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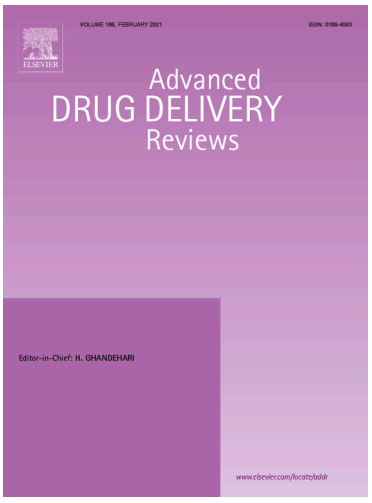

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An overview of *in vitro*, *ex vivo* and *in vivo* models for studying the transport of drugs across intestinal barriers

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Abstract

Oral administration is the most commonly used route for drug delivery owing to its cost-effectiveness, ease of administration, and high patient compliance. However, the absorption of orally delivered compounds is a complex process that greatly depends on the interplay between the characteristics of the drug/formulation and the gastrointestinal tract. In this contribution, we review the different preclinical models (*in vitro*, *ex vivo* and *in vivo*) from their development to application for studying the transport of drugs across intestinal barriers. This review also discusses the advantages and disadvantages of each model. Furthermore, the authors have reviewed the selection and validation of these models and how the limitations of the models can be addressed in future investigations. The correlation and predictability of the intestinal transport data from the preclinical models and human data are also explored. With the increasing popularity and prevalence of orally delivered drugs/formulations, the need of sophisticated preclinical models with higher predictive capacity for absorption of oral formulations used in clinical studies will be required.

Keywords:

Oral delivery, Intestinal barriers, Transport, *In vitro* models, *Ex vivo* models, *In vivo* models

List of abbreviations

AFM	Atomic force microscopy
BBMV	Brush border membrane vesicle
BCRP	Breast cancer resistance protein
BCS	Biopharmaceutics Classification System
BLMV	Basolateral membrane vesicle
CYP	Cytochrome P450
DAMPA	Double artificial membrane permeation assay
EdU	5-ethynyl-20-deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle-associated epithelium
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GST	Glutathione-S-transferase
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
hInEpCs	Human intestinal epithelial cells
HRP	Horseradish peroxidase
HuMiX	Human-microbial cross talk
IBD	Inflammatory bowel disease
iPSC	Induced pluripotent stem cell
IVIVC	*In vitro-in vivo* correlation
LDH	Lactate dehydrogenase
L. rhamnosus	*Lactobacillus rhamnosus*
M cells	Microfold cells
MDCK	Madin-Darby canine kidney
MPPs	Mucus penetrating particles
MRP	Multidrug resistance-associated protein
MRP1	Multidrug resistance-associated protein 1
MTX	Methotrexate
NLCs	Nanostructured lipid carriers
OATPs	Organic anion transporting polypeptides
OCT1	Organic cation transporter- 1
PAMPA	Parallel artificial membrane permeability assay
P_{app}	Apparent permeability
PCL	Polycaprolactone
PD	Potential difference
PEG	Polyethylene glycol
PEPT1	Peptide transporter-1
PET	Positron emission tomography
P-gp	P-glycoprotein
PhIP	Pyridine
pHPMA	Poly(N-(2-hydroxypropyl)-methacrylamide)
PLGA	Poly(lactic-co-glycolic acid)
PVPA	Phospholipid vesicle-based permeation assay
SCC	Short-circuit current
SEM	Scanning electron microscopy
SPECT-CT	Single-photon emission computed tomography/computed tomography

sPLA2	Phospholipases A2
SULT	Sulfotransferase
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TJs	Tight junctions
TMC	N-trimethylated derivative
UGPG	UDP-glucuronosyltransferase
ZO	*Zona Occludens*
3D	Three-dimensional
3Rs	Replacement, reduction and refinement
7H6	Cytoplasmic tight junction-associated protein

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1. Introduction

The oral route is the most preferred administration routes for existing and new chemical compounds, with more than one-half of conventional small-molecule drugs administered orally [1]. Despite innovations and developments in the field of drug delivery, the oral route remains the most popular administration route, and the market share of oral solid-dosage forms is expected to grow to 900 billion US dollars worldwide by 2027 [2]. The high patient compliance, compound stability and low production costs associated with the oral route medications have facilitated their popularity [3-6]. Nonetheless, to achieve effective therapeutic concentrations, the drugs must demonstrate suitable solubility and intestinal permeability. The poor pharmacokinetic properties of newly developed molecules have been among the major challenges faced during the drug discovery and development phases. Therefore, the prediction of intestinal absorption remains one of the key facets in the design and development of drug products as it is one of the determinant factors of the efficacy of the drugs and their delivery system [7, 8].

Drug transport across the intestinal epithelium is a complex and dynamic process that involves numerous mechanisms and pathways. Passive intestinal transport can occur either through the intestinal cells (transcellular transport) or through the tight junctions between adjacent enterocytes (paracellular transport) [9]. Additionally, several energy-dependent (active), carrier-mediated and endocytic pathways are exploited to promote compound permeation across the intestinal epithelium [9-11]. The absorption of a compound by the intestinal epithelium is a multivariate process, making it difficult to use a single model to accurately predict the intestinal absorption of drugs in humans [12]. Furthermore, there are several barriers and factors which may limit the rate and extent of intestinal absorption of orally delivered drugs [5]. The physicochemical properties of the drug compound (such as molecular weight, solubility, lipophilicity, and stability) and formulation design have significant impact on its fate in the GI tract. Additionally, the GI tract also present several physiological and biochemical barriers that influence the intestinal absorption [5, 13]. The mucosal layer and the intestinal epithelium represent physical barriers, whereas the regional differences and harsh gastrointestinal (GI) milieu constitute biochemical barriers [1, 14]. Knowledge of these factors and barriers that influence drug absorption is necessary to develop drug formulations with optimal therapeutic efficiency.

Achieving enhanced oral bioavailability and reducing the variability of bioavailability of currently available and new drug entities is one of the crucial objectives in the pharmaceutical industry [7]. To fill this gap in knowledge, an in-depth understanding of the underlying mechanisms and different factors involved in drug absorption, along with a good predictive model, is needed at different stages of drug development. In the early development phase, analyzing intestinal drug absorption allows researchers to select potential drug candidates with a desired absorption profile. The understanding of the absorption mechanism of the drug candidate using absorption models greatly aids in developing drug delivery systems with optimal features [8]. A number of techniques and models are used to screen and predict drug absorption at different stages of drug discovery and development, including *in silico*, *in vitro*, *ex vivo* and *in vivo* methodologies [3]. However, animal experiments represent intact organisms necessary to simulate the complex interplay of different process which is crucial for studying intestinal drug absorption. Thus, *in vivo* methodologies are widely used despite being expensive, time consuming and poorly correlating with humans [15-17]. Recent developments in molecular and cellular biology have allowed the development of powerful models and tools, especially cell-based *in vitro* models, to study absorption in specific cell lines and specific biological barriers. *In vitro* methods provide less expensive, faster, more ethical and less labor intensive options to evaluate drug absorption [18]. Furthermore, with advancements, *in vitro* methods have been able to incorporate multiple cell lines and different facets (the mucus layer and extracellular matrix) to closely mimic the conditions in the human gastrointestinal tract, thus providing adequate predictability of the potential absorption behavior of the candidate drug in humans [19-22]. Additionally, human cell lines are being used for improving the predictability of *in vitro* transport studies [23, 24]. *Ex vivo* models have greater similarity, including greater complexity, to human conditions for enhancing the predictions of intestinal absorption while retaining advantages such as faster and systematic study, robustness and compatibility with high-throughput processes, making them viable alternative approaches [25-27]. However, the maintenance of tissue viability and integrity throughout an *ex vivo* study is of utmost importance [28, 29]. In both the *in vitro* and *ex vivo* methods, the establishment of system predictability and its correlation with the *in vivo* performance of the drug are crucial. Overall, there are a number of models available to study drug transport, each of which has its own benefits and limitations. In this review, different *in vitro*, *ex vivo* and *in vivo* models used to study drug transport across intestinal barriers are discussed in detail.

2. Gastrointestinal tract

2.1 Anatomy and physiology

The natural progression of orally ingested nutrients in the diet involves breaking down its components, which are then absorbed via the intestinal epithelium. Similarly, orally ingested drugs follow the same route, but most therapeutics are poorly dissolved and/or have low permeability, and these challenging setbacks are based on their own physicochemical properties and on the different barriers encountered in the gastrointestinal tract (GIT) [4, 5]. Thus, it is very important to understand the physiology of the GIT and the different barriers it presents to identify the fate of orally delivered drugs and to develop an effective drug delivery system.

The anatomy and physiology of the human GIT has been discussed in detail in several reviews [30-32]. In addition to its role in digestion and absorption, the human GIT also acts as a protective barrier against unwanted pathogens and toxins and is involved in immune responses. The dynamic nature of the GIT and regional differences in pH, enzyme activity, mucosal thickness, drug residence time and surface area characterize site-specific absorption capabilities [13]. The small intestine is the major site of absorption, accounting for approximately 90% of total intestinal absorption [31, 33]. The GIT comprises four concentric layers that are connected by connective tissue and neural and vascular networks: the mucosa, the submucosa, the muscularis propria, and the serosa [31, 34, 35].

Several distinct cellular mechanisms, such as cell proliferation, differentiation and apoptosis, occur in the intestinal mucosa. Intestinal epithelial stem cells are found at proliferative crypts and differentiate mainly into two types of populations: absorptive enterocytes and secretory cells [36-38]. The different types of cells found in the intestinal epithelium are depicted schematically in Figure 1. Absorptive enterocytes comprise the largest population (more than 80%) of intestinal cells and renew rapidly (life span of 3-4 days). Enterocytes are polarized cells with apical and basolateral sides and are tightly packed into a single layer. The apical layer of enterocytes comprises well-ordered microvilli structures, which significantly increase the surface area, thus enabling augmented absorption of nutrients [39]. Secretory cells include goblet cells, enteroendocrine cells and Paneth cells. Goblet cells are the second most abundant cells in the intestine and are interspersed among enterocytes. These cells are critical for

producing and secreting mucin, which is one of the major parts of the mucus layer [4, 40]. Paneth cells are found in the Lieberkühn crypt base and are critical for secreting proteins that can kill bacteria [26]. Enteroendocrine cells produce and secrete gut hormones in response to stimuli such as rate of nutrient absorption, the composition of the luminal milieu and the integrity of the epithelial barrier [41]. Secreted hormones control intestinal functions, insulin secretion, nutrient assimilation and food intake. In addition, the intestine harbors microfold cells (M cells), tuft cells and cup cells [42]. M cells are found in Peyer's patches, specialized regions in the intestine with no mucus layer protection, and exhibit low aminopeptidase activity. The high endocytic potential of M cells allows the transport of macromolecules, antigens and microorganisms. Furthermore, these cells are also crucial for initiating the mucosal immune response [43]. The specific roles of tuft cells and cup cells are still unknown; however, the involvement of tuft cells in the immune response has recently been identified [44]. The roles of different types of intestinal cells are summarized in Table 1.

Table 1: Different types of intestinal cells and their functions

Cell type	Total fraction	Functions
Enterocytes	>80%	The absorptive cells critical for the absorption of nutrients by the epithelium [39].
Goblet cells	~16%	These cells produce and secrete mucins, which are a major components of the mucus layer and protects the intestinal epithelium from components in the lumen [45].
Enteroendocrine cells	~1%	These cells coordinate gut functioning through specific hormonal secretions, such as glucagon-like peptide-1 (GLP-1), somatostatin, and Peptide YY [41].
Paneth cells	-	These cells secrete antimicrobial proteins (lysozyme and phospholipases A2 (sPLA2) and defensins and are involved in innate immunity [26].
M cells	<1%	These specialized cells are involved in the transepithelial transport of macromolecules, particles, and microorganisms. They also sensors of luminal antigens, triggering immune response [42, 43].
Tuft cells	0.4%	These cells have roles in the immune response by providing a reservoir for chronic norovirus infection and contribute to thymic function [46]
Cup cells	-	The specific function of these cells is not yet known [44].

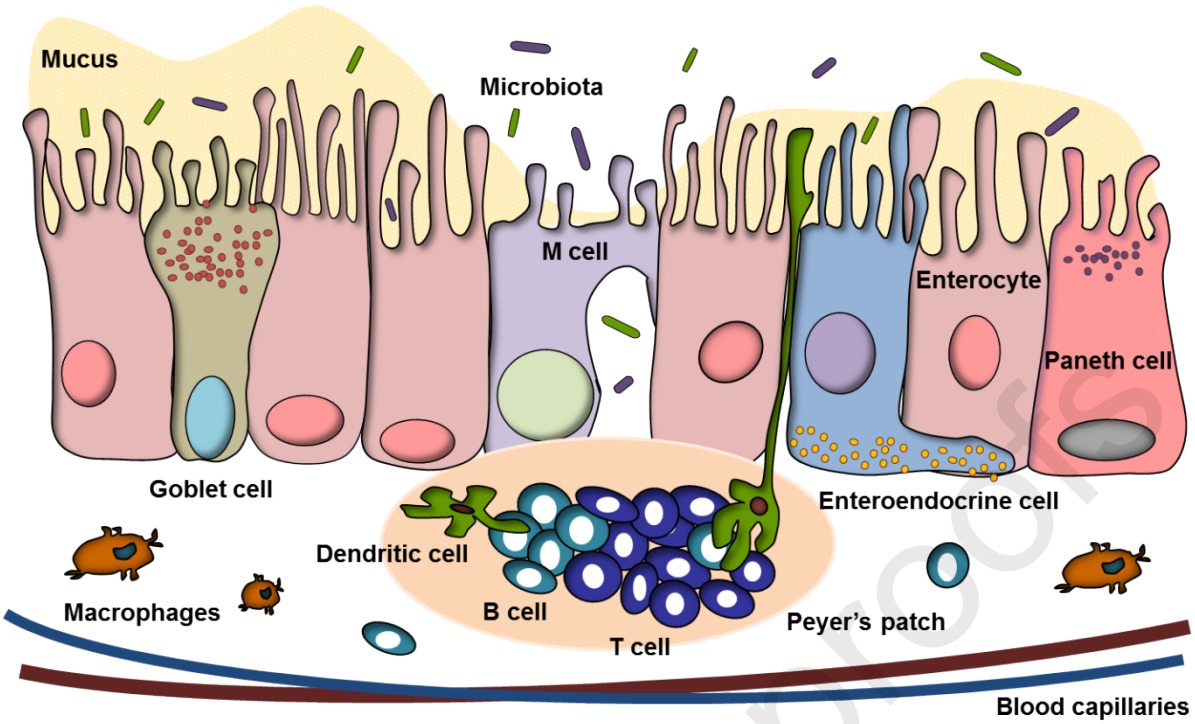

Figure 1: Graphic description of the intestinal epithelial monolayer.

2.2 Drug transport mechanisms

The transport of drug molecules across the intestinal epithelium is a complex process involving several pathways throughout the small and large intestine (illustrated in Figure 2). Transport can be either an active or passive process [47, 48]. Active transport involves the movement of the molecule against a concentration gradient and requires energy consumption (direct or indirect). On the other hand, passive transport follows Fick's law of diffusion and involves the passage of molecules in the direction of a concentration gradient [31, 49]. Passive transport can be either paracellular (between neighboring cells) or transcellular (passage through the cells) transport of drug molecules from the intestinal lumen to enter systemic circulation [31]. The transport pathways the drug molecule follow greatly depend on the physicochemical properties of the drug, such as size, chemical structure, and hydrophilic-lipophilic balance. For example, low molecular weight hydrophilic molecules tend to paracellular route of transport, whereas hydrophobic molecules can cross the intestinal epithelium by partitioning into lipid bilayers [50, 51].

Paracellular passage involves movement of molecules through the narrow and convoluted water-filled intercellular space between adjacent intestinal epithelial cells. The presence of tight junctions between adjoining cells along with the narrow pathway (10 Å) greatly hinders the passage of molecules taking this route [52, 53]. Furthermore, the paracellular space represents a very small fraction of the total intestinal surface area (0.01%-0.1%), suggesting that this pathway offers a limited window for absorption, and is complemented by other transport pathways [54, 55].

Passive transcellular transport is a non-energy-dependent process and is less likely to cause saturation or be inhibited. It might involve diffusive permeability across the cell membrane or it might be catalyzed by transporters in non-energy dependent manner. During transcellular passage, the molecules diffusing across the cell membrane exist as desolvated species [47]. Macropinocytosis and clathrin- and caveolae-mediated endocytosis are examples of active transcellular pathways [56]. Carrier-mediated transport involved in drug intestinal permeability could be via drug transporters (for some small molecules) or can be a receptor mediated phenomenon. Carrier-mediated transport can be either active or passive, depending on the transporters involved. In receptor-mediated transport, the drug acts as a ligand that binds to a specific receptor on the surface of the intestinal epithelial cells. Carrier-mediated transport is a saturable process and depends on the stereochemical specificity of the ligand interacting with the receptor [47]. In endocytosis, molecules enter the cell after being engulfed by membrane-attached vesicles that are pinched off from the apical membrane [57]. It enables the cell to engulf micron-sized particles. The high transcytosis capability of M cells was demonstrated in an *in vitro* system, where intestinal models of M cells showed five-fold higher transport than intestinal models with only enterocyte-like cells [58]. The fate of the drugs undergoing cellular internalization in these pathways is highly dependent on ligand-receptor binding combination [59].

The membrane transporter family are classified into adenosine triphosphate (ATP)-binding cassette (ABC) transporters and the solute carrier (SLC) transporter superfamily [4, 60]. ABC family includes efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRP) on both apical and basolateral side of the intestinal epithelia [10]. These transporters secrete molecules into the intestinal lumen, thus greatly interfering with drug absorption, which in turn reduces drug bioavailability. SLC transporters include oligopeptide transporter (PepT1/SLC15A1), the apical sodium-dependent bile acid transporter

(ASBT/SLC10A2), the sodium-dependent vitamin transporter SVCT1 (SLC23A1), the sodium-dependent multivitamin transporter (SMVT/SLC5A6), the monocarboxylate transporter MCT1 (SLC16A1), amino acid transporters (LAT1/SLC7A5 and $ATB^{0,+}$/ SLC6A14 and the organic cation/carnitine transporter OCTN2 (SLC22A5) [4, 10]. SLC transporters are crucial for uptake and transport of a number of drug molecules (such as acyclovir, saquinavir and docetaxel [61].

