hammar, R., Sundbom, M., Skogar, M., Graf, W., Webb, D.-L., Hellström, P. M., Artursson, P., Pelaseyed, T., Di Martino, M. L., Sellin, M. E. (2024) Maturation of human intestinal epithelial cell layers fortifies the apical surface against *Salmonella* attack. *Manuscript, in revision.*

II. **Ceylan, M.,** Tzioufa, F., Di Martino, M. L., Lopes, A. C. C., Eriksson, J., Vo, D. S., Hjelmqvist, D., Hammar, R., Gardner, I., Sundbom, M., Skogar, M., Hellström, P. M., Webb, D.-L., Karlgren, M., Lundquist, P., Sellin, M. E., Hubert, M., Artursson, P. (2025) Human jejunal enteroids for studies of intestinal epithelial drug transport and metabolism. *In manuscript.*

III. **Ceylan, M.,** Hammar, R., Jonasson, E., Poetz, O., Hammer, H. S., Sundbom, M., Skogar, M., Hellström, P. M., Webb, D.-L., Sellin, M. E., Artursson, P., Lundquist, P. (2025) Freshly isolated human jejunal enterocytes for presystemic drug metabolism studies. *In manuscript.*

IV. **Ceylan, M.,** El Sayed, M., Bergström, C. A. S., Karlgren, M. (2025) Advancing drug efflux model: co-culture of CRISPR-edited MDCK II clones expressing human MDR1 and BCRP. *In manuscript.*

List of additional papers not included in the thesis

I. Yee, S. W., Ferrández-Peral, L., Alentorn-Moron, P., Fontsere, C., **Ceylan, M.,** Koleske, M. L., Handin, N., Artegoitia, V. M., Lara, G., Chien, H.-C., Zhou, X., Dainat, J., Zalevsky, A., Sali, A., Brand, C. M., Wolfreys, F. D., Yang, J., Gestwicki, J. E., Capra, J. A., Artursson, P., Newman, J. W., Marquès-Bonet, T., Giacomini, K. M. (2024) Illuminating the function of the orphan transporter, SLC22A10, in humans and other primates. *Nature Communications*, 15(1):4380.

II. Geiser, P., Westman, J., **Ceylan, M.,** Bosman, W., von Beek, C., Artursson, P., Kjellén, L., Pelaseyed, T., Sellin, M. E. (2025) A naked host cell membrane is the optimal surface for targeting by *Salmonella*. *In manuscript*.

Thesis defence

This thesis will be presented and publicly defended on April 11, 2025, at 9:15 am in room A1:107a at the Biomedicinskt Centrum (BMC) of Uppsala University in Uppsala, Sweden.

Faculty examiner (opponent)

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Henrik Johansson
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Chair of the public defense

Per Larsson
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Contents

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ADME	Absorption, distribution, metabolism, and elimination
ABC	ATP-binding cassette
AO	Apical-out
BO	Basal-out
CYP	Cytochrome P450
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
ELC	Elacridar
ENR	EGF, Noggin, and R-spondin
ENR-RT	ENR medium supplemented with RANK ligand and Tumor necrosis factor
HBSS	Hank's balanced salt solution
IEC	Intestinal epithelial cells
LC/MS-MS	Liquid chromatography-tandem mass spectrometry
LY	Lucifer yellow
MDCK	Madin-Darby canine kidney cells
MED-FASP	Multi-enzyme digestion filter-aided sample preparation
NaBut	Sodium butyrate
ODM	Organoid differentiation medium
OGM	Organoid growth medium
PBPK	Physiologically based pharmacokinetic
PhA	Pheophorbide A
Rho123	Rhodamine 123
SLC	Solute carrier
S.Tm	*Salmonella enterica* Typhimurium
SULT	Sulfotransferase
TPA	Total protein approach
UGT	UDP-glucuronosyltransferase

Introduction

Exploring oral drug absorption is essential, as this route of administration is the most preferred by patients and healthcare practitioners due to its convenience and versatility.[1] By 2032, the market for oral solid-dosage forms is expected to double, potentially reaching $1 trillion globally.[2] However, accurately predicting the absorption of orally administered drugs remains a challenge due to the complex interaction of their physicochemical and absorption characteristics.[3]

In the realm of drug development, understanding the ADME (Absorption, Distribution, Metabolism, and Elimination) properties of drug candidates is crucial, as these factors influence how a drug behaves in the body. Although the stomach serves as the initial site for drug absorption, it is the small intestine that proves to be the primary region due to its vast surface area, increased permeability, enhanced blood flow, and transit time.[4]

To address the complexities of oral drug absorption, *in vitro* models of the human small intestine are fundamental tools.[5,6] These models, which range from traditional cell cultures to advanced three-dimensional (3D) organ-on-a-chip technologies, recapitulate intestinal conditions to examine drug absorption processes. Moreover, insights from *in vitro* studies contribute to physiologically based pharmacokinetic (PBPK) modeling.[7] PBPK models use information from *in vitro* assays to simulate ADME processes across different populations, accounting for factors like age, sex, genetics, and health conditions. These models can optimize dosing, reduce side effects, and improve therapeutic outcomes by providing a comprehensive understanding of drug behaviour in various physiological contexts.

This thesis characterized *in vitro* models of the human small intestine using a proteomics approach and functional assays, focusing on their ADME features. The applicability of these models in drug development is assessed through investigations of drug metabolism and transport studies.

The human small intestine

The small intestine is a vital organ, serving as a key site for nutrient and drug absorption while also playing a significant role in communication with other bodily systems and hosting a diverse microbiome that contributes to overall health. Measuring approximately 7 meters in length, the small intestine is divided into three functional parts: the duodenum, jejunum, and ileum.[8] The jejunum, in particular, is recognized as the primary site for drug absorption due to its significant length—ten times longer than the duodenum—and its greater density of villi compared to the ileum, which increases its absorptive surface area.[9]

The epithelial barrier of the jejunum consists of a single layer of intestinal crypt-villus units located on the luminal side of the intestinal wall (Figure 1).[10] These intestinal epithelial cells (IECs) originate from intestinal stem cells located at the base of the crypts, allowing for the continuous replenishment and renewal of the epithelial lining necessary for maintaining gut integrity.[11,12] Among the IEC population, six distinct mature cell types can be identified, categorized into absorptive (enterocytes and M cells) and secretory (Paneth, goblet, enteroendocrine, and tuft cells) lineages.[13] Enterocytes are the predominant cell type in the small intestine and are specialized for nutrient and drug absorption. They feature microvilli on their apical surface, known as the brush border, which increases the surface area available for absorption. The enterocyte membrane contains various transporters and channels that facilitate the uptake of nutrients, electrolytes, and drugs, including monosaccharides, amino acids, fatty acids, and specific ion channels that regulate electrolyte balance.[14] Furthermore, enterocytes contain Phase I and II metabolic enzymes that play important roles in the first-pass metabolism of orally administered drugs.[15]

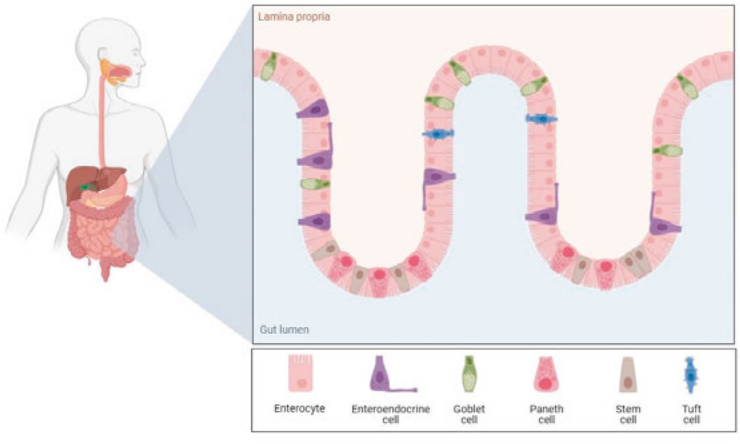

Figure 1: Overview of the intestinal epithelial structure featuring crypts and villi. (Created by Biorender)

Small intestinal drug disposition

In the small intestine, drug disposition involves an interplay of transport mechanisms and metabolic processes that dictate the fate of orally administered drugs. When a drug reaches the intestinal tissue, its absorption can occur via passive or active pathways.[16] Passive paracellular permeability allows low molecular weight hydrophilic drugs to move between cells through the intracellular space, which are limited by tight junctions.[17] However, the majority of drugs, particularly lipophilic ones, permeate transcellularly by traversing cell membranes through passive diffusion.[18] This transcellular route is crucial given its extensive surface area compared to paracellular pathways.

