Models for drug absorption from the small intestine: where are we and where are we going?

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Teaser: We offer a critical analysis of the most widely used absorption models and the current trends in the development of new efficient and relevant models.

The small intestine is a complex organ with movements, flora, mucus and flows. Despite this, the most widely used absorption models consider the organ a cylindrical monoepithelial tube. This review presents the recent evolution of models to take into consideration the complex nature of gut physiology. The most commonly encountered issues are ethical (*in vivo* models) and differences in drug transport as a result of a modified expression of drug transporters or metabolic enzymes compared with human (*in vitro* and *in vivo* models). Finally, this review discusses the way forward to reach an ideal equilibrium between reproducibility, predictability and efficiency for predicting permeability. The features of an ideal model are listed as a guideline for future development.

Glossary

ABC: ATP-binding cassette

API: active pharmaceutical ingredient

BCS: biopharmaceutical classification system

CAT: compartmental absorption and transit model

CYP: cytochrome P450

DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

EGF: epithelium growth factor

FAE: human follicle-associated epithelium

GST: glutathione S-transferase

MATE: multidrug and toxic compound extrusion transporters

MD: molecular dynamics

MDCK: Madin–Darby canine kidney

MGS: metagenomic shotgun sequencing

MM: molecular modeling

NAT: *N*-acetyltransferase

OAT: organic anions transporters

OATP: organic anions transporting polypeptides

OCT: organic cations transporters

OCTN: zwitterion transporters

P-gp: P-glycoprotein

PAMPA: parallel artificial membrane

PB-PK: physiology-based pharmacokinetics

PEPT: peptide transporters

PVPA: phospholipid vesicle-based permeation assay

QSAR: quantitative structure–activity relationship

Ro5: Rule of Five

SLC: solute carrier family

SULT: sulfotransferase

TEER: transepithelial electrical resistance

TMD: transmembrane domains

UGT: UDP-glucuronosyltransferase

Highlights:

- It is of primary importance to obtain relevant models in drug discovery
- Physiology and biology of the oral route is complex to model
- *In silico* models allow high-throughput screening but can be less relevant
- *In vivo* models raise ethical issues
- *In vitro* complex models are a good compromise between relevancy and throughput

Introduction

The oral route is the most common and practical way to administer drugs to the body; even if certain problems remain, especially for anticancer agents [1]. Unfortunately, not all drugs are good candidates for oral administration. The Biopharmaceutical Classification System (BCS) proposed by Amidon *et al.* in 1995 [2] shows that solubility and permeability can be used to determine whether a drug is a good candidate for oral administration. Similarly, the Rule of Five (Ro5; see Glossary for list of abbreviations used in this review) proposed by Lipinski *et al.* in 1997 [3] is also a quick way to assess the suitability of a drug for the oral route. The BCS and Ro5 are related to chemical properties of drugs. They are ways to predict a good absorption process, helping to reach a better bioavailability of the drug and often a low interpatient variability, which means reliability. These positive features are coined in the term drugability, which reflects the fact that the drug is a good candidate for the oral route. Then, the active drug must be formulated to obtain an oral dosage form. In the early stages of drug development, *in vitro* and/or *in vivo* models are extensively used to determine the best formulation of the drug product. Ideally, those models must be easy to implement, relevant, simple, cost effective, accurate and compatible with high-throughput screening. Some of those features are difficult to obtain altogether. As a matter of fact, the complexity of the absorption process makes it impossible for the models to be relevant and to remain simple. These models must also be suitable to assess the absorption of new formulations such as nanomedicines. Besides predicting the extent of drugs absorbed, models are also used to explore the very different barriers to cross and the complex mechanisms of this transport process. Models are also used to study the stability

and the behavior of the formulation. A lot is therefore expected of the absorption models, which is why many techniques are used to construct these models from *in silico* models, based on mathematical analysis and on chemistry properties, to *in vivo* models, often based on molecular imaging. *In vitro* models based on cell cultures are a good compromise between simplicity and relevance and are therefore widely used. In the present study, using the physiology and molecular biology of the gastrointestinal tract as a starting point, we would like to propose a critical analysis of the most widely used *in vivo*, *in vitro* and dynamic absorption models. Subsequently, the current trends in the development of new, efficient and relevant models will be explored to propose the crucial points to consider in the way of innovation in this field. The features of the ideal model to study drug absorption will be presented as a conclusion to this work.