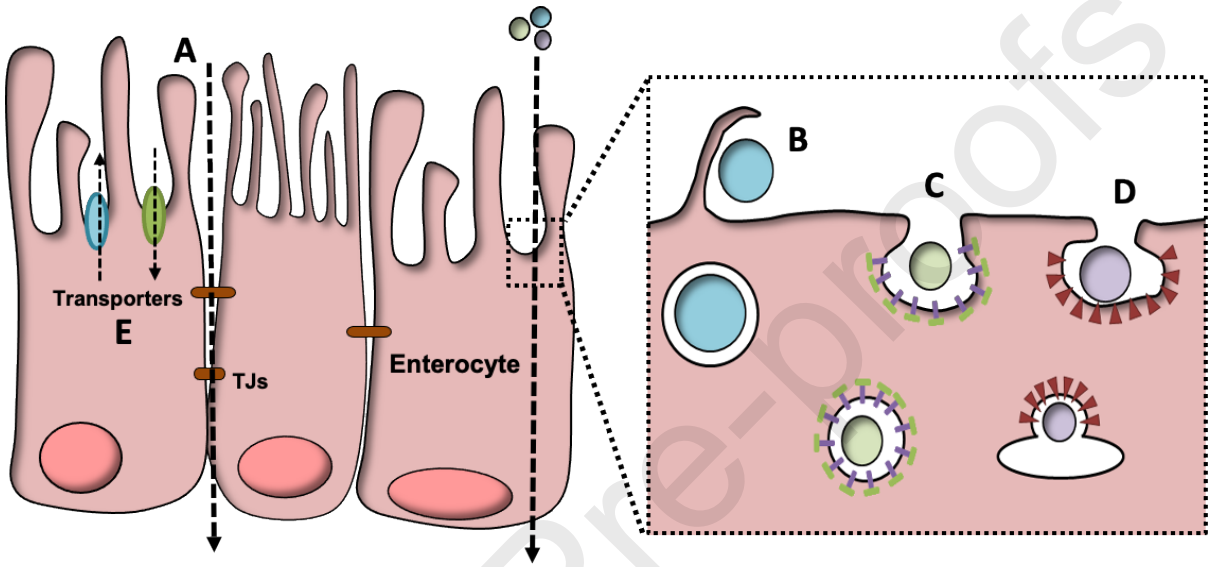

Figure 2: Schematic representation of transport mechanisms across the intestinal epithelium. (A) Paracellular transport; (B) macropinocytosis; (C) clathrin-mediated endocytosis; (D) caveolae-mediated endocytosis; and (E) transporter-mediated transport.

2.3 Intestinal barriers to oral drug delivery

To successfully achieve therapeutic concentrations after oral administration, the drug molecule must overcome several intestinal barriers. The first barrier is the biochemical barrier present in the GI milieu, followed by the protective mucosal layer and the physical epithelial barrier [31, 53].

Biochemical barrier: The biochemical barrier includes the pH variations found throughout the GIT and the presence of a complex intestinal lumen [55]. The wide pH variations in the GIT range from highly acidic pH (pH 1.5 - 3.5) in the stomach to near neutral pH (pH 6.6 - 7.5) in the small intestine, decreasing to pH 6.4 in the cecum. These vast pH variations challenge the integrity and stability of the drug molecules in the GIT. Additionally, complex components of

the intestinal lumen, including proteolytic and digestive enzymes, bile salts and pancreatic secretions, present harsh conditions that additionally impact the solubility and stability of the drug molecules [62, 63]. Enzymatic degradation can occur at different sites, such as the intestinal lumen, brush border, cytosol and lysosomes [62].

Mucosal barrier: The mucus layer covering the intestinal epithelium presents another limiting barrier for intestinal drug absorption. The mucus layer is a highly hydrated and viscoelastic fluid that acts as a protective barrier for the underlying intestinal epithelium [45, 55, 64]. It allows free passage of permeable nutrients, water, and small molecules but hinders the entrance of pathogens and foreign particles [1]. The mucus layer is comprised of distinct layers. Glycocalyx, membrane-attached mucin, is the layer residing on top of the intestinal epithelium, where it serves as a docking system for the second layer comprising mucus [65]. The mucus layer is comprised of mucin glycoproteins, enzymes, electrolytes and water, which acts like a protective gel-like structure. The epithelium secretes bicarbonate ions into the mucus gel layers, creating a pH gradient across the layer with near-neutral pH at the epithelial surface. This bicarbonate rich mucus layer acts a protective barrier against luminal acid [66]. Moreover, bicarbonate ions also play a vital role in regulating the viscosity of mucins and mucus by controlling their swelling and dispersion behaviors [67]. The mucus layer acts as a protective shield for the intestinal epithelium and defends it against pathogens, the GI milieu and foreign particles. Mucus is highly hydrated complex heterogeneous mixture of mucin fibers, lipids, proteins, carbohydrates, cell debris, bacteria, etc. [68]. Mucin fibers are the main components of the mucus layer and are secreted from goblet cells (specialized intestinal epithelial cells) [69]. Mucin fibers are glycoproteins that are rich in negatively-charged glycosylated regions and hydrophobic domains. Mucin fibers are entangled and crosslinked with each other by disulfide linkages and hydrophobic interactions, resulting in a dense porous structure with the ability to sterically block large molecules/particles [45, 70, 71]. The thickness of the mucus layer varies along the length of the GIT, with the thickest layers found in the gastric (170 μm) and colonic (100 μm) regions. The jejunum region has the thinnest mucus layer (10 μm) [1]. The dynamic nature of mucus secretion involves its continuous renewal, in which the old layer is recycled, digested or removed [45]. This clearance of mucus, along with the complex nature of the mucus layer, is a major limiting factor to achieve optimal drug absorption.

Physical barrier: The intestinal epithelium presents a physical barrier against the transport of drug molecules. The small intestine is a highly absorptive surface that acts as the major site of

absorption. The different types of cells found in the GIT have been discussed in the previous section. The tight junctions (TJs) or *Zona Occludens* (ZO) between the intestinal cells ensure the integrity of the epithelium [72, 73]. TJs act as rate-limiting barriers for paracellular diffusion across the intestinal epithelium, as they limit the passage of particles with sizes greater than 2 nm. TJs are complex structures comprising transmembrane integral proteins (claudins and occludins), intracellular plaque proteins (ZO-1, ZO-2, ZO-3, cingulin, and 7H6), and regulatory proteins [5, 72]. The organized interactions between these components and the architecture of the actin cytoskeleton are essential for the assembly and functioning of TJs [5, 72, 73].

Efflux transporters are other limiting barriers for the absorption of orally delivered drugs. There are numerous efflux transporters that are found abundantly on the apical membrane of enterocytes, such as P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP 2) and breast cancer resistance protein (BCRP). These efflux transporter along with metabolizing enzyme cytochrome P450 (CYP) have been identified as one of the major limiting factors for intestinal absorption [10, 74]. In addition to the barriers in GIT, other limiting factors have considerable effects on oral drug bioavailability, such as first pass metabolism by the liver, rapid elimination from the circulation, the immune response, and loss by unwanted uptake by non-target cells.

2.4 Factors influencing intestinal permeability

There are several factors that determine the intestinal permeability of orally delivered drugs. These factors are associated with the GIT (physiological, anatomical and biochemical aspects) and the physicochemical properties of the drugs and drug delivery systems. The physiological, anatomical and biochemical factors that influence intestinal absorption are discussed in detail in the previous section and are summarized in Table 2. There are several physicochemical properties of the drug molecules (molecular size, water solubility and dissolution profile, and hydrophilic-lipophilic balance) and formulation than have an impact on their fate *in vivo* [10]. The involvement of several factors makes prediction of oral absorption from a drug formulation very complicated [75, 76]. The drug solubility/dissolution and permeability of drug across the GI membrane are the fundamental processes that regulate the oral drug absorption.

The solubility of drug in GI milieu is a perquisite for oral drug absorption, as such poor aqueous solubility often leads to poor oral bioavailability. Poor aqueous solubility is a common

limitation of new chemical entities, and are usually overcome by solubility enhancing formulation approaches such as lipid-based systems, cyclodextrins, nanoparticles, co-solvents and amorphous solid dispersions [25, 77, 78]. The solubility and dissolution of drug molecules depend on the pH and nature of the luminal content and gastrointestinal residence time [16, 26, 79, 80]. Intestinal permeability refers to how easily a drug molecule can pass through the intestinal wall. Mathematically, intestinal permeability is directly related to drug's partitioning between GI membrane/milieu, which in turn is dependent on drug solubility. Thus, there is an interplay between the solubility and permeability, the two major factors that influence the oral drug absorption, and it is not enough to consider them separately.

Lipophilicity is one of the major factors that is used for predicting absorption. Previously, the octanol-water partition coefficient (Log P) was used as a tool for predicting absorption process. However, it is now understood that using LogP alone is not accurate, as it oversimplifies a complex process of drug transport across biological membrane [12]. The drug influx and efflux process include both passive and carrier-mediated route. For drug molecules which are transported mainly via passive route, there is a relationship between permeability and lipophilicity. However, a more diverse set of molecules has been shown to follow other mechanisms in combination with passive transport for drug absorption, in which case the correlation between permeability and lipophilicity is deficient [12].

The hydrophilic-lipophilic balance of the molecule determines how the drug interacts with the lipid bilayer of the intestinal epithelium, thus impacting its permeation [79]. For transcellular passage of drugs, the drugs must demonstrate sufficient hydrophilic-lipophilic balance to ensure partitioning of the drug from aqueous GI milieu to lipid bilayer of the cell membrane. This route is mainly used by hydrophobic molecules. Drug molecules with low permeability are only partially absorbed in the lipid bilayer of the membrane [12, 76]. There are several formulation approaches used to improve the permeability profile of such drugs such as using permeation enhancers, tight junction modulators and surfactants [4, 25, 81]. Small and ionizable drug molecules can transit through tight junctions, which is limited to molecules lower than 200 Da due to small pore sizes [50, 51, 82]. In the case of protein and peptide therapeutics, several additional factors impact oral absorption, such as molecular weight, three-dimensional conformation, charge distribution and aggregation potential [34, 83].

Therefore, by understanding and identifying the different physicochemical properties of drugs and physiological factors that affect drug absorption, scientists can develop advanced and efficient formulations that can maximize drug bioavailability.

Table 2: Factors influencing the fate of drug absorption

GI aspects: Physiological	Transit time Gastric emptying Fluid dynamics Physiological response to feeding Membrane receptors Membrane transporters
GI aspects: Anatomical	Gut mucosa
GI aspects: Biochemical	Luminal complexation Gut metabolism Liver uptake
Physicochemical properties of drug molecule	Solubility at GI pH Intestinal permeability Lipophilicity (pKa) Molecular weight and size
Physicochemical properties of protein/peptide drugs (additional factors)	Immunogenicity Three-dimensional structure Aggregation Charge distribution
Formulation aspect	Dosage delivery system Release profile Absorption enhancers Solubilizers Enzyme inhibitors (proteins)

In addition to the relation between the physicochemical properties of the compound and the absorption profile, the type and characteristics of the drug delivery system also influence the absorption of an encapsulated drug molecule [9]. Dissolution of the drug based on its formulation and the GI milieu is one of the highly relevant factors that is used for *in vitro-in vivo* correlation of GI absorption and bioavailability. The dissolution of a drug depends on the region of the GIT where the drug is released, as varied pH profiles in different regions can greatly affect the solubility of a drug. Similarly, fasted or fed state of the organism can also influence the resultant absorption of a drug [16, 80]. Furthermore, the use of either conventional or advanced drug delivery systems (targeted delivery systems, smart delivery systems, micro- and nanosystems) can greatly influence how the drug interacts with GI components. Therefore, drug delivery systems are designed and optimized to overcome limitations associated with drug

molecules [4-6]. For instance, a drug molecule with high solubility and low permeability can be formulated for a system containing absorption enhancers that can significantly increase intestinal permeation [84]. Overall, there are several factors that can influence the intestinal permeability of drug molecules, and they should be considered when developing dosage forms and regimens.

3. General descriptions of intestinal drug transport models

The identification of the intestinal absorption behavior of selected drugs is an essential step in the development of their oral dosage forms. Prior to clinical translation, the effect of the intestinal barriers (physical, biochemical and mucosal) on the oral drug delivery systems are evaluated using a versatile range of laboratory techniques, including *in vitro*, *ex vivo* and *in vivo* methods [3]. These methodologies have become indispensable tools for predicting the intestinal permeability of drugs and ultimately bioavailability.

In vitro models are commonly used during the initial stages of drug selection and innovative formulation development for predicting their potential behaviors *in vivo*. *In vitro* techniques for transport studies are primarily divided into non-cell-based models and cell-based models. The main advantages of these models include cost-effectiveness and relatively easier system establishment, and they provide a system with some resemblance to the human GIT environment. *In vitro* techniques also offer rapid predictions of the potential interactions and fate of the tested product in the GIT *in vivo* [85]. For example, the Caco-2 monolayer has been considered as the reference *in vitro* tool to predict intestinal transport, as it allows easy and rapid evaluation under different testing conditions and with various parameters. The *in vitro* permeability values of passively absorbed compounds measured on the Caco-2 monolayers have been demonstrated to correlate well with human intestinal permeability *in vivo*. To be specific, the permeability coefficients 1) are $> 1 \times 10^{-6}$ cm/s when drugs are completely absorbed in humans; 2) are between 0.1×10^{-6} to 1.0×10^{-6} cm/s when drugs are absorbed to > 1% but < 100%; 3) are $\leqslant 1 \times 10^{-7}$ cm/s when drugs are absorbed to < 1% [86]. However, for the drugs partially transported by carriers-mediated pathway the correlation was much less [87, 88]. An *in vitro* Caco-2-based model also enables the gathering of important information (e.g., transport mechanisms and associated toxicity) regarding drug permeability across the polarized epithelium, which improves insight into drug design and development [42, 89]. *In*

vitro techniques are also beneficial from an ethical point of view, as they prevent the unnecessary use of animals at different stages of drug development. Nevertheless, none of the current *in vitro* models can entirely simulate the integrated environment of the human gut. Each of the established *in vitro* models is partially devoid of the anatomical and physiological features of the intestine (e.g., improper integrity of the mucus layer [90] and deficient effect of intestinal peristalsis [91]). Other major drawbacks of the *in vitro* models are the significant inter- and intra-laboratory variability resulting from differing culture conditions and cell passages and the lack of interindividual differences [79, 92, 93]. Thus, the *in vitro* drug permeability profile offers only limited information and is suitable merely for the initial phase of pharmaceutical research. More detailed information regarding how drugs cross intestinal barriers must be obtained from *ex vivo* and *in vivo* models.

Tissue-based *ex vivo* models, including the everted gut sac models [94], the Ussing chamber method for isolated intestinal mucosae [95, 96], and rat/mouse intestinal loop/perfusion techniques [97], provide alternative strategies useful for elucidating the fate of drugs crossing the intestine. Intestine-based *ex vivo* techniques are relatively inexpensive and simple, offering a compromise between an expensive and complex *in vivo* model and a simple *in vitro* model. These models more closely mimic the physiological conditions due to the preservation of tissue integrity and viability and the use of replaceable biomimetic buffer, thus providing more detailed information on how drugs and/or other formulations will behave in the *in vivo* environment compared to that obtained with *in vitro* methodologies. *Ex vivo* transport studies use animal tissues (e.g., rat, mouse, pig, rabbit, dog or monkey [98-101]) to predict human intestinal absorption *in vivo*. Additionally, in these tissue-based techniques, different intestinal region can be used [102-104], which provides further information on how drugs and drug delivery systems behave at specific intestinal regions. Notably, the transport data generated from these species do not necessarily reflect the true drug permeation behavior in the human GIT. The use of resected human gut tissues from surgeries in *ex vivo models* reflects the actual human *in vivo* conditions more closely, since these tissues maintain the morphological structure of the intestine, the metabolism of various GI enzymes, and the expression of different transporter proteins [96], and they have become increasingly common for use in *ex vivo* permeability studies [95, 96, 105, 106]. However, in contrast to other species and standardized conditions, the state of excised human gut tissues, such as extent of pathological change, and the differences in physiological conditions (e.g., gender, age and diet), may preclude the acquisition of systematic data about intestinal transport [5, 107]. The use of *in vitro* and *ex vivo*

techniques inherently raise questions regarding the validity of inferring *in vivo* conditions; thus, findings on intestinal transport based on these systems need to be verified.

In vivo animal tests are a crucial stage in the development of drug products in preclinical studies and are considered as valuable and powerful tools to assess intestinal absorption of a specific dose of drugs and/or formulations in a living organism; however, they are expensive and time-consuming. The common animal models used for evaluating the performance of oral dosage forms mainly include rats, mice, rabbits, pigs and dogs [16, 108]. The *in vivo* intestinal transport behavior of drugs/formulations in animal models are generally obtained by analyzing and evaluating a considerable amount of *in vivo* data, especially their pharmacokinetic and pharmacodynamic parameters. Other important *in vivo* data, such as the toxicities, distribution, etc., are also crucial parameters that reflect the efficiency of intestinal transport after oral delivery. However, due to the differences between experimental animals and humans in terms of the physiology, anatomy, diet and gut microflora, etc. [16], *in vivo* experimental models are not identical to humans. Nonetheless, the substantial data gathered from these species are sufficient to predict the intestinal transport of drugs and their efficacy in related disease treatments in preclinical phases.

Despite the availability of a wide range of preclinical methodologies used for the evaluation of the intestinal transport of drugs (as mentioned above), each model has its own advantages and disadvantages (summarized in Table 3). These models (*in vitro*, *ex vivo* and *in vivo*) are usually developed to study the interaction with one or more intestinal barriers [5], including the intestinal milieu, the mucus layer, tight junctions of the epithelium, intestinal epithelial cells and the subepithelial tissue. At each stage of preclinical studies, proper selection of experimental techniques to determine intestinal transport capacity is critical to ensure the best prediction of clinical translation potential. In the subsections below, the authors review currently available models used for the evaluation of intestinal permeability and transport *in vitro*, *ex vivo* and *in vivo*.