Transporters also play a pivotal role in drug disposition, with active uptake and efflux transporters regulating the movement of drugs across enterocyte membranes.[14] Solute carrier (SLC) transporters, such as PEPT1 and MCT1, facilitate the uptake of specific substrates like dipeptides and monocarboxylic acids. On the other hand, ATP-binding cassette (ABC) transporters, Pgp/MDR1 and BCRP, actively efflux drugs back into the intestinal lumen, thus affecting drug bioavailability.

Following absorption, drugs may undergo metabolism within the enterocytes, where drug-metabolizing enzymes such as cytochrome P450 enzymes (CYPs), UDP-glucuronosyltransferases (UGTs), and sulfotransferases (SULTs) playing significant roles.[15,19,20] These enzymes facilitate Phase I and Phase II reactions, transforming drugs into more polar metabolites that can be easily excreted.

The processes of absorption, metabolism, and the individual variability in enzyme expression and activity—including genetic polymorphisms—can result in significant differences in drug bioavailability among individuals. Quantification of specific proteins in *in vitro* systems and *in vivo* tissues can give more accurate predictions of *in vitro* to *in vivo* translations[7] (Figure 2).

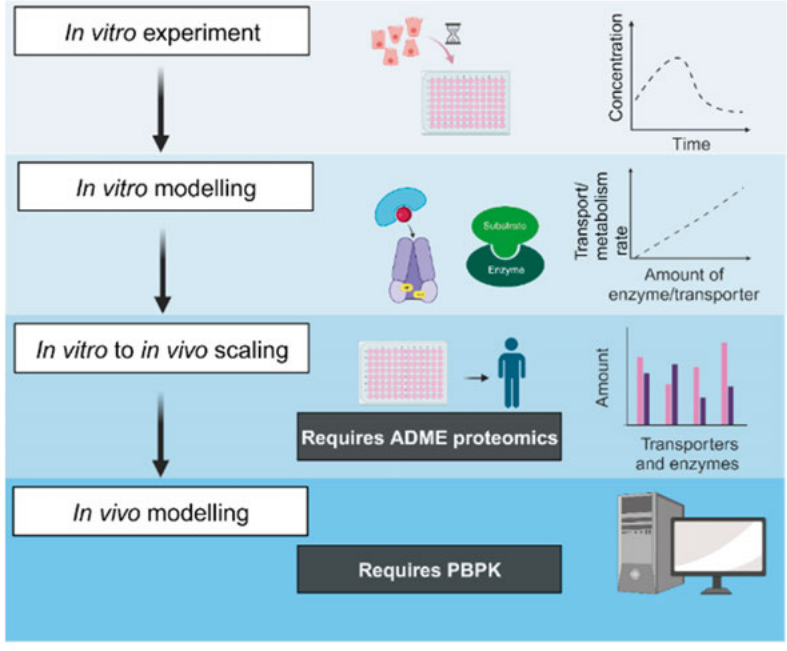

Figure 2. *In vitro* to *in vivo* translations: the role of quantitative ADME proteomics. (Created by Biorender, adapted from Heikkinen et al. 2015)

In vitro models of human small intestine

Several *in vitro* models of human intestine have been developed with the aim of enhancing the study of ADME in the small intestine. These models are generated from either cell lines or tissue sources. Some commonly used intestinal models are Ussing chamber[21], precision-cut tissue slices[22], Caco-2[23], MDCK [24], human primary enterocytes[25], organoids derived from pluripotent stem cells[26], organoids derived from adult stem cell[27], and Gut-on-a-chip[28]. Although each model has different limitations, they also have many advantages making then suitable as intestinal ADME models.[29]

Enteroids

Enteroids, derived from adult stem cells, were among the first organoids successfully developed *in vitro*[30] and recapitulate many features of the complex structure and function of the *in vivo* small intestine tissue.[31] To establishment enteroids, crypts isolated from human biopsies are embedded in an extracellular matrix, resulting in the formation of 3D cystic organoids with distinct budding structures and a lumen. This structure mimics the villus-crypt axis of the intestine, where the budding structures resemble the crypts containing

Lgr5+ and Paneth cells, while the areas between them mimic the villi which consist of terminally differentiated cells that are shed into the lumen and there undergo anoikis. These enteroids exhibit a basal-out polarity, requiring microinjection techniques to access the apical side. However, it has been shown that once Matrigel is removed, suspended enteroids display an apical-out polarity[32], making it easier to access the apical side without microinjection and allowing researchers to directly add compounds of interest to the culture media. Additionally, dissociating the basal-out enteroids into single cells allows for the generation of 2D monolayers, which provide access to both the apical and basal sides.[33] Notably, additional cell types, such as immune cells, neurons, and fibroblasts, can be introduced into enteroid cultures to explore interactions between different cellular compartments, thus creating complex co-culture systems.[34] Enteroid cultures can be maintained long-term while preserving genetic and morphological stability.[35] These features make enteroids a valuable tool for patient-specific modeling in drug development and clinical applications.[36] In terms of ADME, several studies have assessed the usefulness of enteroids in both 2D[37–40] and 3D[41–43] formats, utilizing them to characterize the disposition of small molecules and evaluate their safety profiles. However, no studies have yet examined the global proteome of both basal-out and apical-out enteroids while comparing them to their tissue of origin. Additionally, further investigation is needed for transport studies by live-cell imaging and metabolism studies to support PBPK modeling in these models.

Freshly isolated enterocytes

Isolating enterocytes from human tissue is one of the most accurate methods for capturing native protein expression, making it ideal for studying intestinal metabolism. In 2017, an enzymatic approach was used for this isolation, successfully demonstrating drug metabolism with cryopreserved enterocytes[44] However, this method often results in limited cell viability and may affect essential membrane proteins. To address these challenges, a less invasive approach is necessary to maintain cell viability and functions. In 2014, a gentle "shake-off" procedure was introduced for isolating epithelial cells from the colon, providing a promising alternative for cell isolation[45]. However, this method has not yet been applied to isolate enterocytes from the human small intestine or to conduct metabolism studies with freshly isolated enterocytes. This model has the potential to significantly enhance the predictive accuracy of drug metabolism studies that are more representative of *in vivo* conditions.

MDCK co-culture

The Madin-Darby Canine Kidney (MDCK) cell line is widely used as an *in vitro* model for studying epithelial cell function and drug absorption pro-

cesses, particularly in the context of the renal and gastrointestinal tracts. Originating from a normal kidney of cocker spaniel[46] (exhibiting characteristics of papillary adenocarcinoma), MDCK cells possess a high degree of polarization, forming tight junctions that mimic the barrier properties of epithelial tissues[5]. They can also be modified by knocking down the endogenously expressed canine transport proteins, allowing for the expression of human proteins following transfection.[47] This characteristic makes them particularly valuable for drug transport studies, as they allow researchers to assess the permeability, absorption, and efflux of pharmaceutical compounds. MDCK cells are mainly utilized in transwell assays to evaluate drug permeability and active transport. In 2022, Keemink et al. showed their usefulness as an absorptive epithelial layer in the *in vitro* lipolysis-permeation assay with lipid-based formulations.[48] However, to date, no studies have explored the use of MDCK cell lines that express human transporters within the enabling absorption device. This can serve as a valuable tool for evaluating advanced drug delivery systems in pharmaceutical research.

Mass spectrometry-based proteomics

Studying proteins is essential for understanding biological functions, particularly because they represent the culmination of the central dogma of molecular biology, where DNA is transcribed into RNA and then translated into proteins.[49] Proteins perform most of the molecular functions in the human body, and this makes them crucial for pharmaceutical and clinical applications.[50]

Mass spectrometry-based proteomics is an analytical technique used to study the protein composition of biological samples, providing insights into cellular functions, interactions, and metabolic pathways[51] (Figure 3). The high sensitivity and specificity of mass spectrometry enable the identification and quantification of proteins in complex mixtures, allowing researchers to explore the proteomic landscape of various biological systems, including tissue samples and *in vitro* cell culture models. Conducting quantitative proteomics analysis of drug metabolizing enzymes and transporters in both humans and preclinical models provides essential physiological insights that enhance the translation of *in vitro* and preclinical findings to human applications. Furthermore, integrating this information into PBPK modeling is becoming increasingly critical for ensuring safe, effective, timely, and cost-efficient drug development.[52]

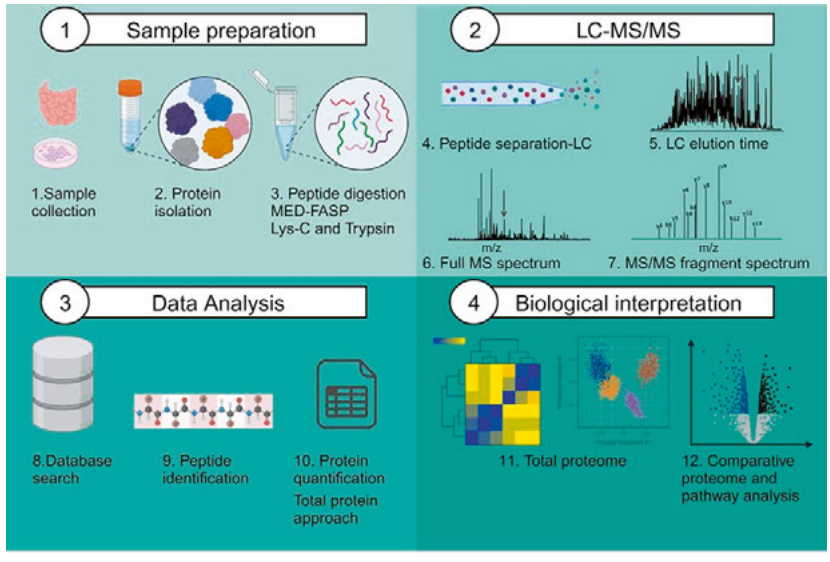

Figure 3. Mass spectrometry based proteomics. (Created by Biorender, adapted from Xie et al. 2011)

Aim of the thesis

The overall aim of this thesis is to develop and characterize *in vitro* models of the human intestine using a comprehensive global proteomics approach, complemented by functional assays with a focus on ADME features. This research aims to demonstrate the potential of these models in drug studies, ultimately providing more physiologically relevant *in vitro* systems that enhance our understanding of intestinal drug disposition.

The specific aims are:

- To analyze the global proteome of 2D enteroid monolayer models, with a particular emphasis on stem cell and differentiation markers, actin regulatory proteins and the ADME related proteins and therefore assess their maturation for bacterial infection and drug studies (Project I).

- To evaluate how closely human intestinal 3D enteroid models resemble *in vivo* intestinal features, through global proteomics and functional analysis, thereby demonstrating their suitability for research on drug metabolism and transport (Project II).

- To improve the cell isolation method for enterocytes from freshly collected jejunum biopsies in order to evaluate their usefulness for drug metabolism studies (Paper III).

- To evaluate the usefulness of the co-culture system consisting of two different MDCK cell lines — one expressing human BCRP and the other expressing human Pgp/MDR1 — in drug transport and absorption studies.

Methods

Enteroids

Human enteroid establishment

Enteroids were established from jejunal tissue resected during bariatric surgery. All donors gave their informed written consents for tissue samples. The procedures were approved by the local governing body (Etikprövningsmyndigheten, Sweden) under license numbers 2010-157, 2010-157-1, 2020-05754, and 2023-01524-01.

In Paper I and II, human jejunal adult stem cell lines were established as described previously in Sellin lab.[53] The jejunum tissue was processed to remove muscle layers, followed by mincing and dissociation to obtain single crypts. These crypts were cultured in Matrigel domes and initially supported by organoid growth medium containing ROCK Inhibitor Y-27632. After two days, the inhibitor was removed, and the medium was routinely changed every 2-3 days to promote growth. Enteroids were expanded through subculturing at days 8-10. At passage 2, the newly formed enteroids were cryopreserved in DMEM F12, with 10% fetal bovine serum, and 10% dimethyl sulfoxide for long-term storage and future experiments.

Culture maintenance

In Paper I and II, to weekly maintenance of the enteroid cultures, the Matrigel domes were dispersed through repeated gentle pipetting with cell dissociation reagent.[53] After centrifuge, the pelleted enteroid fragments were washed with DMEM F12 supplemented with 0.25% BSA and then centrifuged and resuspended in a mixture of Matrigel and organoid growth medium (OGM) at a 3:1 ratio. This was divided into three domes per well in 24-well plates and cultured at 37°C with 5% CO_2. The OGM medium was refreshed every 2 to 4 days.

Enteroid-derived monolayer culture

In Paper I, enteroid monolayers were established based on the protocol.[54] 3D enteroids kept in Matrigel domes with OGM was dissociated into single cells at day 7 to 9 after passaging. The monolayers were confluent within 3 days. For a stem cell/progenitor cell phenotype, monolayers were maintained in

OGM until analysis on days 3-7. For monolayer maturation towards an enterocyte phenotype, the medium was replaced with the differentiation medium containing EGF, Noggin, and R-spondin (ENR) or organoid differentiation medium (ODM). For M-cell-like phenotype, the medium was replaced with ENR medium supplemented with RANK ligand and Tumor necrosis factor (ENR-RT). Monolayer differentiation was allowed for 4 additional days after reaching confluence in OGM.

Enteroid suspension culture

In Paper II, apical-out and basal-out enteroid suspension culture was generated day 3 post splitting.[32] Briefly, the Matrigel domes were broken by gently pipetting and were transferred to a Falcon tube and incubated on ice for ~1 h on a nutating table. Enteroid pellets were washed with ice-cold DMEM F12 + 0.25% BSA. Afterwards, the pellets were re-suspended in ODM. The enteroid suspensions (500 µL per well) were transferred to pre-cooled 24-well ultra-low attachment plates, after which enteroids reversed the configuration and became apical-out. To maintain the basal-out configuration of the enteroids in the suspension cultures, 7.5% Matrigel was added to the medium until use. All suspension cultures were maintained at 37°C in 5% CO_2, and the medium was replaced every second day.

To simplify the description of the different growth conditions leading to different types of enteroids, the nomenclature has been simplified as in Table 1, where the first letter indicates organoids grown in Matrigel (M), basal-out (B) or apical-out (A) configuration, the second letter indicates the organoid growth medium (G) or organoid differentiation medium (D), and the number four or six indicates the number of days the organoids were cultured before use.

Table 1. Nomenclature of organoids in different culture conditions.

	Enteroids in organoid growth medium (OGM)		**Enteroids in organoid differentiation medium (ODM)**	
	Day 4	**Day 6**	**Day 4**	**Day 6**
Basal-out in matrigel	MG4	MG6	MD4	MD6
Basal-out in suspension	BG4	BG6	BD4	BD6
Apical-out in suspension	AG4	AG6	AD4	AD6

Primary enterocyte cell isolation

In paper II and III, we used freshly isolated primary enterocytes from human jejunal tissue samples.

Jejunal tissue samples were collected in ice-cold, oxygenated Krebs-Henseleit buffer and transported to the lab. The mucosa was isolated by dissection, washed, and cut into ~1 cm² pieces. Primary enterocytes were dissociated by incubating the tissue pieces in Krebs-Henseleit buffer with DTT and EDTA. First, the samples were nutated at 350 rpm on ice for 30 minutes, then incubated for an additional 60 minutes at 37°C on an orbital shaker. The supernatant was filtered to remove debris, and the cell suspensions were enriched and cleaned of unwanted cell fractions using a Percoll® density gradient. After centrifugation, the enriched enterocytes were washed and resuspended in ice-cold DMEM. Cell viability was assessed to be greater than 85% using the acridine orange and propidium iodide method in a Nexcelom Cellometer Vision.

MDCK cell lines with human transporters

In paper IV, MDCK$^{cMDR1\text{-}KO}$, MDCK-hMDR1$^{cMDR1\text{-}KO}$ and MDCK-hBCRP$^{cMDR1\text{-}KO}$ cells were cultivated according to previously established protocols.[47,55,56]. All cell lines were cultured at 37 °C with 5% CO_2. Sub-culturing was performed twice a week.

For MDCK-hMDR1$^{cMDR1\text{-}KO}$/MDCK-hBCRP$^{cMDR1\text{-}KO}$ co-cultures cells were seeded in six different ratios of the MDCK-hMDR1$^{cMDR1\text{-}KO}$/MDCK-hBCRP$^{cMDR1\text{-}KO}$ cell clones. Cells were seeded on 12 mm Transwell membrane inserts for transport studies and for global proteomics, on 75 mm Transwell membrane inserts for enabling absorption studies using the ENA device, and on 35 mm µ-Dish for fluorescent imaging. For all co-culture experiments cells were pre-treated 24 hours prior to the downstream assays with sodium butyrate (NaBut) by adding 10 mM sterile-filtered NaBut to the culture medium.

Proteomics analysis

Global proteomics

In Paper I, II and III, enteroids, enterocytes and jejunal tissues were lysed in Tris/HCl lysis buffer containing DTT and SDS. The multienzyme digestion (MED) filter aided sample preparation (FASP) method was performed using trypsin and Lys-C peptidase to digest proteins to peptides.[57] Proteomic analysis was performed on a Orbitrap Q Exactive HF mass spectrometer which was coupled to a nano–liquid chromatography and MS was set to a Top-N method (Data Dependent Acquisition). An EASY-spray C18-column (50 cm, 75 µm inner diameter) was used to separate peptides in acetonitrile/water gradient.