Table 3: Summary of the benefits and limitations of *in vitro*, *ex vivo* and *in vivo* models for evaluating the intestinal transport of drugs

Models	**Benefits**	**Limitations**
In vitro	Low cost relative to *in vivo*; ease of system establishment; no ethical considerations; control of experimental conditions; feasibility of	Lack of actual anatomy or physiology of the intestine; large inter- and interlaboratory

	transport mechanism study	variability; lack interindividual differences
Ex vivo	Maintain the integrity of the intestine; availability of human intestinal segments; different segments of the intestine available	Relatively complex system establishment; uncontrolled experimental conditions; tissue viability; static system lacking blood supply.
In vivo	The gold standard in preclinical phases; intact physiological processes and disease features	Time-consuming and expensive; ethical considerations; species differences between humans and experimental animals

4. *In vitro* models

In the early stages of oral dosage form development, *in vitro* permeability assays represent valuable and vital techniques to characterize the transport capacity of the drug/formulation across intestine barriers. Currently used *in vitro* models for intestinal transport studies are either based on biomimetic membranes (non-cell-based transport models), such as phospholipids/phospholipid vesicle-coated filter or cells (cell-based transport models), such as Caco-2 cells, TC7 cells, and Madin-Darby canine kidney (MDCK) cells [3, 21, 25, 109, 110]. Despite these models only partially mimicking the physiological features of the intestine, they still have use in studies of mechanism of transport across epithelia. In addition, the economic and ethical benefits render *in vitro* models valuable as decision-making tools during initial drug development stages when used to evaluate the transport efficacies and mechanisms of drugs/formulations in the intestine.

Understanding the transport process of drugs/formulations crossing into the intestine is crucial to the development of drugs. The basic transport processes include transcellular and paracellular pathways, which can be evaluated using *in vitro* models. The apparent permeability (P_{app}) is the most commonly used parameter to predict the ability of drugs to cross the gut barrier via the. The P_{app} is expressed as cm/s and is calculated by equation (1).

$$P_{app} = (dQ/dt) \times (1/(A \times C_0)) \qquad \text{Equation (1)}$$

where dQ/dt (mol/s) is the drug transport rate from the donor to the receptor chamber, A (cm^2) is the area of the membrane, and C_0 (mol/L) is the initial drug concentration in the donor

chamber [111-113]. Transepithelial electrical resistance (TEER), is a generally exploited noninvasive method for quantitatively measuring the integrity of TJs in live cells [114].

In recent decades, a large number of research publications have revealed the intestinal processes of drug/formulation transport using different *in vitro* systems. Table 4 summarizes examples of the *in vitro* models used for investigating intestinal transport with oral delivery systems.

4.1 Non-cell-based transport models

Non-cell-based transport systems were developed as alternative *in vitro* permeation evaluation tools because they allow rapid evaluation of intestinal drug transport [115]. They have attracted considerable attention for drug development and have become common tools to investigate the passive intestinal absorption of drugs/formulations. Current non-cell-based transport models mainly include parallel artificial membrane permeability (PAMPA) [116], vesicle-based permeation assay (PVPA) [117] and PermeaPad® [110]. These models are based on artificial biomimetic membranes. They are more suitable for intestinal transport of drugs using high-throughput screening [116], although there are limited studies on the intestinal transport of drug formulations [118, 119]. They were developed to overcome the limitations of *in vitro* cell-based models, including 1) time-consuming and expensive model establishment, 2) incompatibility with certain pharmaceutical excipients due to the sensitivity and viability of cultured cell lines, and 3) large inter- and intra-laboratory variability due to different culture conditions, various cell passages and technical issues. In this section, the authors review the most commonly used non-cell-based transport models.

4.1.1 PAMPA

PAMPA is based on a filter infused with phospholipids in an organic solvent to mimic the lipid composition of the intestinal membrane [109, 115]. Egg lecithin, a mixture of lipids primarily containing phosphatidylcholine (an important component of the phospholipid portion of the cell membrane), has been used to simulate the phospholipid components of mammalian membranes [120]. Kansy *et al.* initially used 10 % egg lecithin and n-dodecane to develop the original PAMPA for measuring the intestinal permeability of various compounds with a wide range of physicochemical properties [120]. The experimental data obtained from PAMPAs showed that the *in vivo* absorption ability of approximately 80% of the tested compounds was

accurately predicted. During recent decades, several PAMPA variants have been developed by adjusting the composition and concentration of the phospholipids, the type of organic solvent, the pH of the donor/acceptor medium, the material of the hydrophobic membrane, and the presence of a sink in the acceptor chamber. These variants have been extensively used in the rapid screening of drug permeability of the human intestine. Examples include the hexadecane membrane-PAMPA [121], biomimetic-PAMPA [122, 123], dioleoyl phosphatidylcholine-PAMPA [124], Double-Sink ™ PAMPA [116] and precoated PAMPA [125].

When transported through cells via a transcellular route, drugs/formulations must cross two lipid bilayer membranes, the apical cell membrane and the basal cell membrane. However, in most PAMPA models, the tested drugs/formulations involve only a single permeation step across a single lipid filter. Considering this, Kataoka *et al.* recently established a double artificial membrane permeation assay (DAMPA) containing an intracellular compartment to mimic the intracellular space between membranes to investigate the intestinal permeability of 20 compounds with different physicochemical properties [126]. When compared to those of conventional PAMPAs, the results of the DAMPAs showed improved accuracy of predictions of drug intestinal transport, to a certain extent. Moreover, controlling the environmental conditions of the biomimetic intracellular compartment of a DAMPA may be a potential tool for evaluating certain mechanisms of specific formulations (e.g., the hydrolytic activation of prodrugs) during intestinal transport processes [126].

PAMPAs have become an effective *in vitro* alternative tool to predict the intestinal passive permeability in the pharmaceutical industry. Although the drug transcellular permeability data obtained by PAMPAs largely correlated with those measured in cell-based models (e.g., a Caco-2 monolayer), this simplified approach to determining permeability predicted neither the paracellular or active transport of drugs nor did it account for the membrane retention of lipophilic compounds. Moreover, since there is no physical boundary separating the donor medium from the lipophilic artificial membrane, the possibility that the barrier components dissolve and/or emulsify into the medium must be carefully considered [115].

4.1.2 PVPA

Since PAMPA models are based on a simple phospholipid/organic solvent-coated filter serving as a permeability barrier, they lack biomembrane-like structures, resulting in poor biomimetics. To make an artificial biomimetic membrane that better simulates the structures of the intestinal

epithelium, Flaten *et al.* developed a liposome-saturated filter membrane as an advanced *in vitro* non-cell-based model, PVPA [127]. Originally, the membrane comprised phospholipid-made liposomes (mainly composed of phosphatidylcholine) deposited into the pores and onto the surface of a nitrocellulose filter support. The authors have used the PVPA model to rapidly predict the passive transport of different drugs and formulations (e.g., micelles, liposomes and solid solutions) through the intestinal epithelium [127-132]. These studies showed that under comparable conditions, the PVPA model seemed to mimic *in vivo* transport better than the PAMPA model and to perform as well as the Caco-2 cell model.

A good *in vitro* transport model should demonstrate a high correlation between its predicted drug permeability values and the true *in vivo* permeability data. For this purpose, the same group who developed the PVPA model improved the biomimetic properties of this liposome-based membrane by using negatively charged liposomes that more closely mimic the lipid composition of intestinal cells [133]. Moreover, by supplementing the model with an additional layer of porcine mucus on top of the liposome-based membrane, they recently developed a novel mucus-PVPA model that has proven to be a reliable tool for permeability screening of drugs/formulations, particularly for transmucosal drug delivery systems [134].

Although these PVPA models have high similarity with the intestinal epithelium structure, a laborious preparation procedure is generally needed to improve the stability and short shelf life of these models [127, 133, 134], thus limiting their application to drug development.

4.1.3 PermeaPad®

In 2015, di Cagno *et al.* developed another biomimetic membrane, PermeaPad®, a fast, economical and reliable means of determining passive transcellular drug transport [135]. The authors measured the permeability coefficients of various drugs with different properties by using PermeaPad®. The study demonstrated a good correlation between the tested permeability values obtained from using PermeaPad® and the permeability values from PAMPAs and/or Caco-2 cells as described in the literature [135]. In contrast to the PAMPA and PVPA biomimetic barriers, the PermeaPad® biomimetic barrier is not based on a filter support but is constructed as a sandwich-like structure consisting of a layer of dry phospholipids (soybean phosphatidylcholine S-100) wrapped in two support layers (cellulose-hydrate membranes) [135]. Moreover, the sandwich-like structure prevents the erosion of the wrapped lipid layer and the leakage of lipids into the aqueous environment [115, 135]. Due to its unique design,

the PermeaPad® barrier exhibits stronger resistance to pH changes and aggressive additives (e.g., cosolvents) compared to the PAMPA and PVPA models. In recent years, the PermeaPad® model has been used to conduct transport studies of several formulations, including lipid-, polymer- and surfactant-based formulations [136-139]. For these studies, the PermeaPad® model was prepared manually in the laboratory. Interestingly, a more recent study reported a new PermeaPad® format, a 96-well PermeaPad® plate, that was produced on an industrial scale [110]. The 96-well PermeaPad® plate considerably improved the throughput, allowing the measurement of drug permeability in a fast and reproducible manner [110, 140-142].

Although the non-cell-based transport models reviewed above were considered promising tools in specific cases of oral drug development, these models still need to be optimized and improved to ensure widespread application. The intestinal epithelium is a complex environment and involves complicated transport mechanisms (including active transport, passive transport and paracellular transport). Therefore, simulating a simple bilayer structure of the intestinal cell membrane provides very limited information and are entirely based on passive transcellular diffusion. In other words, the currently available *in vitro* non-cell-based models lack TJ structures, organelles and the expression of receptors and transporters; thus, they fail to capture other important intestinal transport mechanisms of drugs/formulations (e.g., paracellular and active drug transport). Overall, the application of current non-cell-based transport models for predicting drug/formulation transport across intestine barriers is limited to passively-permeating small molecules that dissolve in plasma membrane lipids.

4.2 Cell-based transport models

A wide variety of cells line the luminal surface of the intestinal epithelium, including enterocytes, goblet cells, M cells and dendritic cells (Figure 2). To simulate the intestinal epithelium *in vitro*, various immortalized cell lines derived from tumoral and healthy tissues of animals and humans have been successfully employed. In addition, some primary cells derived from human or animal intestines (e.g., primary human intestinal epithelial cells (hInEpCs)) have also been exploited as cell models to study the transport of drugs crossing into the intestine [143]. Among these cell models, different cell lines mimic the different heterogeneous compositions of *in vivo* intestinal barriers. For instance, Caco-2 cells grow into a polarized monolayer acquiring enterocyte-like morphology, whereas HT29-MTX cells grow into a monolayer of polarized goblet cells that produce a mucus layer. The *in vitro* cell-based

models range from simple monoculture models to three-dimensional (3D) multiculture models. A simple model can be designed to simulate only one of the barriers associated with a single type of epithelium. On the other hand, to establish an *in vitro* model that closely resembles the human intestinal epithelium, coculture-/triple-culture models, 3D culture models and microfluidics-based systems have been successively developed. Although these sophisticated models provide more predictive results, to some extent, the use of these complex *in vitro* systems can be justified only after the *in vitro-in vivo* correlation is improved substantially. Currently *in vivo* cell-based models include Transwell®-based systems [5], membrane vesicles [144] and microfluidics-based systems [145].

Cell-based *in vitro* models use for conducting mechanistic transport studies when the involved barriers, cells and protein expression levels and function are accurately characterized [146-149]. The widely-used techniques for elucidating the drug transport mechanism across the intestinal epithelium include chemical inhibitors, quantitative reagents, qualitative markers, radio- and fluorescent-labelled drugs and genome editing (e.g., specific genes knocked-out or -in cells) [150-153]. Recent advances have allowed development of wide range of transgenic cell lines, where in specific transporters, receptors and components of endocytic pathway are knocked down or overexpressed, which is a more efficient approach to find drug transport mechanism, as compared to chemical inhibitors.

4.2.1 Transwell®-based cell models

Transwell®-based systems are potent *in vitro* tools for studying cargo permeability. The Transwell® intestinal transepithelial apparatus consists of a thick polyester/polycarbonate membrane (10 μm) with a range of pore sizes ideal for use with cell cultures (0.4-3.0 μm). Single or multiple cell lines are seeded on the membrane of the insert, which separates the apical compartment from the basolateral compartment, corresponding to the intestinal lumen and submucosa, respectively, as schematically demonstrated in Figure 3. The compartmentalized model simulates the intestinal epithelium in a relatively realistic manner. Transwell®-based cell models include monoculture models (e.g., an enterocyte-like Caco-2 model), coculture models (e.g., mucus-secreting models and follicle-associated epithelium models), triple-culture models (e.g., Caco-2, HT29-MTX, and Raji B triple-culture models), 3D models (e.g., collagen-based 3D coculture models), and “inflamed” intestinal models (Caco-2 and proinflammatory factors or PMA-differentiated THP-1 co-culture) [11, 20, 25, 154].

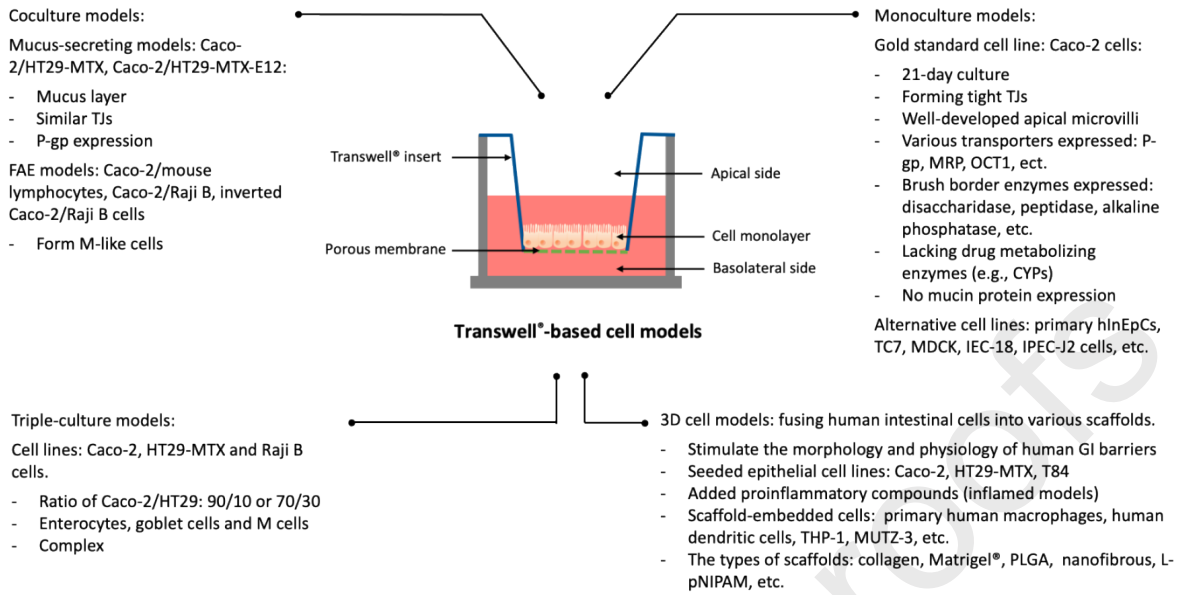

Figure 3: Schematic illustration of Transwell®-based cell models and a summary of Transwell®-based cell models.

Monoculture models

Caco-2 is the most widely used cell line to simulate the intestinal epithelial barrier. The descriptions, utilization, advantages and limitations of Caco-2 cells compared to model human enterocytes to evaluate the intestinal transport of drugs/nanocarriers have been thoroughly reviewed elsewhere [3, 5, 11, 155]. Although originally derived from human colorectal adenocarcinoma cells, Caco-2 cells can spontaneously differentiate and polarize in culture with features similar to the enterocytes of the small intestine. For the establishment of an *in vitro* enterocyte-like model, Caco-2 cells are seeded and grown on the membrane of Transwell® inserts with a density of ~$1x10^5$ cells/cm^2 and cultivated for 21 days [156]. The differentiated and polarized Caco-2 monolayer is characterized by apical microvilli and the formation of TJs between two adjacent cells [157]. In addition, Caco-2 cells also express various enzymes that are typically expressed in intestinal cells, such as disaccharidase and peptidase. Caco-2 cells have widely been used in the assessment of intestinal transport of drugs and formulations [88, 151, 158]. Many examples have demonstrated the Caco-2 monolayer as a good *in vitro* cell model to predict the absorption of orally administered drugs through measurements of permeability coefficients for the monolayers. During recent decades, Caco-2 monolayers have been used to study nanoparticle-based drug delivery systems, especially those devoted to the transport of fragile or hydrophobic molecules across the intestinal barrier. Multiple studies have

focused on elucidating the relative transport mechanism behind these designed nanosystems (e.g., micelles, lipid-based nanoparticles and polymeric nanoparticles) [11].

The well-established protocol for culturing Caco-2 cell monolayers follows a 21-day procedure [89, 159]. Multiple modified protocols have been developed to simplify and shorten the process of Caco-2 cell culturing, such as supplementing and/or modifying the composition of the cell culture medium with growth factors [160-162], hormones [160, 161], and/or puromycin [163]), sodium butyrate [162], or changing the cell-seeding density [164, 165]. By implementing these changes in the culturing process, the modeling cycle can be reduced from 21 to 7 days, even as few as 3 days. Nevertheless, these accelerated models may not allow time for TJs to form between the cells, which may express fewer efflux pumps, resulting in a deviation in the expression of transporters and enzymes, and/or changes in cell morphology [161, 164], which limits the feasibility of these models for the evaluation of intestinal transport. Therefore, in the future, more efforts are needed to develop accurate Caco-2 models with shortened culture duration that do not compromise the advantageous features of the original Caco-2 cell monolayer. Currently, Caco-2 models with shortened culture duration are mainly used in the rapid determination of the intestinal permeability of candidates [166].