Proteins were identified using MaxQuant software[58] and the total protein approach (TPA) was used as the quantification method.[59]

In Paper IV, MDCK cells cultured on Transwell membrane inserts where used for global proteomics analysis. Global proteomics sample preparation and sample run were performed at the Proteomics Biomedicum Core Facility, Karolinska Institutet. Briefly, samples were lysed and sonicated. Protein concentration was determined by using the BCA Protein Assay Reagent. For proteolytic digestion trypsin was used. The peptides were separated on a 50 cm long EASY-Spray C18 column connected to an Ultimate 3000 nano-HPLC. Mass spectra were acquired on a Q Exactive HF quadrupole-Orbitrap hybrid mass spectrometer. The tandem mass spectra of the top 17 precursor ions were acquired. Acquired raw data files were analyzed using MaxQuant with the human and canine proteome reference from UniProtKB. TPA was used as the protein quantification method.[59]

In all papers, global proteomics data were processed for bioinformatics and statistical analyses in Microsoft Excel, Perseus, R or GraphPad Prism[60]. Functional annotation clustering were performed using DAVID[61] and PANTHER[62] databases.

Targeted proteomics

In paper III, targeted proteomics sample preparation and sample run were performed at SIGNATOPE. Protein quantification was conducted in accordance with the previously published methodology.[63,64] Trypsin was used to digest the lysate. Surrogate peptides and internal standard peptides were precipitated using triple X proteomics antibodies. Subsequently, the peptides were eluted and quantified using a modified version of the previously described LC–MS methods[65,66], which describe 6-minute parallel reaction monitoring methods. The raw data were processed using TraceFinder. Peptide amounts were calculated by forming the ratios of the integrated peaks of the endogenous peptides and the isotope-labeled standards.

Microscopy studies

Various microscopy techniques were used across the studies, including bright field microscopy, scanning electron microscopy, transmission electron microscopy, live microscopy, and fluorescence microscopy.

Bright field microscopy

In all Papers, *in vitro* models, including enteroids, freshly isolated enterocytes, and MDCK cell lines, were monitored by bright field microscopy.

In Paper II, enteroid size and shape descriptors for both Apical-out and Basal-out were approximated using bright field images. Images were segmented and masked using Fiji ImageJ[67]. Shape descriptors were acquired by measuring the masks of the segmented organoids.

Scanning electron microscopy

In Paper II, enteroids were imaged by field-emission scanning electron microscopy by Umeå Centre for Electron Microscopy at the Chemical Biological Centre. Briefly, enteroids were fixed overnight at 4°C. The samples adhered to poly-D-lysine coated coverslips, which were then washed, dehydrated with ethanol, and critically point dried. Coverslips were mounted on aluminum stubs, coated with 5 nm of platinum, and analyzed using field-emission scanning electron microscopy (FESEM, Carl Zeiss Merlin) at 5 kV and 120 pA.

Transmission electron microscopy

In Paper II, enteroids were imaged by field-emission transmission electron microscopy by Umeå Centre for Electron Microscopy at the Chemical Biological Centre. Briefly, enteroids were fixed and after washing, samples were stained with potassium ferricyanide in OsO_4 buffer, further stained with tannic acid in MQ water, and uranyl acetate, then dehydrated with ethanol. Samples were infiltrated with Spurr's resin and polymerized overnight at 65°C. Ultrathin sections (70 nm) were placed on formvar-coated grids and analyzed with a Talos L120C at 120 kV, with micrographs captured using a Ceta 16M CCD camera using Velox software.

Live-cell microscopy

In Paper II, live-cell microscopy is used in barrier integrity, SLC mediated and ABC mediated transport studies. Live-cell imaging was performed on a custom-built microscope based on an Eclipse Ti2 body (Nikon), using 10x/0.45 Plan Apo air objective (Nikon) and a back-lit sCMOS camera with pixel size 11 µm (Prime 95B; Photometrics).[53] The imaging chamber was maintained at 37°C, 5% CO_2 in a moisturized atmosphere. Bright-field images were acquired by differential interference contrast, and fluorescence imaging by the excitation light engine Spectra-X (Lumencor) and emission collection through quadruple band pass filters (89402 & 89403; Chroma) and a spinning disk module (X-light V2, Crest optics).

Fluorescence microscopy

In Paper II, enteroids were fixed at room temperature in a paraformaldehyde solution (pH 7.4). Primary antibodies (anti-zonula occludens-1, anti-CYP3A4,

anti-ABCG2/BCRP, anti-ABCB1/Pgp/MDR1, and anti-SLC27A4/FATP4) were used. Enteroids were resuspended in the primary antibody staining solution and incubated overnight at 4°C. After washing enteroids with DPBS, fluorescence-labeled secondary antibodies (Alexa Fluor 488 and Alexa Fluor 568) and phalloidin-Alexa Fluor 660 were added and incubated overnight at 4°C. After additional washes with DPBS, the enteroids were transferred to a microscopy slide. VECTASHIELD® Vibrance™ Antifade Mounting Medium with DAPI was added to sample, and a coverslip was placed on top. Imaging was conducted using a Zeiss LSM800 confocal microscope with a 20x or 40x oil immersion objective.

In Paper III, the apoptosis activity was measured in freshly isolated enterocytes using fluorescent markers for activated caspase-3 and -8. This was done by using the CaspGLOW Fluorescein Active Caspase Staining Kits from Bio-Vision, following the protocol provided by the supplier. The enterocytes samples were incubated at a temperature of 37 °C for a maximum duration of 2 hours. After incubation, samples were analyzed using a Cellometer Vision CBA.

In Paper IV, MDCK cells were dyed with fluorescent cell trackers in accordance with the manufacturer's instructions (ThermoFisher Scientific). MDCK-hMDR1$^{cMDR1\text{-}KO}$ cells were labelled with CellTrackerTM Red CMTPX, whereas MDCK-hBCRP$^{cMDR1\text{-}KO}$ cells were labelled with CellTrackerTM Green CMFDA. Dyed cells seeded in a 35 mm μ-Dish and incubated for an hour at 37°C, 5% CO_2. After washing, cells were imaged using a The Nikon Eclipse Ts2R-FL fluorescence microscope. Images were processed using Nikon Imaging Software.

Drug transport studies

Barrier integrity in enteroids

In Paper II, for the Lucifer yellow barrier integrity study, enteroids were washed with DMEM F12 and resuspended in DMEM F12 containing 100 μM Lucifer yellow (LY). Samples were observed for 2 hours after addition of the LY dye at 37°C and 5% CO_2 using live cell time-lapse microscopy. In the control experiments, the enteroids were permeabilized by addition of EDTA. Quantification of LY fluorescence was performed using Fiji ImageJ. Data were analyzed with one-way ANOVA Kruskal-Wallis test and multiple comparison analysis was conducted between time points in both integrity and control populations.

SLC mediated uptake transport in enteroids

In Paper II, enteroids were washed with DMEM F12, resuspended in DMEM F12 containing 2.5 µM C1-BODIPY-C12 with 5 µM BSA and incubated for 10 min at 37°C.[68] Afterwards, the enteroids were washed and resuspended in DMEM F12 with HCS LipidTOX™ Deep Red Neutral Lipid Stain. Live cell imaging was performed at 60X magnification using confocal microscopy (37°C, 5% CO_2). From the population, 10 representative enteroids were imaged at 60X to visualize accumulation of C1-BODIPY-C12 within lipid droplets.

ABC mediated efflux transport in enteroids

In Paper II, BCRP activity was measured using the BCRP substrate pheophorbide A (PhA) as previously described.[47] In brief, apical-out enteroids were incubated with 1 µM PhA, with or without 2 µM of the BCRP inhibitor KO143. After 18 h incubation, enteroids were washed with PBS. Thereafter, relative fluorescence was measured using a Tecan plate reader using excitation and emission wavelengths of 395 nm and 670 nm, respectively. Results were compared to negative (MDCK cells without hBCRP) and positive (MDCK cells overexpressing hBCRP) controls using values obtained from the published data in Wegler et al. 2021.

In Paper II, enteroids were strained and incubated in DMEM F12 with 10 µM of the fluorescent P-gp substrate rhodamine 123 (Rho123) at 37°C, 5% CO_2. In control experiments, the enteroids were pre-treated with 2 µM of the P-gp inhibitor elacridar (ELC) for 1 hour. After incubation, the enteroids were washed and resuspended in DMEM F12 and subsequently live imaged at 4X and 60X magnifications. Images were analyzed by Fiji ImageJ. Data analysis was performed using GraphPad Prism.

ABC mediated efflux transport in MDCK cells

In Paper III, for drug efflux assays MDCK-hMDR1$^{cMDR1\text{-}KO}$ and MDCK-hBCRP$^{cMDR1\text{-}KO}$ cells were seeded at a density of 500,000 cells per 12 mm Transwell insert. The cell medium was changed 24 hours prior to the experiment, and experiments were performed four days post-seeding. Transepithelial electrical resistance was measured to confirm cell monolayer integrity. Drug substrates for hMDR1 and hBCRP, including prazosin, labetalol, dantrolene, ritonavir, fluvastatin, and verapamil (1 µM), were used as previously described (Wegler et al., 2021). Drug concentrations were analyzed by LC-MS/MS. For NaBut pre-treatment, 10 mM NaBut was added to the culture medium 24 hours prior to the assays. Statistical comparisons were performed using the Student's t-test.

Drug absorption studies in MDCK cells

A MDCK monolayer, comprising 74% MDCK-hBCRP$^{cMDR1\text{-}KO}$ and 26% MDCK-hMDR1$^{cMDR1\text{-}KO}$ cells (in total 7.5 million cells), was cultured on transwell filters for 4 days. To enhance transporter expression and function, cells were incubated with 10 mM NaBut for 24 hours before the studies. Permeation studies were conducted as previously outlined, using Titrando equipment for automated pH titration with stirring at 450 rpm in the donor compartment, while a magnetic stir bar was used in the receiver chamber.[69] The donor medium was prepared with FaSSIF powder in a buffer containing Tris-maleate, NaCl, and CaCl2, adjusted to pH 6.5. The receiver compartment contained HBSS buffered with HEPES and 4% BSA, pH 7.3-7.4. Membrane integrity was monitored by assessing lucifer yellow permeation and donor pH. Concentration of 12 µg/mL Amorphous ritonavir[70] was added to the donor chamber, and samples were collected from both compartments until 45 min. The samples were analyzed by LC-MS/MS.

Drug metabolism studies

In Paper II and III, enteroids and enterocytes were incubated with several compounds including terfenadin, midazolam, dabigatran etexilate, diclofenac, phenacetin, bufuralol, and mephenytoin. As negative control, cells were heated at 95°C for 5 min. Samples were collected at 0, 30, 60 and 120 min and lysed with acetonitrile containing 10 nM warfarin. The protein content of the pellets was quantified using the tryptophan method. Compound concentrations were determined by LC-MS/MS using an Acquity UPLC coupled to a XEVO TQ triple-quadrupole mass spectrometer with positive electrospray ionization.

Metabolic clearance measurement

In Paper II and III, the elimination rate constant in metabolic stability and uptake experiments was calculated from the first-order elimination equation:

$$[S^t] = [S^0] \times e^{-k \times t} \qquad \text{Eq.1}$$

where $[S^t]$ is concentration of substrate at a given time point t, $[S^0]$ is the initial concentration of substrate, and k is the elimination rate constant, which was determined by nonlinear regression within a time point interval representing the initial elimination rate.[71]

In vitro intrinsic clearance for the compounds was calculated from:

$$CL_{int} = k \times V \quad \text{Eq.2}$$

where V represents the volume of incubation.

PBPK simulation

In Paper II, the Simcyp simulator V23 r2 was used for PBPK simulations by Certara Predictive Technologies. The baseline midazolam simulation used an unmodified midazolam file within the simulator with a 2 mg oral dose simulated across 10 trials of 15 subjects (ages 18-55, 19% female). Observed data was extracted from various studies.[72–75]

For simulations using apical-out enteroid data, the baseline model's intestinal metabolism was replaced with intrinsic clearance (µl/min/million cells) values from apical out enteroids, adjusted for protein abundance and binding.[76] The final CL_{int} used in the model was 177.6 (CV=11%) (µl/min/mg microsomal protein in the intestine). In the simulation where no intestinal metabolism was considered the CL_{int} in the intestine was set to 0.

For terfenadine a PBPK model was developed and the input parameters in the model are listed in Table 2 below. ADAM model[77] was used for terfenadine absorption with the drug assumed to be in solution all the time. Permeability was predicted using the mechanistic permeability model.[78] Distribution of terfenadine was modeled with a minimal PBPK setup[79], and elimination was assigned to be primarily via CYP3A4 (fm ~0.98). The binding in the intestine (fu,gut) was set to 0.15, aligning with an Fg of ~0.35.

Table 2. Input parameters for the terfenadine PBPK model

Parameter	**Value**	**Source**
MW	471.7	PubChem
Log P	5.01	[80]
pKa	8.6 (base)	
BP	0.78	Predicted in the Simcyp simulator
fu, plasma	0.03	[81]
Absorption	ADAM model (solution)	
Fu,gut	0.15	estimated
Elimination – HLM (ml/min/mg microsomal protein)	13342	CYP3A4
Elimination – HLM (ml/min/mg microsomal protein)	272	Non-CYP3A4 metabolism

Results and discussion

Characterization and usefulness of 2D enteroids as an *in vitro* model of human intestine (Paper I)

In this study, we investigate the influence of intestinal epithelial cell (IEC) maturation on the invasion of *Salmonella enterica* Typhimurium (S.Tm). The active invasion of IECs represents a key event in the infection cycle of many gut pathogens. By establishing intestinal epithelial monolayers derived from human enteroids using the recently developed Apical Imaging Chamber, we cultured these monolayers with different mediums to mimic immature progenitor cells (OGM), maturing enterocytes (ENR), and a state enriched with M-cell-associated transcripts (ENR-RT). Global proteomic analysis revealed a total of 7328 proteins, with quantification of 5978 proteins across samples. Principal component analysis demonstrated clear separation of protein expression profiles among the different monolayer types, highlighting the distinct differentiation states.

Notably, IEC layers maintained under immature progenitor conditions were permissive to S.Tm invasion, whereas maturation towards an enterocyte phenotype significantly reduced the frequency of S.Tm-induced epithelial entry structures and lowered invasion efficiency by up to tenfold. This phenotypic shift correlates with altered expression of actin regulatory proteins involved in the invasion process, alongside an increased reliance on the S.Tm TTSS-1 effector SipA for successful entry.

Furthermore, IEC maturation was associated with the upregulation of cell surface mucins, such as MUC1 and MUC13, and shifts in glycocalyx composition, as confirmed by multiple lectin stainings. Time-lapse imaging illustrated the presence of apical S.Tm entry structures in both immature and maturing IEC monolayers, yet with a markedly lower frequency and reduced surface area in maturing layers. Specifically, a ~30% reduction in entry structure surface area was observed in ENR monolayers compared to OGM monolayers.

Enzymatic treatment of the apical surface with StcE mucinase converted maturing IEC layers back to the S.Tm-invasion permissive state observed in immature counterparts, establishing a causal relationship between the complex mucin-linked glycocalyx formed during IEC maturation and decreased susceptibility to S.Tm invasion.

Further, we analyzed ADME proteins and it revealed that their expression levels were notably low in the 2D intestinal epithelial monolayers compared to *in vivo*. Due to low amount of key transporters and enzymes necessary for drug absorption and metabolism, 2D monolayer may not accurately reflect the ADME related functional characteristics of human small intestine. The underrepresentation of ADME proteins suggests that these 2D monolayers may have limited utility in predicting the pharmacokinetic behavior of compounds and highlights the need for more sophisticated model systems that better mimic the *in vivo* intestinal epithelium.

Conclusively, these findings showcase how the maturation state of human IECs dictates susceptibility to invasion by S.Tm, emphasizing the need for further research into the biochemical interactions between bacterial virulence factors and the mature intestinal epithelium. Future studies incorporating physiological variables, such as luminal flow and microbiota, are necessary to fully elucidate the mechanisms underlying IEC maturation and its implications for gut health and disease.

Human jejunal enteroids as an advanced 3D model for intestinal ADME research (Paper II)

For many applications, easy access to the apical surface of the intestinal epithelium is an advantage or even a prerequisite. We therefore established conditions for complete reversion of jejunal enteroids from the native basal-out (BO) to apical-out (AO) orientation and followed their differentiation by global proteomics into matured villus-like enteroids. We compared the differentiation over time with Matrigel-imbedded and suspended enteroids in the native BO orientation. For comparative purposes, we used the same lineage of primary epithelial jejunal cells as in our previous studies in the 2D format.[54] We also included healthy jejunal villus tissue and freshly isolated jejunal villus enterocytes from this tissue as references.

To establish organoid cultures, human enteroids were expanded in Matrigel domes with OGM and subsequently maintained in either OGM or ODM, which lacks stemness-promoting factors.[53] For suspension cultures, enteroids were removed from Matrigel and kept suspended for proliferation in OGM and for differentiation in ODM. BO enteroids were supported by Matrigel to maintain BO orientation. In contrast, polarity reversal to AO orientation was achieved by suspending the enteroids in media without additional Matrigel.[32] Principal component analysis revealed clear differences between the growth conditions, where a time-dependent separation of Matrigel-grown enteroids, from basal-out to apical-out enteroid suspensions is evident. As expected, Matrigel-grown enteroids retained proliferative capacity (MKI67; marker of proliferation Ki-67) and expressed markers of an immature phenotype (e.g.,

OLFM4, SOX9, BMP7) while the expression of most enterocyte markers was below the limit of detection. The expression pattern of AO enteroids at day 6 of differentiation was close to that of freshly isolated jejunal villus enterocytes in that most proliferation and stem cell markers were below detection levels, while the markers for absorptive cells, such as brush border enzymes (e.g., ANPEP, SI, ALP1), were increased to jejunal tissue levels. The expression of markers for goblet, enteroendocrine and Paneth cells were either below or close to the limit of detection, underscoring the dominance of the absorptive enterocyte cell linage. Suspended BO enteroids represented an intermediate state compared to Matrigel-grown enteroids and apical-out enteroids.