Alternative human and nonhuman cell lines have also been used as models of absorptive intestinal epithelial cells and alternatives to Caco-2 cells to overcome the heterogeneity of Caco-2 cells [167] or to better reproduce *in vivo* intestinal phenotypes [143]. The TC7 human cell line, isolated from late-passage Caco-2 cells, has been used to measure drug intestinal transport [168-170]. In terms of the most representative function of entero-epithelial cells in the small intestine, TC7 cells are a more homogeneous population with better developed TJs [167] than the parental Caco-2 cells. Additionally, TC7 cells express CYP enzymes, particularly CYP3A [171]), which is highly expressed in the intestinal epithelium and is involved in the metabolism of many therapeutics (approximately 50 %) [172]). In contrast, the extensively used Caco-2 cell line lacks the expression and functions of CYP enzymes [173]. Turco *et al.* recently reported a study in which the intestinal permeability of nearly 30 synthetic and natural compounds was tested, and the results indicated that TC7 cells provide reliable absorption results for compounds transported via passive diffusion. Notably, specific compounds are particularly suitable for testing with TC7 monolayer, including poorly absorbed and highly lipophilic drugs or drugs with mediated transporters or involved in first-pass metabolism [168]. Kauffman *et al.,* recently discovered that two other human intestinal cell

lines, primary hInEpCs and induced pluripotent stem cell (iPSC)-derived intestinal cells, are attractive alternatives for use in furthering the understanding of drug transport [143]. In addition, various non-human cell lines, such as the MDCK cell line (originating from canine kidney) [174], the IEC-18 cell line (originating from rat intestine) [175], and the IPEC-J2 cell line (originating from porcine intestine) [176, 177], have also been used as *in vitro* cell models to predict the behavior of drugs and/or formulations in the human intestine. However, the use of these nonhuman cells has been limited. Notably, IPEC-J2 cells better mimic human physiology than the other available nonhuman cell lines [176]. Moreover, suitable polarized IPEC-J2 monolayers can be formed with low or high TEER by adjusting the type of serum added to the culture medium [178]. The use of IPEC-J2 cells as mature *in vitro* transport models is still under investigation, and further studies are needed to establish it as a feasible alternative for the evaluation of intestinal permeability [176, 177].

Coculture models

Although intestinal epithelial cells are mainly composed of enterocytes, other cell types also play important roles in intestinal adsorption and transport. The mucus layer, secreted by goblet cells, is one of the major barriers to the transport of drugs/formulations across the human intestine. Lesuffleur *et al.* discovered that HT29 cells (originating from human colorectal adenocarcinoma) cultured in a medium containing methotrexate (MTX) were able to differentiate into mature goblet cells capable of producing mucus [179]. One of the key limitations of enterocyte-like monolayers is the lack of a protective mucus layer, which has been addressed through the use of cocultured Caco-2 and HT29-MTX cells at a ratio of 90:10 on Transwell® membranes. The coculture condition results in the formation of an intestinal monolayer with mucus, thus mimicking the true human intestinal conditions more closely. However, the Caco-2/HT29-MTX coculture model does not form TJs as tightly as they do in Caco-2 cell monocultures, thus increasing the paracellular transport pathway. The loose TJs in the coculture model more closely resemble those in the small intestine *in vivo* [180]. This coculture model has been largely used to evaluate the ability of goblet cell-targeting nanocarriers [181, 182], mucus penetrating particles (MPPs) [183, 184] and mucoadhesive systems [185, 186] aimed at improving the absorption of drug cargo. The major drawback of the Caco-2/HT29-MTX coculture model is the nonuniformity, both in terms of mucus layer coverage and mixing of two different cell lines, which leads to the formation of mucus-free patches in the coculture model. In contrast, a subclone of the HT29-MTX cell line, the HT29-

MTX-E12 cell line is able to form confluent monolayers with suitable TJs and a continuous mucosal layer with a thickness similar to human intestinal mucus [187]. This subclone has also been used to form co-culture model with Caco-2, and has been widely used to investigate the impact of intestinal mucus on the transport of drugs/formulations [188-190]. In particular, HT29-MTX-E12 shares many similarities with gastric cells, as its mucus composition is more similar to that of stomach mucin than to that of intestinal mucin [191]. The differentiated HT29-MTX-E12 cells mainly express MUC5AC, MUC1 and MUC 6. In contrast, there is only trace amounts of intestinal mucin MUC2 expressed on the differentiated HT29-MTX-E12 cells [192].

M cells are specialized intestinal cells that are mainly found in the follicle-associated epithelium (FAE) of Peyer's patches in the small intestine [4, 193]. Although M cells are found in small proportions in the human GIT (<1%), they perform as efficient antigen deliverers and have the ability to transport particulate matter [194]. Owing to these high transcytotic ability, M cells have been exploited in oral delivery systems (e.g., vaccines) [4, 195], thereby leading to the requirement of cocultures of Caco-2 cells and lymphocytes for the development of *in vitro* cell models that present the M cell phenotype. The initial M cell-like model was developed by Kernéis *et al.* [196]. This model was established by adding primary lymphocytes (isolated from mouse Peyer's patches) into the upper chamber of a Transwell® system with the basolateral side filled with differentiated Caco-2 monolayers (14 days). After maintaining the culture for several days, the culture in the Transwell® system was able to produce the main features of M cells and the FAE [196]. The main limitations of the Caco-2/mouse lymphocyte coculture model are the lack of uniformity and the use of nonhuman lymphocytes. To overcome these limitations, Gullberg *et al.* developed a new M cell-like coculture system based on normally oriented Transwell® inserts, in which human Burkitt's lymphoma Raji B cells were seeded into the basolateral compartment and a monolayer of two-week-old differentiated Caco-2 cells on the apical side [197]. des Rieux *et al.* used this model to study the impact of M cells on the intestinal transport of drug-loaded nanoparticles. This study emphasized the importance of M cells in the transport of oral delivery systems across intestine barriers [198]. To favor a closer contact between Raji B cells and Caco-2 cell in the monolayer, the authors inverted the orientation of the insert 3-5 days after seeding the Caco-2 cells on the apical side of the membrane. After approximately 10 days, Raji B cells were seeded on the basolateral side of the inverted inserts. Ultimately, the inserts were used in their normal orientation to conduct transport studies [22, 195]. The results of the morphologic analysis confirmed that the inverted

FAE model led to approximately 15-30% cells being morphologically similar to M cells. This improved model offers some advantages, such as 1) cocultures using classic differentiated human cell lines, 2) not using primary cells (avoiding animal use) and 3) reducing the high variability between experiments and different laboratories [22, 195]. Currently, the FAE coculture models have been used to investigate the transport mechanism of various nanoparticulate systems, including nontargeted/targeted polymeric nanoparticles and lipid nanoparticles [199-202]. The researchers found that the tested polymeric nanoparticles made from carboxylated polystyrene [195], PLGA [201], β-glucan [203], chitosan [201] or its N-trimethylated derivative (TMC) [201], increased the M cell-specific transport within the FAE to a differing degrees. The researchers further grafted different ligands (RGD [202], RGD peptidomimetic [201] and Glycine-Arginine-Glycine-Aspartic Acid-Serine (GRGDS) [204]) onto the surface of drug carriers to further increased drug transport via M cell targeting. For instance, Lee *et al*. found GRGDS-conjugated β-glucan carriers (GRGDS-BG) had more targeting affinity for Caco-2 cell/Raji B cell co-culture model than for Caco-2 monoculture model [204]. Due to the highly efficient M cell targeting, antigen PR8-loaded GRGDS-BG demonstrated significant antibody concentration (IgA/IgG) in serum, intestine, and mucus after 21 days of first oral dose [204]. Additionally, in contrast to polymeric nanoparticles, submicron nanostructured lipid carriers (NLCs) failed to increase the drug permeability of M cells [199].

Triple-culture models

Although coculture models have been extensively used as *in vitro* tools for the evaluation of intestinal transport, neither mucus-secreting nor FAE models simulate the intestinal epithelial layer entirely. Considering the importance of three main types of epithelial cells in intestinal physiology (e.g., absorptive enterocytes, mucus-secreting goblet cells and antigen-delivering M cells), Prof. Sarmento's group developed a triple-culture *in vitro* cell model with three human cell lines (Caco-2, HT29-MTX and Raji B cells) to investigate the intestinal transport of insulin in solution and encapsulated within nanoparticles [205, 206]. They found that the normally oriented triple-culture model with a Caco-2:HT29-MTX cell ratio of 90:10 exhibited a more physiological, functional, and reproducible *in vitro* intestinal transport model compared to those comprising different cell-seeding orientations and ratios [205, 206]. The triple-culture model has been successfully used to evaluate the intestinal permeability of several drug molecules, including protein drugs such as insulin, delivered by nanoparticles [207, 208]. Recently, Prof. Sarmento's group assessed the intestinal permeability of 12 model drugs in

Caco-2 cell culture monolayers, Caco-2/HT29-MTX cell coculture monolayers and a triple-culture Caco-2/HT29-MTX/Raji B cell model [20]. The findings of this study suggest that the sophisticated triple-culture model could be a suitable tool for elucidating the comprehensive transport mechanism of drugs [20]. Schimpel *et al.* also developed a triple-culture Caco-2/HT29-MTX/Raji B cell model using a Caco-2:HT29-MTX cell seeding ratio of 70:30 to investigate the intestinal transport of polystyrene nanoparticles with sizes of 50 and 200 nm, respectively [209]. The resulting permeability data indicated a good correlation between the *in vitro* triple-culture cell model and an *ex vivo* porcine intestinal mucosa model, suggesting that this triple-culture Caco-2/HT29-MTX/Raji B cell model is also a reliable *in vitro* model to study particle uptake [209].

3D cell models

3D intestinal transport models that simulate the morphology and physiology of human gastrointestinal barriers have recently attracted the attention of the scientific community and are expected to become standard approaches to studying intestinal transport. Despite the limited number of studies performed thus far, 3D intestinal cell models developed to date are typically based on human intestinal cells fused into different scaffolds (promoting cell proliferation and differentiation [210, 211]) on the apical side of Transwell® membranes. The establishment and improvement of 3D intestinal cell models are realized by fusing different scaffold-embedded cells or changing the type of scaffold used.

Collagen gel has been the most commonly used scaffold material for constructing 3D intestinal models. Leonard *et al.* developed a 3D "inflamed" intestinal cell model by incorporating primary human macrophages and dendritic cells into a collagen scaffold and seeding three epithelial cell lines (Caco-2/HT29-MTX/T84 cells) on top of the collagen scaffold to form a differentiated monolayer. Then, proinflammatory cytokines (e.g., interleukin-1β) were added to the apical compartment of a Transwell® system [212]. The "inflamed" 3D model has been used to evaluate the potential efficacy of budesonide-encapsulated nanocarriers (PLGA nanoparticles and liposomes) for the treatment of inflammatory bowel disease (IBD) administered via the oral route [213]. In a more recent study, the same group reported another variation to this model wherein one single-cell line (Caco-2 cells) was seeded on the top of the collagen scaffold containing two other cell lines (THP-1 human macrophages and MUTZ-3 dendritic cells) [214]. Li *et al.* also used collagen gel as a scaffold to develop a 3D intestinal mucosa model comprising a coculture Caco-2/HT29-MTX cell monolayer and two types of

stromal cells (fibroblasts and immunocytes) incorporated into the collagen gel [215]. Compared to the classic 2D Caco-2 cell monoculture model, this model more closely mimics the native intestinal layer (e.g., it has a higher correlation coefficient), representing a better predictive tool for the study of drug intestinal transport [215]. Overall, these 3D intestinal models that use collagen gel as a scaffold may be promising tools for studying drug transport in the intestine. However, one should take into account certain limitations, such as the culturing of Caco-2 cells on top of the collagen scaffold, shortened height of villi and the formation of multiple layers as cells penetrate the matrix due to the degradation of collagen during the long-term culturing period (21 days) [216].

To establish improved 3D intestinal models, other scaffolds have been exploited to ameliorate the limitations of the collagen scaffold-built models. For instance, the architecture of villi in 3D intestinal models can be reproduced using polymeric scaffolds. Costello *et al.* used biopolymeric PLGA as a scaffold with villus-like features [210]. This study showed that culturing Caco-2 and HT29-MTX cells on top of PLGA scaffolds simulates the native morphology and differentiation observed in the human intestine [210]. Patient *et al.* also found that nanofibers can be used as 3D scaffolds. The transport model established using 3D nanofibrous scaffolds more closely mimicked native intestinal tissue and was particularly suitable for evaluating passive intestinal epithelial transport [217]. In addition, more recent studies have demonstrated that L-pNIPAM hydrogel scaffolds not only capitalize on the 3D structure of human intestinal villi but also support the long-term coculture of a 3D model (up to 12 weeks), suggesting that it is a promising scaffold for developing robust *in vitro* 3D intestinal models for studying drug transport in normal intestine and/or in abnormal intestine expressing a chronic disease (e.g., IBD) [218, 219].

The complicated *in vitro* cell-based transport models reviewed above, including co-cultured models, triple-cultured models and 3D models, have been widely used for the prediction of intestinal transport of oral formulation since these models show high relevant physiological features of human intestine [220-223]. We have collected some examples in Table 4.

4.2.2 Brush border membrane vesicles (BBMVs) and basolateral membrane vesicles (BLMVs)

BBMV models are high-throughput models used in physiological studies and drug discovery and development to evaluate drug uptake by enterocytes, drug stability, enzyme interactions or

transport mechanisms [34, 107]. BBMV models are based on isolated apical membranes from different parts of the GIT, thus allowing evaluation of apical membrane transport without the influence of the basolateral membrane or regional differences in the GIT. The purified fraction of apical membranes is extracted from a homogenate of frozen inverted intestine [107, 224, 225]. BBMVs have been successfully extracted from different sources, including the human GIT. The purification of BBMVs is performed using calcium ions, which can easily remove microsomal fragments by forming large aggregates that can be separated using slow speed centrifugation [224, 226]. BLMVs, on the other hand, contain only purified fractions from the basolateral membranes of the intestinal epithelium [227]. The orientation and functionality of the vesicles can be assessed by enzyme markers and specific carriers, respectively [107]. Drug uptake is evaluated by quantifying the amount of drug in both vesicles and the medium. In one study, 23 reference drugs were evaluated for accumulation in BBMVs prepared using rabbit small intestine. The drug accumulation data demonstrated good correlation ($R^2 = 0.853$) with human oral absorption data [228]. Despite having all the components of the epithelial membrane, these models provide incomplete information about the absorption process both for movement into and out of cells via the apical and basolateral membranes. Thus, transport mechanisms such as paracellular transport cannot be studied with these models [107]. Moreover, intraday variations in vesicle formation and drug leakage from the vesicles are some of the drawbacks associated with these systems [95]. In addition, evaluating highly lipophilic compound with these models can lead to false positive results due to high non-specific binding to the lipid membranes [229]. Therefore, the possibility of such misleading results should be carefully considered. However, the advantages associated with these models, such as the quick and easy-to-use protocol, the possibility of performing high-throughput screening and the use of human intestinal tissues make these models good options for screening drug absorption during the early phases of drug discovery and development.

4.2.3 Microfluidics-based systems

Conventional *in vitro* cell-based models, including 3D intestinal models, are produced in a static environment; that is, these models lack the dynamic and active microenvironment observed *in vivo*. To ameliorate these limitations *in vitro*, microfluidics-based systems, including gut-on-a-chip and human-microbial cross talk (HuMiX), have emerged as cell-culture models for studying drug transport across intestine barriers. These models utilize

microfluidic technology for *in vitro* cell culture, thus reproducing the 3D topology, dynamic environment and gut microbiome observed in the human intestine [230, 231].

The most common design for gut-on-a-chip microdevices consists of a porous membrane that supports a monolayer of intestinal epithelium cells and separates two compartments, simulating the intestinal lumen and blood circulation, respectively. Kim *et al.* developed a human gut-on-a-chip system that mimicked intestinal luminal fluid flow and peristalsis-like motions (Figure 4) [230]. This chip supplies low shear stress flow and cyclic strain to Caco-2 cells that are seeded on extracellular matrix-coated porous membranes during cell culture, thus promoting differentiation of the monolayer cells, forming villi-like folds and increasing the intestinal barrier function. Moreover, this system was also able to achieve coexisting microbial flora and epithelial cells for at least one week [230]. The development and morphologic identification of the Caco-2 monolayer on this chip has also been described. The system exposes cultured cells to physiological peristalsis-like motions and liquid flow, inducing the Caco-2 cells to spontaneously develop 3D intestinal villi (Figure 4B), basal proliferative crypts (Figure 4C) and four different intestinal cells (absorptive cells, mucus-secreting cells, enteroendocrine cells, and Paneth cells) (Figure 4D**)** [145]. In another work, these authors demonstrated that this human gut-on-a-chip can be established as a stable ecosystem since commensal microbes and immune cells can be cultured to coexist with intestinal epithelial cells for a relatively long period (from many days to weeks) [232]. By mimicking the destructive effects of pathogenic bacteria on the intestinal villi *in vitro*, they created a human intestinal disease model (IBD; gut inflammation-on-a-chip) [233]. The same group also found that other patient-specific disease models can be established by modeling the intestinal disease pathophysiology on the chip [234]. In addition to seeding Caco-2 cells, human pluripotent stem cells derived from intestinal organoids or human primary epithelial cells isolated from healthy regions of intestinal biopsy samples have recently been cultured on the porous membrane of gut-on-a-chip microdevices [235, 236]. Notably, these models can be used to analyze how selected drugs are transported through normal and/or pathological intestines in a controlled manner, which is not possible using other existing *in vitro* models or *in vivo* animal models.

Using gut-on-a-chip to establish an intestinal ecosystem is challenging, as the cocultured commensal and/or mutualistic microorganisms only grow under aerobic conditions [232, 233], which limits the applications of this model to some extent. To overcome these limitations, Shah *et al.* developed another modular microfluidics-based model named HuMiX that consists of

three co-laminar microchannels: a medium perfusion microchamber, a human epithelial cell culture microchamber and a microbial culture microchamber (Figure 5) [231]. This system mimics the intestinal human-microbe interface by coculturing representative human epithelium cells (Caco-2 cells) and microbial cells, *Lactobacillus rhamnosus* (*L. rhamnosus*) GG (a commensal facultative anaerobe), or *Bacteroides caccae* (obligate anaerobe) [231]. The transcription, metabolism and immune response data from Caco-2 cells cocultured with *L. rhamnosus* GG can be replicated *in vivo* [231]. Although the HuMiX model was originally developed to study host-microbial interactions and is still under the stage of development and refinement, it exhibited the potential to be an advanced *in vitro* model for drug discovery, drug screening, and drug transport.

Although a growing number of studies have recently described the establishment and improvement of these advanced *in vitro* models, to our knowledge, there are no examples of these approaches being utilized to investigate the drug intestinal transport of oral delivery systems. These advanced microfluidic devices exhibit striking similarities with the physiology of the epithelium, the dynamic microenvironment and the microbiome coexisting in the human gut. Thus, they bridge the gap between conventional cell culture and animal models and are expected to serve as useful tools for future research on drug transport across intestine barriers.

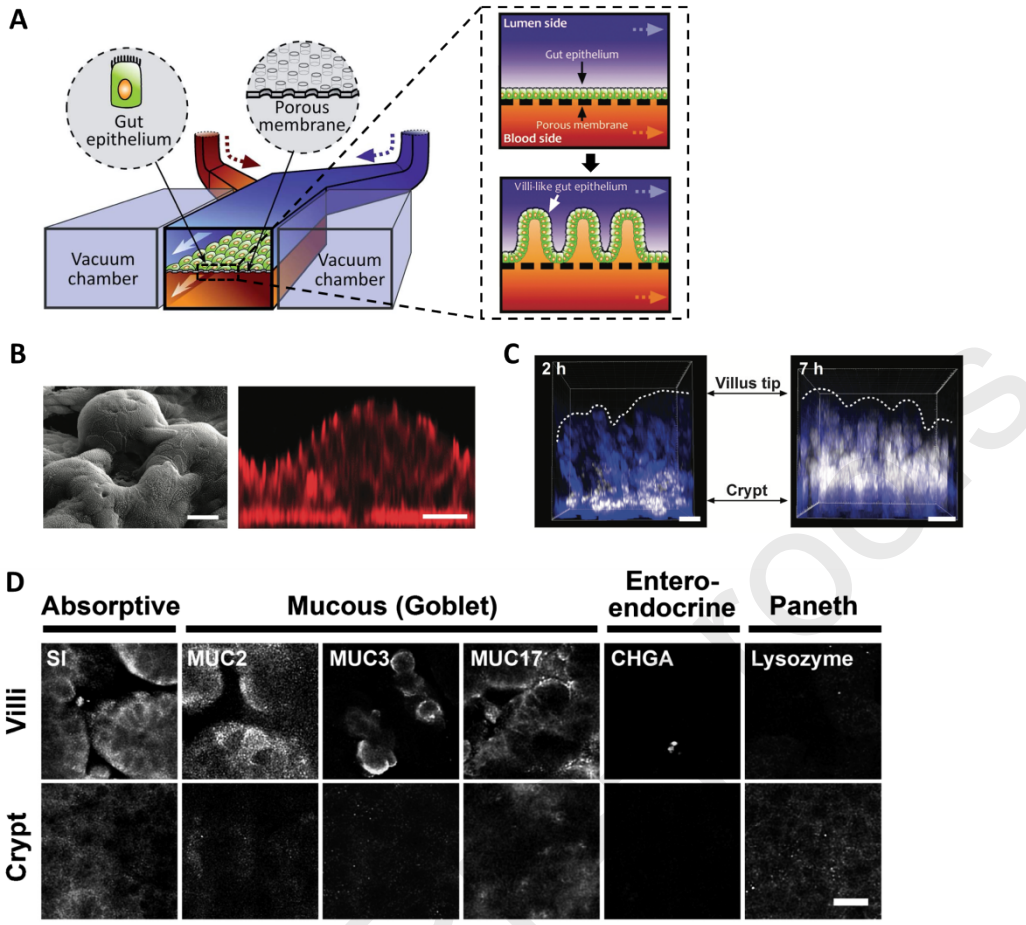

Figure 4: Human gut-on-a-chip device mimicking the intestinal luminal fluid flow and peristalsis-like motions and forming the intestinal villi (including various intestinal epithelial cell subtypes) and basal proliferative crypts. (A) Schematic illustrations of the device simulating intestinal luminal fluid flow and peristalsis-like motions. (B) The transformation of a planar intestinal epithelium into 3D villus structure as captured in a scanning electron microscopy (SEM) image (scale bar: 10 mm) and a confocal image (tight junction protein ZO-1 (red); scale bar: 25 mm). (C) Regeneration of basal proliferative crypts. Fluorescence microscopic basal crypt after a 2 h EdU (5-ethynyl-20-deoxyuridine) labeling pulse (left) and 5 h after EdU was washed out (right) (scale bar: 20 mm). Nuclei were stained with Hoechst 33342 (blue). Positively proliferating cells were labeled with EdU. The white dashed line represents the upper lumen boundary of villi. (D) Reconstitution of multiple differentiated intestinal cell types. Confocal images of differentiated Caco-2 cell villi and crypt regions, labeled with specific markers of absorptive enterocytes (sucrase-isomaltase), mucus-secreting goblet cells (mucin 2, 3, and 17), enteroendocrine cells (chromogranin A) and Paneth cells (lysozyme) (scale bar: 25 mm). Reproduced, with permission, from ref. [145, 230]. (The details and explanations of this figure legend can be found in the web version of these articles.)

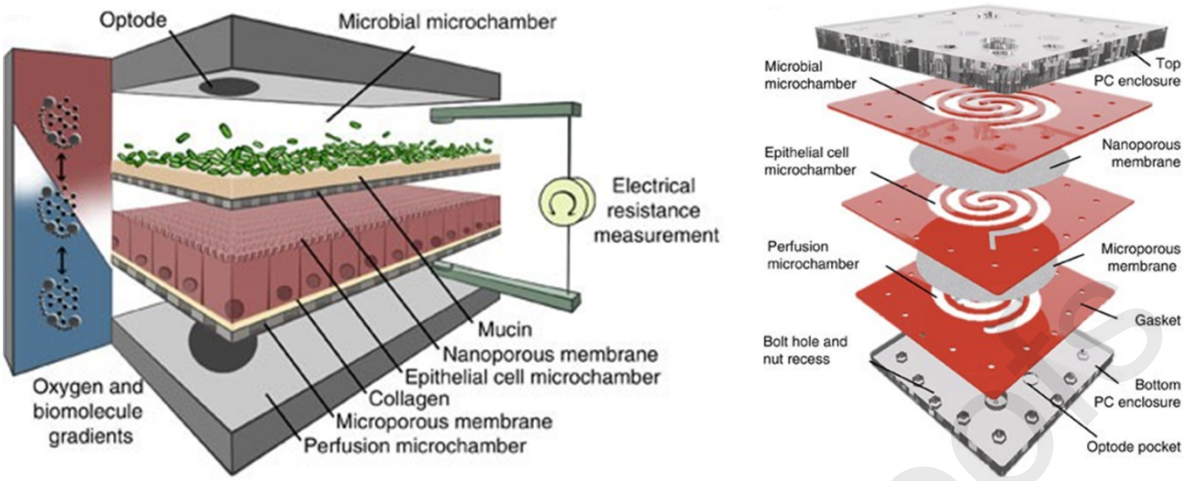

Figure 5: Scheme of the HuMiX model mimicking the intestinal human-microbe interface with cocultured gut epithelial cells and bacterial cells. Reproduced, with permission, from ref. [231].

4.3 Practical aspects of *in vitro* transport models

Table 5 summarizes the current *in vitro* models used for drug transport studies. The degree to which these models simulate true human GI conditions determines the accuracy of the predicted human data. Therefore, the validity of these models is essential when they are established and used for the study of the transport of drugs across intestine barriers.

The non-cell-based transport models are based on the simple idea of using biomimetic artificial membranes to mimic the intestinal epithelium barrier. The existing non-cell-based models are usually validated by comparing the correlation between the permeability values obtained from the tested model and Caco-2 or human absorption values [125, 127, 135, 237].

Various cell lines have used to establish different *in vitro* cell-based transport models. These cell lines have their own unique morphological and functional features, as well as specific cell markers, which simulate different intestinal cells, such as enterocytes, goblet cells and M cells. The validation of *in vitro* models established by different cell lines includes the integrity of the monolayer, the characteristics of differentiation and the functionality of the model, which can be evaluated using a wide variety of techniques. Among these morphological and functional parameters, it is easiest to determine the integrity of the model by measuring TEER. In general, tighter TJs form between epithelial cells, resulting in higher TEER values. Since TJs regulate

the transport of molecules via the paracellular pathway, the P_{app} of small molecules (e.g., lucifer yellow [238], mannitol [239], and FITC-dextran [240]) that are known to pass through paracellular transport can be used to validate the integrity of the cell membrane. The differentiation characteristics and the specific features of the cell lines in the model are examined using specific intestinal cell markers and evaluating the cell morphology. There are several techniques that could be used to assess cell morphology, such as immunofluorescence cytochemistry, immunohistochemistry, transmission electron microscopy (TEM), SEM, and atomic force microscopy (AFM). In addition, model functionality is mainly assessed by the expression and activity of specific efflux transporters on the cell surface (e.g., P-gp, breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP)), which can be confirmed by immunofluorescence staining and/or chemo-competitive inhibitors. Validation studies of the aforementioned *in vitro* cell-based models can be found elsewhere [21, 195, 206, 241]. Pereira *et al.* validated the cell morphology of Caco-2 and Caco-2/HT29-MTX cell coculture models by using TEM technology [21]. They found that the apical microvilli and TJs were observed in the TEM image of 21 day-differentiated Caco-2 monolayers. Some residual mucus was detected on the surface of microvilli in the coculture model. In this coculture model, they visualized intracellular granules, which are similar to *in vivo* mucin granules in goblet cells [21]. des Rieux *et al.* validated the inverted Caco-2/Raji B cell coculture model using various techniques, including TEER and P_{app} value determination, and immunohistochemistry, immunofluorescence cytochemistry, TEM and SEM images [195]. Considering these validation studies, the authors showed that, although it may overestimate the number of M cells, the inverted Caco-2/Raji B cell coculture model is a useful tool for studying the impact of M cells on nanoparticle transport [195]. Zhang *et al*. recently developed a novel *in vitro* 3D intestinal model consisting of an epithelium, subepithelial fibroblast network and extracellular matrix [242]. The TEM images of this model revealed structures similar to those of the intestine. The validation of this new 3D model was confirmed with the TEER values and transporter (P-gp and BCRP) activities, which were closer to the physiological characteristics of the human small intestine [242]. The most advanced *in vitro* intestinal models, gut-on-a-chip and HuMiX, exhibit similar characteristics to the physiology of intestine, such as peristalsis-like motions, villi-like folds and the intestinal human-microbe interface [145, 231].

The use of a proper *in vitro* transport model can provide useful information during the initial drug/formulation development phase, which may help to assess associated risks, as well as save time and expense. The information provided in Table 5 is helpful for selecting appropriate *in*

vitro models according to the needs of the experiment and to obtain valuable preclinical data. There are two crucial principles that need to be considered when selecting *in vitro* models. First, the properties of the drugs, excipients, carrier materials should be considered as some tested drugs/formulations because they 1) may harm the intestinal barriers *in vitro*; 2) may fail to be transported by specific cell lines; and 3) may be strongly adsorbed by the membrane or plastic components. In addition, the purpose of the experiments is another important consideration in the selection of models. For instance, the least expensive artificial membrane-based *in vitro* transport models are used not only in the high-throughput screening of drugs, but also in understanding the influence of GI fluids on drug transport. The biomimetic GI fluids generally cover a wide range of pH (2-8) and contain phospholipids, bile salts, enzymes and/or various fatty acids that are present in human GIT [186, 243].These factors and components play important roles in the intestinal transport of oral drugs/formulations. It is well known that these well-developed biomimetic media as apical transport buffer could compromise the integrity of these biological barriers [244]. In contrast to the cell-based transport models, the non-cell-based transport models are good alternatives for the study the impact of GI fluids on the intestinal transport of some specific drugs. On the other hand, to optimize the tested drug/formulation, it is necessary to investigate the comprehensive transport mechanism. At that level, choosing a valid cell-based *in vitro* model is crucial. Additionally, some other factors that need to be considered are 1) the expression of enzymes, transporters and receptors; 2) the simulation of physiological and pathological conditions; and 3) the impact of the gut microbiome on drug transport.

Table 4: Examples of intestinal drug transport studies evaluated in *in vitro* models.

Drugs	Drug delivery systems	Assessment models	Performed studies	References
Aciclovir	Self-microemulsifying drug delivery systems	PAMPA	Permeability study	[245]
Budesonide	Multistage silicon-PLGA particles	Caco-2 inflamed 3D cell model	Permeability study; cytokine analysis	[223]
Curcumin	α-Lactalbumin nanotubes	Caco-2/HT29-MTX-E12 3D co-culture cell model	Permeability study	[189]
Ferulic acid	Chitosan-coated PLGA nanoparticles	Caco-2 monoculture; Caco-2/HT29-MTX/ Raji B triple-culture model	Permeability study	[246]
GLP-1	A Recombinant Lactococcus Lactis	MDCK monoculture model	Permeability study	[247]
GLP-1/DPP-4 inhibitor	Multifunctional tailorable composite systems	Caco-2/HT29-MTX/ Raji B triple-culture model	Permeability study; DPP4 enzymatic activity study	[186]

Insulin	Chitosan nanoparticles	Caco-2 monoculture model	Permeability study; transport mechanism studies	[248]
Insulin	Trimethyl chitosan nanoparticles	Caco-2/HT29-MTX co-culture model	Permeability study; TJs opening mechanism study	[182, 249]
Insulin	Trimethyl chitosan nanoparticles	Caco-2/HT29-MTX-E12 co-culture model	Permeability study; TJs integrity study	[113]
Insulin	pHPMA-coated NPs	HT29-MTX-E12 monoculture model	Permeability study; Mucus effect study	[222, 250]
Insulin	Solid lipid nanoparticles	Caco-2 monoculture model	Permeability study	[251]
Insulin/exenatide	Silica nanoparticles	Caco-2 monoculture model	Permeability study; transport mechanism studies	[252]
Insulin	-	Caco-2/HT29-MTX 3D co-culture model	Permeability study	[21]
Naproxen, indomethacin and metronidazole	Chitosan-coated and PEGylated liposomes	mucus-PVPA	Permeability study	[134]
Ovalbumin	pH-responsive bacterial nanocellulose/polyacrylic acid hydrogel microparticles	Caco-2/HT29-MTX/ Raji B triple-culture model	Permeability study; TJs opening study	[221]
Paclitaxel	PEG-b-PCL micelles	MDCK monoculture model	Permeability study	[253]
PR8	GRGDS-conjugated β-glucan nanoparticles	Caco-2/Raji B co-culture model	Permeability study	[204]
Carnitine	-	Mouse intestinal brush-border and basolateral membrane vesicles	Uptake and transport study	[254]
siRNA	Lipidoid nanoparticles	Caco-2 monoculture model	Permeability study; TJs integrity study	[255]
Silymarin	Nanostructured lipid carriers	PAMPA; Caco-2 monoculture model	Permeability study	[256]
-	Fluorescent latex microparticles	Caco-2/mouse lymphocytes, Caco-2/Raji B and inverted Caco-2/Raji B co-culture models	Permeability study	[257]

Table 5: A summary of different in vitro models for drug transport studies

In vitro models			Complexity	Intestinal barriers	Advantages	Disadvantages
Non-cell-based models	PAMPA		Low	A filter support soaked with phospholipids dissolved in an organic solvent	Easy to use High-throughput screening	Poor biomimetic membrane Barrier components may dissolve and/or emulsify into the aqueous medium Measures only passive transcellular transport
	PVPA			A filter support onto which phosphatidylcholine liposomes are deposited	Easy to use High-throughput screening High similarity with the biostructure of the intestinal epithelium	Laborious preparation procedure Poor storage stability Shows general poor resistance to additives Measures only passive transcellular transport
	PermeaPad®			A sandwich-like structure consisting of a layer of dry phospholipids wrapped in two support layers	Fast and economical tool High-throughput screening Good resistance to pH changes and aggressive additives Industrial scale manufacturing Good storage stability (at least one year)	Measures only passive transcellular transport
Cell-based models	Transwell®-based cell models	Monoculture models		Single-cell enterocyte-like cell lines seeded on a polycarbonate membrane	Gold *in vitro* transport model Relatively easy to use For studying transport mechanism	Lacking mucus layer and M-like cell Underestimates paracellular transport Large inter- and intra-laboratory variability
		Coculture models		Enterocyte-like/goblet cell-like cell or M cell-like cell lines seeded on a polycarbonate membrane	Compensatory mucus layer or M cells Reliable paracellular transport For studying transport mechanism	Lacking some main types of intestinal cells Large inter- and intra-laboratory variability
		Triple-culture models		Enterocyte-like/goblet cell-like/M cell-like cell lines seeded on a polycarbonate membrane	Three main types of intestinal cells Reliable paracellular transport For studying transport mechanism	Time consuming Large inter- and intra-laboratory variability
		3D cell models		Different human cell lines fused to various scaffolds on a polycarbonate membrane	Relatively complete intestinal morphology and physiology Can simulate pathological intestine (e.g., IBD) For studying transport mechanism	Easily degrades specific scaffolds Time-consuming Large inter- and intra-laboratory variability
	Membrane vesicles	BBMV (BLMV)		Isolated and purified human intestinal epithelial cell, either brush border or basolateral side	Allows the study the interaction with specific membrane of intestinal epithelia Study interaction of drugs and formulations at cellular level Specific transporters can be isolated and used to evaluate the interaction with drug Very small quantity of drugs is required. The vesicles can be cryopreserved	It is not possible to isolate completely pure vesicles Isolation process can damage transporters, enzymes associated with the membrane Sensitive analytical method is needed
	Microfluidics-based systems	Gut-on-a-chip	High	Different human cell lines seeded on a porous membrane in microdevices	Reproduces the 3D topology, dynamic environment and gut-microbiome in human intestine For studying drug transport through the normal and/or pathological intestine in a controlled	No practical application for drug development Time-consuming and expensive Complex techniques
		HuMiX				

5. *Ex* vivo models

Ex vivo studies are based on experiments/measurements performed on tissue extracted from organisms in a controlled external environment that resembles the natural conditions [26]. *Ex vivo* models are considered to be a compromise between *in vitro* and *in vivo* models. One major advantage of using functionally isolated tissues as part of an intact mucosa is the presence of the entire intestinal epithelium with a mucus layer and the expression of transport and drug metabolism proteins [258, 259]. Thus, this model allows higher interplay and cross talk among the cellular components and mimics the *in vivo* condition more closely than *in vitro* models [260]. Furthermore, *ex vivo* models can be used as alternative models to perform experiments that cannot be performed in living organisms because of ethical considerations [261]. Another advantage of this system is that the use of excised human tissues can provide more in-depth knowledge about the intestinal absorption process of the tested compound [5]. *Ex vivo* experimentation for orally delivered systems have included evaluating drugs in terms of intestinal absorption, interaction with the intestinal epithelia and the mucus layer and immunomodulatory responses. Some of the common intestinal *ex vivo* models include everted rings and sacs, diffusion and perfusion models [107, 259]. A detailed introduction of each type of *ex vivo* model is given in the subsections below, and the advantages and disadvantages of different models are also summarized in Table 6.