The time dependent gradual transformation towards a more differentiated phenotype is observed in growth conditions by analyzing cell markers such as MKI67 and Aminopeptidase N in enterocytes and jejunal tissue. Principal Component Analysis reveals distinct differences between Matrigel-grown enteroids and differentiated apical-out enteroids. Enrichment analysis revealed that biological processes enriched in matrigel-grown enteroids were associated with proliferation, including RNA and DNA replication, regulation, and cell division. In contrast, pathways enriched in differentiated apical-out enteroids were related to xenobiotic metabolism and epithelial cell differentiation.

As in any cells, the intestinal epithelium needs to take up nutrients to proliferate, differentiate and exert their functions. Further, the enterocyte monolayer is responsible for the selective absorption of nutrients from food, such as lipids, sugars, amino-acids, vitamins and trace elements. SLC transporters comprises the largest group of membrane transporters. In our study, we quantified several SLC transporters in enteroids and primary enterocytes. We also focused on clinically relevant ABC transporters that influence the pharmacokinetics and hence the effects of different drugs.[14] Overall, expression levels of both transporter families were lower in Matrigel-grown enteroids compared to suspended enteroids.

When new drug candidates are filed for evaluation and approval by regulatory agencies, studies of drug transport and drug interactions with clinically relevant transporters are recommended.[14] Among these, Pgp/MDR1 and BCRP are particularly relevant in the human small intestine. Therefore, we investigated their function in enteroids. Immunostaining confirmed the correct localization of BCRP at the apical side, where it overlapped with the actin belt of the microvilli and terminal web. While the live-cell microscopy assay developed for Pgp/MDR1 provided a detailed fingerprint of functional heterogeneity within the enteroid population, obtaining mean values is often sufficient for transporter assays. To evaluate BCRP functionality, we loaded AO enteroids with the fluorescent BCRP substrate pheophorbide A[82] in both the presence and absence of the BCRP inhibitor KO143.[83] Accumulation of pheophorbide A was measured under both conditions, and the ratio of the two efflux values was used as an indicator of efflux efficiency. Interestingly, the

efflux ratio observed in the AO enteroids was higher than that previously reported in MDCK cells overexpressing BCRP.[47] This finding indicates that BCRP effectively expels its substrate in the enteroid model.

Moreover, the enzyme expression studies demonstrated that optimally differentiated suspension enteroids exhibit an enzyme expression profile closely resembling that of freshly isolated jejunal enterocytes and jejunal tissues. This led us to investigate the function of CYP3A4, the most clinically relevant drug-metabolizing enzyme, using two orally administered drugs whose pharmacokinetics are strongly influenced by intestinal CYP3A4 metabolism: terfenadine and midazolam. The majority of the enterocytes in the AO enteroids had a strong intracellular CYP3A4 staining. Terfenadine, which is rapidly metabolized by CYP3A4 to fexofenadine was efficiently cleared from the medium when added to the enteroids, while fexofenadine simultaneously appeared in the medium. The intrinsic clearance of AO enteroids was about six times lower than that of freshly isolated enterocytes, but after scaling with CYP3A4 expression, the difference was reduced to threefold. PBPK modeling of terfenadine, where we incorporated AO enteroid metabolism rates corrected for CYP3A4 expression, was performed to evaluate the effectiveness of the model in assessing intestinal metabolism. The simulation showed the *in vivo* results following a 60 mg dose of terfenadine to 140 virtual male subjects. The simulation based on the AO enteroid data gave a slight overprediction of the plasma concentration as compared to the clinical data, but the results remained well within the confidence limits of the virtual population. Similar findings were obtained for the more slowly metabolized drug midazolam. These results indicate that AO enteroids are metabolically active and can be used to predict the impact of intestinal metabolism on systemic drug exposure.

Freshly isolated human enterocytes from jejunal mucosa in drug metabolism studies (Paper III)

This study focused on the isolation and characterization of human jejunal enterocytes to establish a more physiologically relevant *in vitro* model for studying intestinal drug metabolism.

The morphology of isolated enterocytes showed a predominance of a rounded shape rather than the characteristic columnar form, probably influenced by mechanical stresses during isolation. The viability of the enterocytes, averaged at 86.2%, demonstrated a gentle dissociation protocol. Yield per gram of jejunal mucosa was calculated at 35.5 million cells, ensuring sufficient quantities for metabolic studies. Analysis of apoptosis revealed that ~20-30% of cells have caspase 3 and caspase 8 activation, indicating that a significant portion of the cells remained functionally competent following isolation

and throughout subsequent assays. Overall, modifications to the isolation procedure yielded higher viability compared to previous methods. The morphological assessment, coupled with apoptosis analysis, showed that these enterocytes retain key functionalities necessary for metabolic studies.

Proteomic profiling revealed a strong correlation in the expression of key ADME proteins between isolated enterocytes and jejunal mucosa, with a correlation coefficient of 0.86. High levels of CYP enzymes, specifically CYP3A4, along with non-CYP (e.g. carboxyl-esterases) and phase II enzymes (UGTs and SULTs), confirmed the metabolic capabilities of the isolated enterocytes. The expression profile of ADME-related proteins suggests that isolated enterocytes could effectively mimic *in vivo* conditions, offering a significant advantage over traditional cell lines like Caco-2, which exhibit reduced expression of clinically important metabolic enzymes.

Metabolic assays indicated measurable activity across various enzymes, with the highest intrinsic clearance observed for dabigatran etexilate, metabolized by CES, along with notable activities for CYP2C9, CYP3A4, CYP2D6, CYP1A2, and CYP2C19. Although inter-donor variability may introduce some limitations, the overall high metabolic activity and relevant protein profiles make isolated enterocytes a valuable tool for accurate predictions of human presystemic metabolism.

In conclusion, the metabolic function exhibited by freshly isolated jejunal enterocytes supports their potential utility in drug development, risk assessment, and other applications.

MDCK co-culture and drug absorption (Paper IV)

This study focused on the development and characterization of a co-culture system utilizing MDCK II cell lines overexpressing human drug transporters Pgp/MDR1 and BCRP. The addition of sodium butyrate (NaBut) significantly enhanced the activity of these transporters. For instance, the Pgp/MDR1-mediated efflux ratio increased around sevenfold for known substrates like prazosin and labetalol after NaBut treatment. Proteomic analysis revealed Pgp/MDR1 and BCRP concentrations of 6.14 fmol/µg and 0.55 fmol/µg, respectively, following treatment, indicating successful induction of transporter expression.

Various co-culture seeding ratios (e.g. 20 % / 80 % to 68 % / 32 % of the MDCK-hMDR1$^{cMDR1\text{-}KO}$/MDCK-hBCRP$^{cMDR1\text{-}KO}$ cells) were tested, revealing a dynamic influence on transporter activity. At a seeding ratio of 26% MDCK-hMDR1$^{cMDR1\text{-}KO}$ to 74% MDCK-hBCRP$^{cMDR1\text{-}KO}$, both transporters exhibited strong functional activity, reflected by high efflux ratios for their respective substrates dantrolene (BCRP) and ritonavir (Pgp/MDR1). Protein expression levels were confirmed through global proteomic analysis. This selected ratio allowed further assessments of co-culture performance.

The co-culture system was also employed to investigate the absorption of amorphous ritonavir using an enabling absorption device. Ritonavir dissolved in the donor compartment, reaching a concentration of approximately 13 μM after 45 minutes, and demonstrated effective absorption across the co-culture barrier. Importantly, very low permeation of lucifer yellow was noted, indicating the integrity of the co-culture monolayer.

In conclusion, this study successfully established an MDCK co-culture model expressing both Pgp/MDR1 and BCRP transporters, paired with an advanced absorption device, to be used in drug transport and absorption studies for advanced drug delivery systems. This model exhibits significant potential for informing *in vitro* drug development processes and understanding drug absorption in various physiological conditions.

Conclusion

This thesis provides development and characterization of *in vitro* models of the human intestine using a proteomics approach and functional assays with a focus on ADME features. The potential for their applicability in drug studies was assessed through drug metabolism and transport studies.