5.1 InTESTine™

InTESTine™ is a recently developed commercially available physiologically relevant intestinal tissue model developed by TNO [262]. This new system is a predecessor of TIM systems, which only mimic the condition in the intestinal tract. However, the InTESTine™ model uses freshly isolated healthy porcine intestinal tissue from different regions of the animal's GIT. The model promises to be a cost-effective way to study absorption, metabolism and the complex physiology of the intestine. The system is a medium-throughput system that is available in 24- to 96-well plate format. The schematic representation of the InTESTine™ system is shown in Figure 6A. To date, there have been no published investigations demonstrating the use of this system, but it presents a novel way to perform *ex vivo* studies with porcine intestinal tissue [262].

5.2 Intestinal rings/slices

Intestinal rings/slices are intestinal models first described by Otto *et al*. (1954) and were commonly used for analyzing the kinetics of carrier-mediated transport and drug accumulation in enterocytes [107, 263]. In this model, the excised intestine is everted and cut into either rings or slices (2–5 mm in width) and submerged in oxygenated buffer containing the test drug compound. The intestinal rings and segments remain viable for 30-60 min and 2 h, respectively [107, 264]. At the end of the experiment, the drug content is quantified in both tissues and buffer. The intestinal rings are easy to use and can be used to test multiple compounds simultaneously. Ungell *et al.* have demonstrated that uptake in everted intestinal rings is similar to in vivo bioavailability results, under appropriate conditions [265]. Intestinal rings have been shown to have good correlation with *in vivo* bioavailability results. However, this model also has several disadvantages, such as drug absorption from the serosa side of the intestine, limited viability of the tissue, and limited applicable analytical methods [265]. Moreover, this technique cannot easily distinguish between uptake and binding.

With improvements in precision cutting and cryopreservation techniques, precision cut intestinal slices have emerged as a newer generation of *ex vivo* intestinal models used to evaluate absorption, distribution, metabolism, excretion and toxicity [263]. This model is a simple, fast and reliable technique and has a viable duration ranging from 8-24 h, which is significantly higher than that of traditional techniques. The slices should have thicknesses up to 400 μm to ensure adequate oxygen and substrate supply to the intestinal slices [266, 267]. Precision-cut intestinal slices are easy to handle and require only a short training period. A more detailed review of precision cut intestinal slices was published by Li *et al.* [263], and the standardized protocol to prepare precision cut models is described in detail elsewhere [268]. In a specific region, more than 100 slices can be prepared, and they can be used to perform a large number of experiments. Despite having clear benefits, these models are also limited by not enabling the directional transport of drugs to be measured [263].Nonetheless, precision cut models are suitable and useful tools for studying regional differences in the intestine and drug metabolism and the regulation of enzymes and transporters involved in drug disposition [268-271].

5.3 Intestinal sacs

Wilson and Wiseman first introduced everted intestinal sac models using rat and hamster intestines [272]. The everted intestinal sacs were used to determine drug accumulation and drug transport across the intestinal mucosal layer. This model has been extensively used for pharmacokinetic, efflux transport, multidrug resistance, and drug interaction studies [42, 273]. Test compounds can be

monitored on the luminal side and on the serosal side. The intestinal sections are cut into small tubes (2-3 cm long), which are inverted on glass rods and tied on both ends. The mucosal surface is exposed towards the buffer solution present in the receiving compartment, and the serosal layer forms the inside of the sac, which is filled with buffers. The ligated everted sac is placed in a container with buffer and test compound. At the end of the experiment, the drug content is quantified on both luminal and serosal sides, and the weight of the sac is also measured [26, 107, 265]. Another variation to this model is designed using a polypropylene ring at one end of the sac, which makes sampling easier. Under optimal experimental conditions and handling, the integrity of the intestinal tissue is observed for 120 min [274]. The model can be based on intestinal sections with or without the muscularis mucosa stripped. However, the effect of stripping or not stripping must be considered carefully, as models without stripping can provide results that underestimate drug transport due to probable loss due to binding to muscle tissues [273]. There are several advantages associated with this model: it is relatively fast and inexpensive, the mucosal layer is present, no specialized equipment is needed, multiple drugs can be tested simultaneously and the low volume of the serosal compartment allows quantification of poorly permeable drugs [26]. The major drawback of this model is the viability of the tissue (total disruption of epithelia is observed after one hour) and the possible diffusion through the lamina propria. Possible morphological damage during eversion can also greatly impact the validity of the study [273, 275]. Moreover, stress-induced mucus overproduction in everted models and unwanted removal of mucosal layer can also impact the study [12, 276-278]. From a practical aspect, the tissue excision time and preparation must be quick, and the tissue must be submerged in oxygenated buffer to ensure minimal structural damage to the tissue.

Ultimately, to avoid possible structural damage to the tissue, the sac may be formed without eversion. In addition to minimal morphological damage, the non-everted sacs are simpler to use and require a small amount of test compounds. Furthermore, in these modified models, sampling can be performed for different time intervals without disturbing the intestinal tissue [279, 280].

5.4 Diffusion chambers

Diffusion chambers are one of the most common models used for *ex vivo* intestinal experiments. Ussing chambers and Franz cells are the two variants of diffusion chamber models and are briefly discussed in the subsection below.

Ussing chamber

The Ussing chamber was established by Hans Henriksen Ussing in the 1950s, and it has been widely studied since its discovery, with several variants of this model developed and commercially available [281]. This model has been used to evaluate drug transport across numerous types of epithelial tissues from both animal (mouse, rat, rabbits, dogs, rats and monkeys) and human biopsy samples [106, 282, 283]. This technique is used for both free drug solution and oral formulations [282-284]. In this model, intestinal segments are removed and cut open into planar epithelial sheets. The intestinal tissue can include the serosal layer and muscle tissues or they can be removed. The flat tissue is mounted between two half-cells that are both filled with oxygenated buffer solutions. The continuous bubbling of both chambers with carbogen (95% oxygen and 5% carbon dioxide) ensures stabilization of buffer pH, sufficient oxygenation of the tissue and reduces the unstirred water layer [285]. A schematic representation of a Ussing chamber is shown in Figure 6B. The sampling is done from the receiving chamber at regular intervals, which is then replaced by the same volume of fresh prewarmed buffer. The apparent permeability of the tested compounds is calculated based on the calculated rate of transport, exposed area and initial concentration of the test compound. Electrodes between the two chambers allow the continuous measurement of the transepithelial resistance of the membrane, short-circuit current and potential difference, which in turn enables continuous monitoring of the integrity and viability of the tissue [26, 27, 95]. In addition, histological evaluation and lactate dehydrogenase assays can be performed to ensure the viability of the tissue [95, 286, 287]. The Ussing chamber allows the measurement of bidirectional transport of the drugs (absorption and secretion). The use of a Ussing chamber allows measurements of drug absorption in different regions of the gut and under different physiological conditions (pH and simulated media) and the evaluation of transport mechanisms [288, 289]. It also allows the evaluation of different drug transporters on the intestinal epithelium [95]. Moreover, the presence of the mucosal layer allows a closer approximation of the permeability of drugs to *in vivo* data. This model has been successfully used with human intestinal biopsy samples [95, 96, 105, 290, 291]. However, the use of this model is time-consuming (for both tissue preparation and setup) and requires Ussing chambers to perform experiments. Moreover, the underestimation of drug transport has been found for this model, especially for lipophilic drugs [26]. There is also discussion regarding the use of stripped and unstripped intestinal tissue. With unstripped tissues, studies have shown that different types of drugs are impacted differently by the presence of the serosal and muscular layers, as both of these layers depend on the size and lipophilicity of the test compounds [26, 286]. Additionally, this model is low throughput, and there is a possibility that tissue viability is lost during the preparation and mounting stages [292]. Nonetheless, the Ussing chamber can be used as a screening

tool for new drug candidates, because compared to many other methods, the data from this model depending on species and tissue region origin are closely related to the *in vivo* situation in humans.

Franz cells

Franz cells are based on principle a similar to that of the Ussing chamber, but the tissue sections are placed horizontally compared to the vertical arrangement in the Ussing chamber. Initially, the Franz cell model was used for *in vivo* skin and buccal permeation studies; however, its application as a tool for determining intestinal permeability is increasing [293, 294]. Similar to the Ussing chamber, there are temperature-controlled donor and acceptor compartments separated by the tissue. The test sample is introduced in the donor compartment, and sampling is performed from the acceptor compartment at different time intervals. The receptor chamber is continuously mixed using a magnetic stirrer, which significantly reduces the unstirred water layer [26]. This effect can lead to higher permeation of the test compound, as observed for metoprolol, a permeability marker, in Franz diffusion cells compared to that in the Ussing chamber [293].

GI tissue robotic interface system (GI-TRIS)

GI-TRIS is an interfacial device that has been recently developed which ensures long-tern tissue culture and allows high-throughput evaluation of drug transport [295]. In this system, the pig intestinal tissue is set up in a 96 well plate compartmentalized design. In this study, the geometry and compression force have been studied and optimized. The transport study was performed with wide array of model compounds in different intestinal regions and with different incubation parameters. This advanced model allows high throughput investigation of a large number of test compounds through the different regions of the GIT. The robotically handled-tissue culture system would not only help in evaluating drug transport but it can also be a valuable tool for toxicity evaluation, excipient selection, and solubility and dissolution optimization for poorly soluble drugs. Overall GI-TRIS could have a great impact in the advancement and acceleration of drug screening and formulation development process [295].

5.5 Perfusion models

The gut loop model is a simplified perfusion model. In this model, the experimental animal is anesthetized, and a section of the intestine is separated while the link to the blood circulation is maintained [107]. The intestinal segment is washed, and a loop (ca. 10 cm long) is formed by clamping, and then, a known volume of drug solution in physiological buffer is injected in the loop. At the end

of the experiment, the test compound is quantified inside the loop and in the blood samples [3, 7, 284, 296]. This method enables the evaluation of the regional differences in drug absorption in the same animal, thus eliminating intervariability among the test subjects [283]. Furthermore, this simple model does not require expensive equipment and can be performed by researchers trained with *in vivo* animal experiments. However, a large number of animals are needed for this experiment compared to other models. Another major disadvantage of this model is the use of anesthesia and the absence of stirring conditions [107, 297].

The isolated intestinal perfusion model is used for drug absorption studies and has also been used as an *in situ* perfusion model [107]. Unlike the gut loop model, in this model, a 10-30-cm-long segment of the intestine is cannulated at both ends and is perfused continuously with buffer. The rat circulation can also be cannulated via the mesenteric vein and artery, which can provide information regarding the impact of hepatic clearance [34, 292, 298]. Mannitol is used as a permeability marker. This single pass perfusion model has demonstrated good correlation with human oral bioavailability and permeability of different types of drugs [293, 299]. A major benefit of this model is the presence of blood supply, which ensures continuous tissue oxygenation and proper flow features on the serosal side. Moreover, the presence of other components, such as the enteric nerves and enteroendocrine system, gives better control of drug transport and viability. Nonetheless, the use of anesthesia has also been shown to influence drug absorption. This method is very time-consuming and requires a large number of animals, which makes this model unsuitable for screening libraries of test compounds. Several studies have also reported the loss of the drug in the systemic solution due to enzymatic degradation or adsorption onto the plastic components used [107, 259, 300].

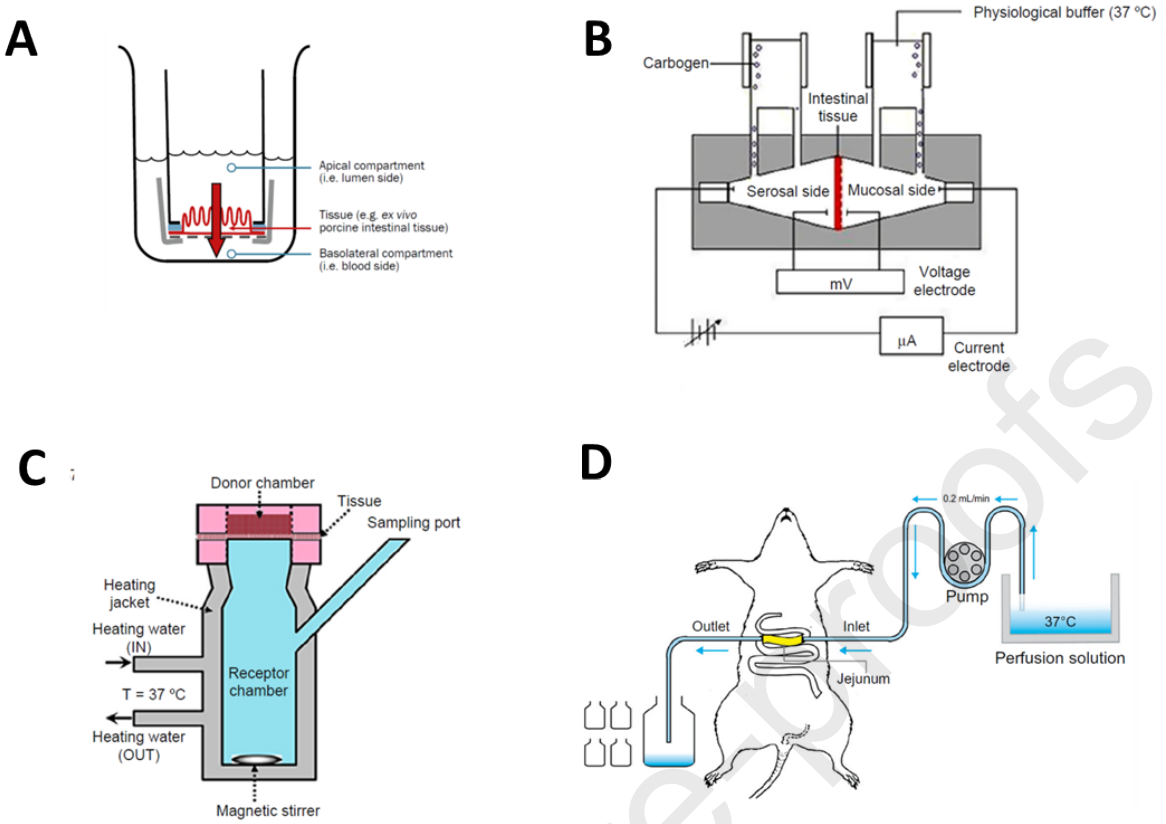

Figure 6: Schematic representation of a (A) InTESTine™ model [262] (B) Ussing chamber, (C) Franz diffusion cell [26], and (D) Single pass intestinal perfusion model, [301]) (Reprinted with permission)

Table 6: A summary of different *ex vivo* models used for drug transport studies

***Ex vivo* models**	**Complexity compared to *in vivo***	**Advantages**	**Disadvantages**
Intestinal rings/slices	Low  High	Easy to use Large number of experiments from one animal High-throughput screening Both animal and human tissue used	Tissue integrity of < 10-20 min Unspecific binding
IntesTINE ™		Easy to use Medium throughput Commercially available	Tissue integrity issues during transportation Difficult to customize Poor stirring condition Possible unspecific binding
Everted Intestinal sacs		Easy to use Directed transport Intermediate number of experiments from one animal High-throughput screening Both animal and human tissue used	Tissue integrity of < 30 min Structural damage to intestinal tissue during eversion Poor stirring conditions
Diffusion chamber: Ussing Chamber		Apparent permeability measurement Continuous measurement of tissue viability Directed transport Regional difference in drug absorption and drug mechanism can be tested Reduced unstirred water layer Both animal and human tissues can be used	Tissue integrity limited to 2-3h Expensive equipment needed Time consuming Less number of experiments per animal
Diffusion chamber: Franz Cells		Apparent permeability measurement Directed transport Regional difference in drug absorption and drug mechanism can be tested Reduced unstirred water layer Both animal and human tissues can be used	Tissue integrity limited to 2h Specialized equipment needed Time consuming Less number of experiments per animal
Gut loop		Directed transport Blood flow maintained	Requires anesthesia and animal surgery Loss of drug in circulation Poor stirring conditions
Intestinal perfusion model		Good stirring Can be performed with or without blood supply Directed transport	Requires anesthesia and animal surgery Time consuming and expensive Less number of experiments per animal Complex process

5.6 Practical aspects of *ex vivo* models

There are several practical aspects of *ex vivo* experiments that need to be considered to ensure the validity and reliability of the results. First and foremost, the source of the animal tissues used is very important. Although human tissues are the most relevant source, a lack of standardization of human tissue samples (age, gender, medication, diet, etc.), inability to perform regional studies, limited availability and ethical aspects greatly diminish its applicability [302]. Rat intestinal tissue is one of the most commonly used tissues for determining intestinal drug permeability, owing to its relatively high correlation with human tissue [303]. In addition, mouse, rabbit, pig, dog and monkey are other sources that have also been used. The similarities in morphological parameters (such as the physiology, anatomy, and intestinal environment) of the excised tissue with its human counterpart is an important criterion to consider when selecting the source of the tissue [12]. However, there are few to no studies that correlate the drug absorption data obtained from an *ex vivo* experiment with data obtained the *in vivo* [26]. In addition to the source, the age and the species of animal selected, the fasted/fed state of the animal, segment harvesting time and method of sacrifice can also influence the study [302]. Moreover, the use of anesthesia during the perfusion experiments affects drug transport. However, for experiments where the tissues are extracted, the use of anesthesia can ensure the integrity of the drug transporter [273]. Following the selection of the source of the tissue, the handling and preparation of the animal tissues are other important aspects. Proper care must be taken during handling of the tissue to ensure minimal structural damage to the tissue. Both stripped and unstripped tissues are used for intestinal studies. Studies have shown that the absence of the muscular layer closely mimics the physiological condition, electrical biases due to sporadic muscle contractions are eliminated, and the viability of the tissue endures because it is adequately oxygenated [304]. Moreover, quick harvesting of the intestinal segments ensures maximum transporter and enzyme activity in the tissue [302].

The experimental setup also be meticulously designed to ensure the validity of the study. The buffer used, equilibration time, viability and integrity marker used, oxygenation of buffer, sink condition, sampling method, drug quantification technique and apparent permeability calculation are some of the important experimental factors that need to be optimized [26, 290]. Krebs-Ringer bicarbonate buffer is the most common incubation buffer used for drug transport studies [285]. This buffer can be supplemented with other compounds where necessary; for example, mannitol can be added for osmotic balance, and bovine serum albumin can be added to eliminate unspecific binding [79]. Other commonly used buffers include 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and phosphate buffer saline solution. For closer resemblance to physiological conditions, simulated gastrointestinal

fluids have also been used to study intestinal transport [305, 306]. However, prolonged exposure to the simulated intestinal medium has been shown to diminish the viability of rat intestinal tissue in a Ussing chamber, as observed by two fold increase in mannitol permeability and substantial damage to microvilli after 120 min of exposure [289, 307]. The morphological characteristics of the isolated intestine are easily damaged by the conditions of the artificial environment (such as the incubation buffer e.g., FASSIF or FESSIF). The viability and integrity of gut tissues before and after the experiment can be monitored by a variety of experimental techniques, such as electrical measurements, lactate dehydrogenase (LDH) leakage, histological tools, and marker molecules (summarized in Table 7). Electrical measurements, including TEER, short-circuit current (SCC), and potential difference (PD), are common approaches to monitoring intestinal integrity and viability in a Ussing chamber model [95]. TEER, SCC, and PD are measured by placing two electrodes in both apical and basolateral chambers. TEER is the measurement of electrical resistance across a cellular monolayer, and it is a sensitive and reliable indicator of the integrity of the tight junctions and of the cell monolayer. TEER measures the ionic conductance via the paracellular pathway in the epithelial monolayer. The factors affecting TEER measurement include temperature, cell passage number, cell culture medium composition, TEER-related mechanoelectronics and shear stress [114]. PD reflects the voltage gradient generated by the tissue, and SCC reflects electrogenic ionic flux across the epithelium [307-309]. Both TEER and PD depends on the expression and conductance of channels present on both apical and basolateral membranes, and the functions of tight junctions that control paracellular ion transport [310]. Based on the Ohm's law, any changes in the ion conductivity across epithelial membrane will influence these electrical measurements. TEER measurement is a non-invasive technique that allows continuous monitoring of the integrity of the membrane and also can give information on paracellular flux [114]. LDH release is used to measure the damage to the intestinal cell membrane [311]. Histological tools can be used to visually identify the morphology of the intestine [312, 313]. The common markers used to assess the functionality of intestinal tissues include passive paracellular transport markers (e.g., mannitol, Lucifer yellow), transcytosis (horseradish peroxidase, HRP), passive transcellular transport markers (e.g., caffeine), and metabolic activity markers (e.g., testosterone and midazolam) [95, 286, 290, 314, 315]. There are no general probes for active transporter, selective probes for specific transporters are used. For instance, digoxin is an important clinical substrate of MDR1 and midazolam is a specific and selective probe for CYP3A [316]. Similarly, dabigatran etexilate (DABE) is a selective and sensitive probe for gut P-gp [317].

Another important factor to be considered is the quantification technique. Since the analytical methods involve very small concentrations of the test compound, the quantification method selected must have a good range quantification limit. It should not be impacted by the different components in the incubation buffer and a high recovery of the drug from the matrix sample must be ensured.

Table 7: Markers used for viability measurement and flux pathways in *ex vivo* models.

Viability/Functional marker	**Measurement type**
Chemical viability markers	
Mannitol	Passive paracellular transport
Caffeine	Passive transcellular transport
Lucifer yellow	Passive paracellular transport
Metoprolol	High permeability marker
Fluorescein	Low permeability marker
Selective probes for specific transporter	Active transport
Lactate dehydrogenase release assay	Cellular membrane damage
Electrical measurements	Integrity of intestinal barrier
Transepithelial electrical resistance	TEER Porcine Jejunum: < 100 Ω cm^2 [290]
	TEER Rat Jejunum: > 40 Ω cm^2 , 70-100 Ω cm^2 [318]
Potential difference	PD Rat small intestine: > -4 mV [319]
	PD Human small intestine: > -4 mV [283]
	PD Human Colon: ≥ -5 mV [283]
Short-circuit current	SCC Human small intestine: ≥ 100 µA/cm^2 [283]
	SCC Human colon: ≤ 120 µA/cm^2 [283]

6. *In vivo* models

The use of human subjects to evaluate the therapeutic efficacy of a drug can provide the most significant information, but the cost, time, throughput and ethical issues related to human studies greatly limit their application until the final stages of drug discovery and development. On the other hand, *in vitro* and *ex vivo* studies consider only limited aspects and thus provide only partial information regarding drug efficacy in humans. Thus, multifactorial *in vivo* studies are used because they allow experiments on intact organisms that embody the complex interplay between different physiological processes [3, 320]. Pharmacokinetics, pharmacodynamics and toxicological studies are key parameters

that are evaluated with *in vivo* studies. *In vivo* studies generate detailed information regarding the therapeutic efficacy of the tested compound and are crucial for making informed decisions for successful clinical application [321]. In addition to pharmacokinetics, *in vivo* studies provide relevant information regarding drug interactions, mechanism of action, and interaction with organ systems compared to *in vitro and ex vivo* systems that do not mimic a living biological system completely [3, 16, 108].

For *in vivo* preclinical studies, the selection of appropriate animal species that closely resemble humans in terms of anatomy and physiological condition is a critical step in the experimental design [16]. In addition, experimental setup parameters, such as sampling technique, dosage form administration and quantification techniques, are also crucial for designing reliable *in vivo* experiments [108]. The differences in animal species and different experimental parameters used for *in vivo* studies are detailed later. The ethical issues related to animal experimentation must be considered, acknowledging the principle of replacement, reduction and refinement (3Rs) investigations, as described in the EU directive 150 2010/63/EU on the protection of animals used for scientific purposes [322]. *In vivo* studies constitute a very important step of drug discovery and development; thus, the validity, reliability, reproducibility and sensitivity of these models are crucial. In this section, the authors strive to present different animal models used in the evaluation of oral drug absorption.

6.1 Animal models

In vivo models encapsulate the complex dynamics of different factors of the GIT that affect drug absorption. Animal models can be homologous (identical to humans), isomorphic (resembling a human disorder) and predictive (allowing the prediction of human disease and treatment) [108]. There is no single animal species displaying gastrointestinal physiology identical to that of humans, which increases the risk of poor predictions of human outcomes [16]. Therefore, the selection of the animal model must be done cautiously. There are a number of physiological features of the GIT influencing oral absorption, such as surface area, intestinal transporters, tight junctions, pH profile, residence time, gastrointestinal fluids, enzyme profile and gut wall metabolism. Some of the commonly used animal species used for drug absorption studies are mouse, rat, pig and dog. Since this review aims to review the *in vivo* models used for drug absorption studies, only relevant animal models are discussed. A more detailed review of this topic was published by Sjogren *et al.* [16]. Table 8 summarizes the comparison of the physiological parameters of humans with those of rat, mouse, dog and pig animal models.

In addition to selecting the species of the animal models, other factors of the animal model need to be considered, such as age, gender, and disease state [323, 324]. For example, in diabetic rats, possible

differences in the metabolic patterns and responses to induced stress were observed between males and females [325]. Similarly, when selecting animal models with human disease, careful consideration must be taken to incorporate as many disease-related features as possible, as animal models rarely completely mimic the corresponding conditions in humans [324]. The confounding effects of the disease, disease heterogeneity, and limited lifespan of animal models are other important aspects, as the obtained results may reflect either the effect of the treatment or complications associated with the disease [323]. Furthermore, to perform animal experimentation, authorization from the respective ethical committee(s) is required. This ensures that ethical considerations have been taken into account including the principle of 3Rs to minimize stress and pain [326].

6.1.1 Mouse and rat models

The most common species used for evaluating orally administered drugs are rats, representing approximately 80% of the total studies [323]. There are several differences and similarities in the gastrointestinal physiology of rats and humans (Table 8). The gastric emptying rate of liquids in rats (15-30 min) in the fasted state is somewhat similar to humans, which is a significant factor when determining drug absorption in Biopharmaceutics Classification System (BCS) class I drugs [327]. Saphier *et al.* demonstrated that commercially available capsules (length 7.18 mm) were retained in the rat stomach, whereas short capsules (3.5 and 4.8 mm in length) in the stomach were eliminated. The study also demonstrated that the rats in the fasted state retained the capsules for a longer time than rats in the fed state [328]. In general, rats are considered to be a good predictive model for oral absorption, as a high correlation between human and rat jejunal permeability has been reported by several studies. The GeneChip technique also demonstrated a high correlation for jejunal permeability ($R^2 = 0.8$) but showed only a moderate correlation for the expression levels of transporters ($R^2 = 0.56$) and no correlation for metabolic enzymes [323, 329]. In addition, the Ussing chamber studies with rat small and large intestine tissues has demonstrated rat colon a useful and predictive for the absorption of drugs from modified release dosage forms [16, 330, 331]. Despite being used as a good predictive preclinical model for GI absorption, there are several limitations associated with the rat model. First, rats are nocturnal animals, and this rhythm has greatly impacted the feeding and dosing regimen. The differences in dietary intake and the susceptibility of rats to coprophagy is also a limitation [323, 332]. Second, the low pH of the small and large intestines of rats can also greatly limit the application of evaluating drugs and their delivery systems [333]. For example, acidic drugs can precipitate in the rat intestine but not in the human intestine. The metabolism enzyme type and distribution also differ between rat and human models, for example, the expression of CYP3A4/CYP3A9 and uridine 5'-

diphospho-glucuronosyltransferase (UDPG) existed 12- to 193-fold differences between them [334], which impact the translation of the effects observed in rats to humans in a relevant manner [335]. In addition, the rat GIT differs from human GIT in aspects such as gut microflora, reuptake and fecal excretion and the absence of a gall bladder in rats [323, 332].

Although mice are among the most extensively used animal models, relevant biopharmaceutical information on the use of mouse models is limited [16]. The anatomical and physiological data of the mouse model compared to humans and other animal models are summarized in Table 8. The GIT of mice shares some similarity with the human GIT; for instance, both have finger-shaped villus morphology [336]. However, similar to rats, mice have lower pH in the small and large intestines than humans, which may have implications for evaluating the absorption of acidic drugs and oral drug delivery systems, especially pH responsive systems. The very low luminal content in the mouse GIT can greatly impact the dissolution of oral dosage forms [333]. In contrast, Escribano and coworkers demonstrated that the mouse model is an adequate tool for the evaluation of intestinal permeability, showing good predictability of absorption in the human GIT [16, 337]. Information regarding the metabolic enzymes in mice is not plentiful compared to other animal models and is mainly limited to ABC transporters and CYP450 enzymes [335, 338, 339]. Furthermore, in both rat and mouse models, one major limitation is the size of the species, which does not allow the use of intact dosage forms, such as tablets or capsules. The inability to administer final intact dosage form orally is a crucial limiting factor for screening formulations in rodents.

6.1.2 Dog model

Dogs are acceptable models for evaluating drug absorption owing to their similarity with humans in terms of anatomy, gastrointestinal motility patterns in the fasted state, residence times and secretory factors [16]. A comparison of dog models and humans based on anatomy and physiological features of the GIT is summarized in Table 8. Briefly, the stomach of dogs is anatomically similar to that of humans based on volume of stomach 0.5–1 L (living beagle), however they differ in terms of pH range and transit times (Table 8) [328]. Studies have demonstrated an unstirred water layer with similar GI thickness in both dogs and humans, which is a critical parameter for rapidly absorbed drugs [340]. Despite having differences in the small intestine, a good correlation has been established between the relative bioavailability of 11 drugs administered to dogs and the human colon [341]. Haller and coworkers studied the gene expression pattern of drug transporters in the livers and intestines of beagles [342]. The study demonstrated similarities in the expression of UGT1A6, ABCC1 (MRP1), ABCG2 (BCRP), ABCB1 (multidrug resistance mutation1, MDR1), SLC15A1 (PEPT1), and SLC22A1

(Organic cation transporter-1, OCT1). However, significant differences were observed in the case of P-gp expression in dogs and humans [342]. Furthermore, the pH profile of dogs in intestine is found to be similar to that of humans, which is crucial when determining the absorption of drug molecules whose properties are pH dependent. Nonetheless, we cannot ignore the gastric pH differences between dog and human in the fasted state, in which the pH of canine stomach was found to be to be on average higher and more variable than in humans [343]. Additionally, the colonic bioavailability of drugs given to dogs have been shown to have good correlation with those of humans ($R^2 = 0.8$) [344]. Dog models have been shown to be reasonable animal models, but there are clear species differences in regional intestinal metabolism that still need to be further evaluated. These models have been used to evaluate the *in vivo* performance of several oral formulations such as modified release multiparticulate systems [345] and nanoparticle based systems [346, 347].

6.1.3 Pig model

Pigs, especially minipigs, are considered alternative translational species for biomedical and pharmaceutical research. Pigs have used increasingly as preclinical models to assess the oral bioavailability of drug products in recent years. The principal advantage of this model is their similarities to humans in terms of their anatomical, physiological and biochemical features of the GIT [348, 349]. For instance, the pH profiles of pigs and humans are fairly comparable, with similar pH ranges and patterns throughout the GIT. This similarity is a unique advantage for this model (versus the canine model), making pig models suitable for evaluating pH-responsive drugs and drug delivery systems [350, 351]. Moreover, when the luminal surface area of the small intestine of pigs and humans were examined, considering both basic cylindrical estimates and the apical 'brush border', comparable absorptive surfaces were estimated for pigs and humans at 168–210 m^2 and 252 m^2, respectively [352, 353]. The small intestine residence time is similar between pigs and humans, but gastric emptying is slower and variable in pigs [350, 354-356]. In addition, the intestinal microbiome in the pig colon and the digestive properties of the small intestine are also considered to be similar to those of humans [357]. Thus, considering the above similarities, pigs can be considered good models for evaluating the absorption profiles of drugs mainly absorbed in the small intestine, with the potential slower gastric emptying rate taken into account. Based on previous literature and a study by Henze et al, despite of low number of studies with only 20 drugs, the porcine model demonstrates good correlation ($R^2 = 0.52$, excluding justifiable outliers) with oral bioavailability in human, which is higher than that of rat ($r^2 = 0.29$, 121 drugs) or dog ($r^2 = 0.38$, 128 drugs) [329, 353, 358]. It has been reported that CYP enzymes in pig showed high similarities with CYP enzymes in human. For instance, enzyme CYP3A4, the most

important enzyme family in man, is relatively well preserved in pigs [359]. Moreover, enzyme CYP1A1, 1A2, 2B, 2E1 and 3A in pig also have no big differences compared to humans [360]. Owing to the similarities in CYP enzymes, pigs are considered good models for studying drugs metabolized by some CYP enzymes that show a very high similarity with human [349]. On the other hand, the pigs differ from humans in metabolizing enzymes CYP2C and CYP2D. These enzymes are critical for the metabolization of 22% (CYP2C) and 12% (CYP2D) of drugs [361, 362]. There are few studies that have used porcine model to study the *in vivo* performance of oral formulations [363, 364]. Therefore, the porcine model is considered a suitable model for preclinical drug absorption studies; however, it needs further optimization for use in investigating intestinal permeability, metabolism and transporters.

Table 8: The anatomical and physiological parameters relevant to drug absorption studies of the human GIT compared to the parameters of rat, mouse, dog and pig models. (Adapted from Sjogren *et al* [16])

	Human	Mouse	Rat	Dog	Pig (Landrace)
pH range	Stomach (fasted): 1-3.5 Stomach (Fed): 3.0-6.0 Small intestine (fasted): 6.0-8.0 Small intestine (fed): 5.0-6.5 Large intestine (fasted): 5.5-8.0 Large intestine (fed): na	Stomach (fasted): 4.0 Stomach (Fed): 3.0 Small intestine (fasted): 5.0 Small intestine (fed): 4.8 Large intestine (fasted): 5.7 Large intestine (fed): 4.5	Stomach (fasted): 4.5-7 Stomach (Fed): na Small intestine (fasted): 4.5-7.5 Small intestine (fed): 3.8- 5.0 Large intestine (fasted): na Large intestine (fed): 6.6-6.9	Stomach (fasted): 1.5-6.8 Stomach (Fed): na Small intestine (fasted): 6.1-7.6 Small intestine (fed): 5.5-7.2 Large intestine (fasted): ca. 6.5 Large intestine (fed): ca. 6.5	Stomach (fasted): 1.2-4 Stomach (Fed): 4.4 Small intestine (fasted): 7-8 Small intestine (fed): 4.4-7.2 Large intestine (fasted): na Large intestine (fed): 6.6-6.1-6.6
Transit time	Stomach (fasted): 10-15 min Stomach (Fed): 0.5-3h Small intestine (fasted): 3-4h Small intestine (fed): 3-4h Large intestine (fasted): 8.0-18.0h Large intestine (fed): na	Stomach (fasted): na Stomach (Fed): 1 h Small intestine (fasted): na Small intestine (fed): 1-2h Large intestine (fasted): na Large intestine (fed): < 3h	Stomach (fasted): 15-30 min Stomach (Fed): 5.4-13.3 h Small intestine (fasted): 60-111 min Small intestine (fed): 150-180 min Large intestine (fasted): < human Large intestine (fed): < human	Stomach (fasted): 2-76 min Stomach (Fed): na Small intestine (fasted): 3-4h Small intestine (fed): na Large intestine (fasted): 10-11h Large intestine (fed): na	Stomach (fasted): 1-28 days Stomach (Fed): na Small intestine (fasted): < 1-3 days Small intestine (fed): na Large intestine (fasted): < 1-3 days Large intestine (fed): na
Length small intestine	3-5m	40.2cm	102-148cm	2.5-4.1m	17-19 cm/kg
Length large intestine	1.5m	8.3 cm	26 cm	Colon: 34-60 cm	4.3 cm/kg
Permeability values	Reference	Similar to human	Less than in humans, good correlation	Higher than human due to shorter small intestinal transit time	Less than in humans due to the slow gastric emptying

6.2. Perfusion models

The gut loop model is a simplified perfusion model. In this model, the experimental animal is anesthetized, and a section of the intestine is separated while the link to the blood circulation is maintained [107]. The intestinal segment is washed, and a loop (ca. 10 cm long) is formed by clamping, and then, a known volume of drug solution in physiological buffer is injected in the loop. At the end of the experiment, the test compound is quantified inside the loop and in the blood samples [3, 7, 284, 296]. This method enables the evaluation of the regional differences in drug absorption in the same animal, thus eliminating intervariability among the test subjects [283]. Furthermore, this simple model does not require expensive equipment and can be performed by researchers trained with *in vivo* animal experiments. However, a large number of animals are needed for this experiment compared to other models. Another major disadvantage of this model is the use of anesthesia and the absence of stirring conditions [107, 297].