The specific conclusions are:

- The amount of barrier integrity-related proteins discovered in 2D monolayers was remarkably similar to human jejunal mucosa. However, the large number of clinically significant ADME proteins were quantified in very low amount in differentiated 2D enteroid model. Our findings demonstrate that differentiated 2D models can be employed for barrier function studies and for ADME studies that focus on the function of a specific ADME protein with sufficient expression in the model (e.g., UGT1A1, ABCC3) (Paper I).
- Enteroids exhibit better differentiation in 3D compared to 2D culture, highlighting the significance of both cell–cell contact and cell shape in driving this process. Moreover, the 3D enteroid cultures maintain an intact barrier function. Further, proteomic analysis reveals a high expression of ADME-related proteins, confirming that all examined transport (SLC and ABC mediated) and metabolism (CYP) pathways in this study are functional (Paper II).
- Enterocytes were effectively isolated from human jejunum tissue with high viability and yield using a gentle shaking method. Proteomic analysis demonstrates an expression profile of Phase I and II metabolic enzymes, closely matching the expression levels observed in the tissue. Additionally, metabolic activity has been confirmed with various substrates for clinically important Phase I and II enzymes (Paper III).
- An MDCK co-culture model expressing both human Pgp/MDR1 and human BCRP transporters was successfully developed, and drug efflux activity was confirmed using a Transwell culture setup. Further, this co-culture has demonstrated its utility as an absorptive monolayer within an enabling absorption device, allowing for the investigation of amorphous ritonavir dissolution and absorption (Paper IV).

Future perspectives

The importance of developing more translatable and personalized model systems can be emphasized by the fact that 90-95% of compounds predicted to be effective and safe in preclinical testing—primarily conducted using cell lines and animal models—fail to show the same results in humans. Indeed, only 1 in 1,000 drug candidates successfully progresses through both preclinical research and human trials to obtain FDA approval. This challenging phase is often referred to as "the valley of death." While no model can perfectly replicate the complexity of human organs, they can still effectively mimic specific functions or biological processes. By understanding how these *in vitro* models are similar to and different from human tissue, we can improve their utility and leverage their advantages in pharmaceutical and medical studies. This thesis highlights the importance of characterizing *in vitro* models of human small intestine and selecting appropriate culture conditions for specific applications, moving us closer to their meaningful use in drug development.

Mass spectrometry-based proteomics is a powerful analytical technique for performing proteome profiling of *in vitro* systems and *in vivo* references. By analyzing the abundance of thousands of proteins in these samples, researchers can gain valuable insights into biological pathways and molecular mechanisms, as well as clinically important ADME proteins such as transporters and drug-metabolizing enzymes. Other omics technologies, including single-cell proteomics, genomics, transcriptomics, and metabolomics, significantly contribute to a comprehensive understanding of biological systems. Single-cell proteomics allows protein expression at the individual cell level, revealing cellular diversity and functions. Genomics identifies genetic variations linked to health and disease, while transcriptomics examines RNA molecules present in a cell to understand gene expression patterns. Metabolomics, on the other hand, focuses on small molecules produced during metabolic processes, providing insight into the metabolic state of cells. Together, these omics disciplines create a holistic view of cellular functions and interactions, capturing the dynamic nature of biological systems. This integrated approach not only enhances our understanding of fundamental biological processes but also supports the development of personalized medicine. Moreover, artificial intelligence will increasingly be used to analyze and integrate the vast amounts of data generated by these omics technologies, allowing for more accurate predictions of *in vivo*.

Incorporating insights from omics studies into PBPK models is crucial for accurately predicting drug behavior in humans and enhancing *in vitro-in vivo* extrapolation in drug development. Utilizing omics data provides a comprehensive understanding of inter-individual variability in drug response, which is influenced by genetic, environmental, and physiological factors. As a result, PBPK models become more capable of predicting pharmacokinetics accurately across different demographic groups. Ultimately, employing knowledge from omics studies in PBPK modeling not only advances personalized medicine by tailoring therapies to individual patient profiles but also enhances the safety and effectiveness of drug development practices. By addressing the complexities of human biology with *in vitro* models, this approach can significantly reduce the risk of adverse drug reactions and increase the likelihood of therapeutic success.

Building on the potential of *in vitro* models, enteroids are invaluable tools in drug development. While 2D cultures have been beneficial for barrier studies, they often fall short in replicating the complexity of *in vivo* systems. In contrast, 3D cultures offer a more accurate representation of physiological conditions. Live-cell imaging techniques applied in these 3D cultures allow researchers to investigate drug transport in real time, as they occur within living cells. Future studies will increasingly focus on enteroids and their interactions with the intestinal microbiome, oxygen levels, vascularization, mucus production and the incorporation of other cell types into the cultures. Notably, future studies can focus on using the freshly isolated enterocytes as an *in vivo* reference for matching donors of enteroids. Additionally, the metabolic function of cryopreserved enterocytes could be studied in the future, further expanding our understanding of drug metabolism in enteroids and enterocytes. This multifaceted approach paves the way for personalized medicine, striving to create a "human intestine in a dish."

Transitioning from the potential of enteroids in drug development, MDCK cell lines remain essential tools for studying drug permeability and transport mechanisms since 1990s. Future research should prioritize evaluating their functionality as an absorptive membrane, complete with a mucus layer, in conjunction with advanced absorption devices. This approach will enhance our understanding of drug absorption and efficacy, providing more accurate insights for optimizing drug formulations in clinical applications.

In this thesis, we explored various *in vitro* models of the small intestine, evaluating their key features, strengths, and limitations in pharmaceutical studies. Finally, we offer insights into how these studies can collaboratively evolve to develop even more sophisticated experimental models of human small intestine. I hope that the work presented here will contribute to new tools for use in drug development in the future.

Populärvetenskaplig sammanfattning

Tänk dig att försöka förutsäga hur en ny medicin påverkar din kropp utan att egentligen förstå hur den samverkar med ditt matsmältningssystem. Det är precis den utmaning forskare möter vid utvecklingen av nya läkemedel. Tunntarmen, som spelar en avgörande roll i upptaget av både näringsämnen och läkemedel, är ett extremt komplext organ. Att exakt kunna förutsäga hur ett läkemedel beter sig där är en av de stora utmaningarna inom läkemedelsforskningen. Den här avhandlingen presenterar forskning som syftar till att utveckla mer avancerade *in vitro*-modeller av tunntarmen – provrörsmodeller som gör det möjligt att bättre förutse hur läkemedel absorberas i kroppen.

För närvarande misslyckas många läkemedel i kliniska prövningar efter till synes framgångsrika prekliniska tester eftersom de modeller och djurmodeller som används före kliniska tester inte tillräckligt återspeglar vad som händer i den mänskliga kroppen. Det är här tunntarmen, med sin komplexa blandning av celler, transportörer och enzymer, spelar en betydande roll.

I denna avhandling undersöktes flera sätt att utveckla och karaktärisera bättre laboratorieodlade modeller av tunntarmen:

- **2D-enteroidmonolager:** Tänk på dessa som förenklade, platta lager av tarmceller odlade i laboratoriet. Även om de är enklare att arbeta med än 3D-modeller saknar dessa modeller den fulla komplexiteten hos den mänskliga tarmen. Denna studie visade att de är bra för att studera vissa aspekter av läkemedelsabsorption men underrepresenterar viktiga processer för läkemedelsmetabolism och läkemedelstransport. Läkemedelsmetabolism, dvs. processerna genom vilka läkemedel bryts ner i tarmen, och läkemedelstransport, som avser läkemedel som aktivt pumpas från tarmen till blodet.
- **3D-enteroider:** Dessa 3D-modeller är miniatyrbollar av tarmen som odlas från stamceller. De efterliknar strukturen och funktionen av tarmepitelet *in vivo* mer noggrant än 2D-modeller och har visat sig vara utmärkta för metabolism- och transportstudier.
- **Nyisolerade enterocyter:** Här har vi isolerat individuella tarmceller direkt från mänsklig vävnad. Även om det är utmanande att studera dem under lång tid på grund av deras begränsade livslängd visade denna forskning att de har hög livskraft och metabol aktivitet omedelbart efter isolering. Framtida studier kommer att utforska metoder för att bevara dessa celler för långvariga studier.

- **MDCK co-kultur:** En överraskande källa för en modell av mänsklig tarm: hundens njurceller. MDCK-celler har varit ett värdefullt verktyg i läkemedelsforskning sedan 1990-talet. Här använde vi modifierade MDCK-celler för att uttrycka två olika aktiva transportörer som finns i tarmcellerna. Transportörerna fungerar som pumpar som fångar läkemedel och transporterar dem tillbaka till tarmens lumen istället för att låta dem tas upp i kroppen. Genom att använda olika ration för de två celltyperna och en specialiserad absorptionsanordning visade vi att dessa modeller kunde användas för att studera läkemedelsabsorption.