The isolated intestinal perfusion model is used for drug absorption studies and has also been used as an *in situ* perfusion model [107]. Unlike the gut loop model, in this model, a 10-30-cm-long segment of the intestine is cannulated at both ends and is perfused continuously with buffer. The rat circulation can also be cannulated via the mesenteric vein and artery, which can provide information regarding the impact of hepatic clearance [34, 292, 298]. Mannitol is used as a permeability marker. This perfusion model has demonstrated good correlation with human oral bioavailability and permeability of different types of drugs [293, 299]. A major benefit of this model is the presence of blood supply, which ensures continuous tissue oxygenation and proper flow features on the serosal side. Moreover, the presence of other components, such as the enteric nerves and enteroendocrine system, gives better control of drug transport and viability. Nonetheless, the use of anesthesia has also been shown to influence drug absorption. This method is very time-consuming and requires a large number of animals, which makes this model unsuitable for screening libraries of test compounds. Several studies have also reported the loss of the drug in the systemic solution due to enzymatic degradation or adsorption onto the plastic components used [107, 259].

6.3. Experimental techniques and parameters

In vivo preclinical studies provide information regarding oral bioavailability, which is an indirect measure of intestinal permeability. In addition, these studies also generate other information, such as pharmacodynamics, imaging, pharmacokinetic profiles and toxicology. This information is key to accurately predicting pharmacokinetics parameters in humans and clinical efficacy of the tested oral

dosage forms. The reliability of these results depends on the accurate selection of the animal model and the experimental setup.

Since no animal model can replicate the human condition perfectly, the selection of a validated *in vivo* model is critical to address the clinical situation. The validation of *in vivo* models can be conducted based on different criteria, including face validity, predictive validity and target validity. Denayer *et al.* offered a detailed summary for each validation criterion of *in vivo* models [366]. In this summary, the validation procedure of *in vivo* models designed to study orally delivered drugs and delivery systems was briefly addressed. For oral drug delivery, the gastrointestinal environment (e.g., enzymes), morphology (e.g., villi) and digestive process (e.g., transit time) of animal models should be validated. Furthermore, the pathophysiological features of the disease induced in the selected model species need to be validated. Theoretically, the more similar a species is to humans, the more likely the pathophysiology of the disease induced in that model species will be similar to the disease in humans [366]. The validation studies in the selected animal models are based on a wide range of biotechnological approaches, such as histological tools, gene analysis and blood tests. In addition, evaluating the intestinal permeability and/or oral bioavailability correlation between the animal models and humans could also be considered as an intuitive validation method for the given model [323, 329]. However, validation studies comparing the intestinal permeability and/or oral bioavailability between *in vivo* animal models and humans are limited.

There are several experimental parameters that need to be considered when designing a reliable experimental setup for oral absorption studies. The administration technique, dosage volumes, sampling techniques, and analytical methods are a few of the key parameters that need to be considered [108]. The selection of the administration technique greatly depends on the type and size of the dosage forms and the experimental objective. The administration techniques used for oral dosing are oral gavage, intragastric and intestinal instillation, and syringe feeding techniques. Oral gavage is one of the most common administration techniques for orally dosing animals, and it can be used to precisely dose both solids and liquid formulations [367]. A range of gavage needles made of different materials and sizes is available from which a selection can be made based on the selected animal species and type of dosage [367]. The size of the capsules has a remarkable influence on their fate after administration [328, 368]. Positive displacement pipetting is another technique that can be used for administering powders [369]. Despite being a noninvasive process with only moderate personnel training required, the use of oral gavage is limited by a restricted dosing volume, which greatly limits its application in the initial phase. Intragastric and intestinal instillation are invasive processes that require highly experienced personnel with the skills to use anesthesia and perform

surgery. The use of anesthesia raises the risks of other confounding effects on physiological parameters such as blood glucose levels, blood pressure and cardiovascular reflexes [323]. Thus, insertion of biocompatible cannula into the intestine allows experiments in unanesthetized animals. Despite having limitations, instillations have been shown to have a high correlation with human oral bioavailability [323, 367].

The evaluation of drug transport and absorption is a component of pharmacokinetics studies, and the measures include absorption, distribution, metabolism and excretion of a compound over a time course. One of the important techniques for evaluating drug absorption is sampling blood from animal models. The tail vein is the most common site for blood samples withdrawal from rats, whereas arterial and venous blood sampling are the most common methods used for dog models [370]. The withdrawn blood samples are treated to avoid coagulation and are then analyzed using appropriate analytical techniques, such as enzyme-linked immunosorbent assay (ELISA), mass spectrometry, a reader for fluorescently labeled compounds and scintigraphy for radiolabeled samples [323]. In addition to blood sampling, intestinal tissues can also be extracted and evaluated for determining the uptake of test compounds. Evaluating intestinal tissues from different regions provides information regarding regional differences in the uptake of the test compounds.

Fluorescently labeled or radiolabeled drugs or formulations can also be used to ensure easier quantification. Nevertheless, the high fluorescence background of tissue homogenates can interfere with the final readouts, resulting in misinterpretations of the results [108]. The labeled drugs (radioisotopes, fluorophores and bioluminescence markers) also allow imaging of whole animals or excised portions to visualize the absorption behavior of the drugs throughout the GIT at different time points. Imaging also allows studying regions for determining drug dissolution, interactions with gastrointestinal components, and the extent it is released from formulations. Radioactive labeling is a noninvasive technique that can be used to visualize the drug absorption process in high definition and real-time after oral administration [371]. The exposure to radiation and instability of radioisotopes are the major drawbacks of this method. Single-photon emission computed tomography/computed tomography (SPECT-CT) and positron emission tomography (PET) are alternative high-resolution techniques that can be used to obtain 3D sensitive and high-resolution imaging, which can help reveal the specific site of drug absorption [372].

7. Correlations between *in vitro* studies, preclinical *in vivo* studies and human data

The oral bioavailability of drugs is the key parameter that determines the fate of orally delivered drugs and is primarily determined by the rate and extent of drug absorption across intestine barriers. Although the physicochemical properties of oral drugs/formulations have an impact on their intestinal transport, this process largely depends on the anatomical (e.g., intestinal mucosal layer, epithelial cells and the expression of receptors and transporters), physiological (e.g., (e.g., gastric emptying times, transit times, acid and the dynamic environment) and biochemical features (e.g., drug metabolism in intestine) of the intestine. It is impossible, impractical, and even unethical to use humans as experimental subjects without any preclinical studies due to the potential for severe, unknown and unwanted adverse effects associated with a drug/formulation. Over the last century, scientists and researchers have developed and used a wide range of preclinical tools to study the transport of drugs across intestine barriers, including *in vitro*, *ex vivo* and *in vivo* models. These models were conceived to overcome issues associated with using human subjects, such as expense, safety and ethical problems. In this review, we have reviewed the development, characterizations and applications of these models in detail. These models have been widely used to predict and/or measure the pharmacokinetic characteristics of new oral drugs/formulations. The accuracy of the prediction of clinical results is mainly determined by the degree to which these models simulate the human environment. In addition, the correlation between preclinical data obtained from these models and human data is critical to drug design and development.

The initial understanding of the ability of drugs/formulations to cross the human intestine barriers is based on *in vitro* studies. Thus, researchers have developed a wide range of *in vitro* models that link the components of human intestine environments and barriers to measure the performances of drugs/formulations to predict their efficacies in the human intestine. The utilization of these models minimizes the likelihood of lead drugs/formulations with poor PK profiles being used for costly *in vivo* preclinical and clinical research. The use of more complex *in vitro* transport models that incorporate multiple physiologically relevant factors has possibly enabled the development of better *in vitro*-*in vivo* correlation (IVIVC). IVIVC serves as a surrogate for *in vivo* pharmacokinetics, thus reducing the number of human studies needed for the development of new drugs/formulations. However, using more complex and advanced *in vitro* models correspondingly makes the process more expensive and time-consuming. Notably, since these models are primarily used as predictive tools in the development of drugs/formulations, the potential products should be validated *in vivo*.

Furthermore, each model has its own unique advantages and disadvantages, as we summarize in Tables 5. In summary, *in vitro* non-cell-based models are mainly developed for high-throughput drug studies. The relevance of the predictions based on these models to clinical trials is very limited. *In vitro* cell-based models are mostly produced by abnormal cancerous cell lines, failing to simulate all the characteristics of the healthy intestinal epithelium. The abnormal cancerous cell lines never completely behave like normal intestinal cells; thus, we cannot expect these models to have high capacity for clinical predictivity. Furthermore, the sensitivity of different cell lines to drug/formulation differs, which affects the clinical prediction accordingly. In addition, the assessment of the concentrations for the drug and excipient (e.g., proteins and lipids) adsorbed onto plastic devices (e.g., cell plates) should be considered when *in vitro* models are used to extrapolate the intestinal permeability of drugs/formulations to humans [373]. To our knowledge, there are currently no available *in vitro* transport models that can adequately replicate human intestinal physiology and environment. Therefore, choosing valid *in vitro* models for the evaluation of intestinal transport of drugs/formulations, which highly correlates with *in vivo* data in preclinical studies, even in clinical trial studies, is imminently needed. For example, Ji *et al.* developed chitosan-coated insulin-loaded nanocomposites for oral delivery [374]. In their study, considering the results obtained from *in vitro* Caco-2 monolayers, the authors found that chitosan-coated nanocomposites harmlessly opened TJs to increase the paracellular transport of insulin better than noncoated nanocomposites. The promising results gained from their study on *in vitro* Caco-2 monolayers prompted them to perform further pharmacological and pharmacokinetic studies on diabetic rats *in vivo*, where the chitosan-coated formulation exhibited a stronger hypoglycemic effect than the noncoated formulation, with increased oral bioavailability, up to 15.19% [374]. The excellent IVIVC in this study not only showed the valid application of the *in vitro* Caco-2 model but also suggested that the oral product may have the potential to be promoted into clinical phases after more follow-up trials.

Tissue-based *ex vivo* models offer a tradeoff between *in vitro* models and *in vivo* models. Since *ex vivo* models exploit living intestinal segments from animals and humans, they offer some unique advantages for predicting clinical outcomes compared with other models. The intestinal permeability data from different intestinal segments provide a strong basis for the design of final market products for human trials. In general, *ex vivo* models mostly exploit the use of tissues from different animals, with those of rats and pigs, compared to those of other animals, being the most similar to tissues of humans [12, 375]. Moreover, many studies have reported that permeability studies generated from rat or pig tissues correlate better with those obtained for human tissue [290, 376]. Importantly, the use of human intestinal tissues to directly perform transport studies eliminates the boundaries between

animal species and humans, providing good clinical predictions [105, 106, 291]. Nonetheless, the availability of healthy intestinal tissue derived from humans is very limited. Most human intestinal tissues exploited in *ex vivo* transport studies present under pathological conditions, which may lead to a large gap between experimental and true clinical data

Animal models with reliable predictability for pharmacology and toxicology are widely used in drug development and have effectively facilitated the clinical translation of drugs. Nevertheless, the overall success rate of drugs/formulations in clinical development remains low, despite a large number of drugs/formulations exhibiting good therapeutic efficacy in animal models. One obvious reason for this outcome is that the use of animal data to predict or estimate the efficacy of drugs/formulations in humans is not necessarily deterministic. When conducting clinical transformation based on animal data, special attention should be paid to differences in body size between experimental animals and humans. Due to the different metabolic requirements associated with different ratios of body weight to body volume, the extrapolation of dosage levels of animals to humans may lead to inaccurate or incorrect predictions. Another reason for the different outcomes is that preclinical animal studies usually begin with a small homogeneous laboratory animal population, such as rats and mice, while clinical studies are usually conducted in heterogeneous human populations. Therefore, in the late stages of preclinical studies, the interspecies differences in the pharmacokinetic parameters, such as metabolism of oral drugs/formulations (e.g., intestinal permeability and liver metabolism) must be carefully considered. In addition, performing pharmacological and toxicological studies in large animals that are more similar to humans is also essential.

8. Conclusion and future perspectives

The therapeutic efficacy of oral drugs is determined by a vast array of factors, from the physicochemical properties of the drug formulations to gastrointestinal barriers. A tremendous amount of research effort has been devoted to designing, characterizing and validating versatile models to evaluate the fate of orally delivered drugs in the GIT. This review details the most commonly used *in vitro*, *ex vivo* and *in vivo* models for studying the transport of drugs across intestinal barriers. Although these models have made great contributions to the development of oral drugs/formulations, the intrinsic defects of these models have led to limited and/or unreliable predictions in clinical studies.

The models in the intestinal transport studies were originally developed to accelerate the development, improvement and clinical translation of orally delivered products. To achieve this goal, current research must be focused on improving current models and/or creating novel biomimetic models. As models continue to evolve, more information about the tested drug/formulation will emerge during the preclinical evaluation, thus improving the decision-making process. In addition, the proper selection of validated models and experimental techniques is also crucial. Another important design challenge, which is often easy to ignore, involves the development of models that are simple and practical. Overall, it is quite clear that the data obtained from the preclinical models greatly impact the development and market translation of oral drugs/formulations. These important issues have been emphasized and discussed in this review.

In addition to the use of classic models (*in vitro*, *ex vivo* and *in vivo*), the emergence of *in silico* models has promoted the development of techniques in the study of drugs/formulation crossing intestinal barriers. *In silico* models use heavy computational resources to provide information on drug/formulation intestinal transport. They can accurately predict the transport mechanism of drugs (e.g., passive diffusion and drug-receptor interaction) by using molecular modeling or quantitative structure–activity relationship approaches or predict the behavior of drugs in intestinal tissues and/or systemic circulation via physiology-based pharmacokinetics models. Considering the importance of *in silico* models in finding drugs that cross intestinal barriers, their use for in-depth study will be a new trend for model development and improvement in the future.

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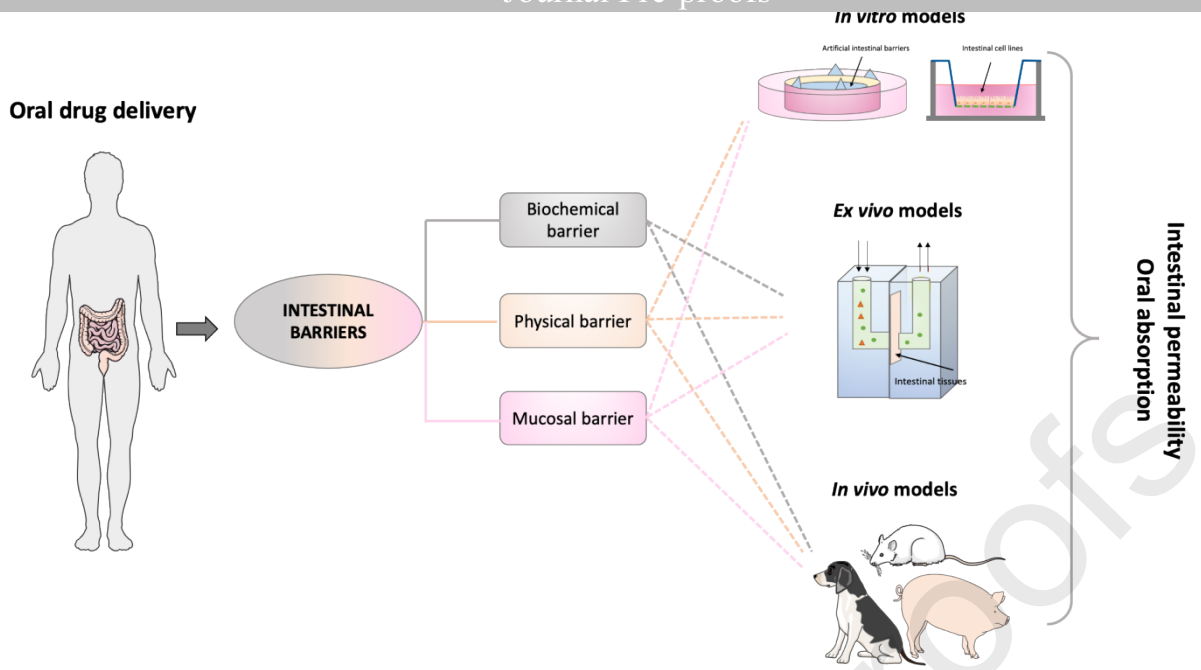

Highlights:

- Oral absorption depends on drug, formulation and gastrointestinal tract
- Different preclinical models are used to evaluate the intestinal drug transport
- Preclinical models greatly impact development and translation of oral formulations
- Advanced preclinical models with better correlation to clinical data are needed