För att besvara viktiga frågor om våra modeller använde vi en kombination av avancerade tekniker. Mikroskopistudier gav insikt i deras struktur och cellulära organisation, medan tester av läkemedelstransport och metabolism visade hur väl modellerna kunde ta upp och bryta ner läkemedel. En avgörande faktor var proteomik – en kraftfull metod som analyserar tusentals proteiner samtidigt – vilket gjorde det möjligt att direkt jämföra våra modeller med mänsklig tarmvävnad. Resultaten visade hur väl modellerna efterliknade den verkliga mänskliga tarmen.

Utvecklingen av mer exakta provrörsmodeller, som de vi presenterar i denna forskning, har stor potential för läkemedelsutveckling. Genom att bättre förutsäga hur läkemedel absorberas och bearbetas kan dessa modeller bidra till säkrare och mer effektiva behandlingar. Framtida forskning kommer att fokusera på att ytterligare förfina modellerna och utforska nya teknologier för att få en ännu djupare förståelse av den komplexa mänskliga tarmen.

Popüler bilim özeti

İlk kez kullanacağınız bir ilacın etkisini hangi yollardan göstereceğini, mide ve bağırsaklarınızla nasıl etkileşime girdiğini, elinizde bilimsel veriler olmadan tahmin etmeye çalıştığınızı hayal edin. Besin ve ilaç emilimi için hayati bir organ olan ince bağırsak, son derece karmaşık bir yapıya sahiptir ve ilacın burada nasıl davranacağını doğru bir şekilde tahmin etmek, ilaç geliştirmede önemli bir engeldir. Bu tez, ilaçların sindirim sistemindeki davranışlarını daha doğru tahmin edebilmek için *in vitro* (laboratuvar tabanlı) model geliştiren araştırmaları sunmaktadır.

Şu anda birçok ilaç, klinik denemelerde başarısız olmaktadır çünkü klinik testlerden önce kullanılan basit modeller ve hayvan modelleri, insan vücudunda gerçekleşen olayları yeterince yansıtamamaktadır. İşte burada, hücreler, taşıyıcı proteinler ve enzimlerden oluşan karmaşık bir karışım içeren ince bağırsak önemli bir rol oynamaktadır.

Bu araştırma, laboratuvar ortamında daha iyi ince bağırsak modelleri geliştirmek ve karakterize etmek için çeşitli yöntemler keşfetmiştir:

- **İki boyutlu enteroid - hücre tabakaları**: Bunlar laboratuvar ortamında yetiştirilen düz ve basit bağırsak hücre katmanları olarak düşünülebilir. 3 boyutlu modellerden daha kolay çalışılabilen bu modeller, insan bağırsaklarının karmaşıklığını tam olarak yansıtamamaktadır. Bu çalışma, bu modellerin belirli ilaç emilim yönlerini incelemek için iyi seçenek olduklarını, ancak ilaç metabolizması ve ilaç taşınması için kritik süreçleri yeterince temsil etmediğini göstermiştir. İlaç metabolizması, ilaçların bağırsakta nasıl parçalandığını ifade ederken, ilaç taşınması, ilaçların bağırsaktan kan dolaşımına aktif olarak pompalanmasına atıfta bulunur.
- **3 boyutlu enteroidler:** Bu modeller kök hücrelerden üretilen ve bağırsakların küçük top şeklinde olan 3 boyutlu modelleridir. İki boyutlu modellere göre, *in vivo* bağırsak örtüsünün yapısını ve işlevini daha benzer şekilde modellerler. Bu modelin aynı zamanda ilaç metabolizma ve taşıma çalışmaları için ideal bir seçenek olduğu bildirilmiştir.
- **Yeni izole edilmiş enterositler**: Bu model sistemi, insan dokusundan doğrudan bağırsak hücreleri izole edilmesi esasına dayanır. Bu hücrelerin kısa ömürleri nedeniyle uzun süreli çalışmalara imkan vermese de izolasyondan hemen sonra yüksek canlılık ve metabolik

aktivite sergiledikleri gösterilmiştir. Gelecekteki araştırmalarımız, bu hücrelerin daha uzun araştırmalara cevap verebilecek şekilde prezerv edilmesine dair bilimsel projeleri kapsayacaktır.

- **MDCK ortak-kültürü**: İnsan ince bağırsağını modellemek için sıradışı bir yaklaşım ise köpek böbrek hücrelerinin (MDCK) kullanımıdır. MDCK hücreleri, 1990'lardan beri ilaç araştırmalarında kullanılmaktadır. Projenin bu basamağında, insan bağırsaklarında bulunan ve kan dolaşımımıza girmeye çalışan ilaçların kana geçişini durdurmak üzere yakalayıp, bağırsaklara geri gönderen taşıyıcıları araştırmak üzere özelleştirilmiş MDCK hücreleri kullanıldı. Farklı hücre oranları ve özel bir emilim cihazı kullanarak, bu modellerin ilaç emilimini incelemek için kullanılabileceğini gösterdik.

Modellerimizin verimliliğini test etmek adına ortaya çıkan önemli soruları yanıtlamak için bir dizi teknik kullandık. Mikroskopi çalışmalarıyla kullandığımız modellerin yapısını ve hücresel organizasyonunu, ilaç taşıma ve metabolizma deneyleri ile ilaçların modeller tarafından ne kadar verimli bir şekilde işlendiğini, ve son olarak proteomik deneyleri ile, modellerimizin gerçek insan bağırsağına benzerliğini kanıtlamak üzere doğrudan karşılaştırma yapmamıza olanak tanıdı.

Bu araştırmada sunulan insan bağırsaklarının daha doğru *in vitro* modellerinin geliştirilmesi, ilaç geliştirme için önemli sonuçlar doğurmaktadır. Geliştirdiğimiz modeller, ilaç emilimi ve işlenmesi konusunda daha doğru tahminler yapılmasını sağlamakta ve sonuç olarak daha güvenli ve etkili ilaçların ortaya çıkmasına katkıda bulunmaktadır. Gelecek araştırmalar, bu modellerin geliştirilmesine ve insan bağırsağını daha iyi anlamak için yeni teknolojilerin keşfine odaklanacaktır.

Popular science summary

Imagine trying to predict how a new medicine will work in your body without truly understanding how it interacts with your digestive system. That is the challenge scientists face when developing new drugs. The small intestine, a vital organ for nutrient and drug absorption, is incredibly complex, and accurately predicting how a drug will behave inside it is a major hurdle in drug development. This thesis presents research focused on developing better *in vitro* (lab-based) models of the small intestine for more accurate predictions.

Currently, many drugs fail in clinical trials after seemingly successful preclinical testing because the simpler models and animal models used before clinical testing do not adequately reflect what happens in the human body. This is where the small intestine, with its complex mix of cells, transporters, and enzymes, plays a significant role.

This research explored several ways to develop and characterize better lab-grown models of the small intestine:

- **2D enteroid monolayers:** Think of these as simplified, flat layers of intestinal cells grown in the lab. While simpler to work with than 3D models, these models lack the full complexity of the human gut. This study showed that they are good for studying certain aspects of drug absorption but underrepresent crucial processes for drug metabolism and drug transport. Drug metabolism, i.e. the processes by which drugs are broken down in the intestine, and drug transport, which refer to drugs being actively pumped from the intestine into the blood.
- **3D enteroids:** These are 3D miniature ball shaped models of the intestine grown from stem cells. They more closely mimic the structure and function of the *in vivo* intestinal lining than 2D models and proved to be excellent for metabolic and transport studies.
- **Freshly isolated enterocytes:** Here we have captured individual intestinal cells directly from human tissue. Although challenging to study long-term due to their limited lifespan, this research demonstrated their high viability and metabolic activity immediately after isolation. Future studies will explore methods to preserve these cells for long-term studies.
- **MDCK co-culture:** A surprising source for a model of human intestine: dog kidney cells. MDCK cells have been a valuable tool in

drug research since the 1990s. Here, we used MDCK cells modified to express two different active transporters, i.e. a sort of pumps that are present in the intestinal cells and capture and pump drugs that try to enter our body back into the intestinal lumen. By using different cell ratios and a specialized absorption device, we here showed that these models could be used for studying drug absorption.

We used a combination of techniques to answer key questions about our models. Microscopy studies revealed the models' structure and cellular organization. Drug transport and metabolism assays showed how efficiently drugs were processed, i.e. taken up and broken down, by the models. Crucially, proteomics—a powerful technique for analyzing thousands of proteins at once—allowed a direct comparison to human intestinal tissue, telling us how closely our models mimicked the real human gut.

The development of more accurate *in vitro* models of the human intestine, as presented in this research, has significant implications for drug development. These models allow for more accurate predictions of drug absorption and processing, ultimately contributing to safer and more effective medications. Future research will focus on further refining these models and exploring the potential of new technologies to better understand the complex human gut.

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