Where are we?

Physiology and pharmacology of the small intestine

Physiology and motility of the small intestine. The main drug absorption steps occur in the small intestine. This organ is a complex tube that can be divided into three different parts with different absorptive capabilities. The first part, the duodenum, is 25–30 cm long and the passage of the drugs through this part is relatively quick, resulting in a poor net absorption of drugs. The second part is the jejunum. Unlike the duodenum, the jejunum is characterized by a highly active peristalsis, favoring absorption. Finally, drugs that have not been absorbed in the jejunum might be absorbed in the ileum (given that the site of absorption mainly depends on the physicochemical properties of the drug). The ileum reveals fewer villi than the jejunum but has a similar absorption capability. Mucus is another fundamental part of the gastrointestinal tract, acting as a mechanical and physical barrier, but also performing the role of a niche for the microbial cells. Microbiotic flora are an essential part of the gut. It is now clear that the gut microbiome plays a key part in digestion but also in the production of enzymes and vitamins and in the regulation of the immune system. Despite the increasing interest in the topic (the number of papers on Pubmed with the keyword ‘microbiota’ reached 5028 in 2015 versus 245 10 years earlier), the microbiota, its metabolic activities and its interactions with the digestive epithelium are still not fully understood. In each part of the intestinal tube, the commensal flora composition

and the mucus bilayer show a high variability. The main characteristics of the small intestine are summarized in Table 1.

Impact of drug transporters. Drug absorption is mainly subject to significant transporters, even if some drugs can be absorbed by passive diffusion, and to the effects of metabolism. The fate of these drugs is represented schematically in Figure 1a. The paracellular transport, which is related to molecules below 3.6 Å or 250 Da, occurs through tight junctions between epithelial cells along the intestinal mucosa and shows an important intra- and inter-individual variability [4]. However, it has been previously shown that bigger structures such as nanoparticles can cross the tight junction structures [5]. Nevertheless, several current drug classes cross the epithelial cells by transcellular transport, either by passive transcellular transport (only small lipophilic drugs) and/or by carrier-mediated transport (antivirals, penicillins, statins, etc.) [6].

Role of transporters in the absorption of drugs. Transporters are usually classed into two categories. The first category is the solute carrier (SLC) family, which mediates the uptake of drugs. The ATP-binding cassette (ABC) family is the second category and gathers efflux transporters (Figure 1b). Most of the SLC transporters are secondary active transporters, for which transport is driven by various energy-coupling mechanisms [7]. This category is divided into the SLCO superfamily (former SLC21), which gathers the organic anions transporting polypeptides (OATP), the SLC22 superfamily, which gathers organic cation transporters (OCT) and zwitterion transporters (OCTN), the SLC47 superfamily, which gathers the nucleotide transporters (ENT and CNT), and peptide transporters (PEPT).

- Few members of the SLCO superfamily are found in the intestine. Although OATP2A1 and OATP4A1 are ubiquitous and transport mainly prostaglandins and bile salts, respectively, only OATP1A2 can have a role in transporting drugs. Situated at the apical side of the enterocytes, its substrates are bile salts, thyroid hormones and xenobiotics (antibiotics, anticancer drugs, antifungals, β-blockers, statins).
- In the SLC22 superfamily, all the members share a common structure: 12 α-helical transmembrane domains (TMDs). This family actively participates in small intestinal absorption but also in hepatic and renal excretion of drugs. In the intestine, the most common SLC22 transporters are OCT1, OCT2, OCT3, OCTN1, OCTN2 and Octn3.

153 - OCT1 is a well-known transporter of metformin, quinidine and type 1 cations
154 (such as dopamine, choline and *N*1-methylnicotinamide) by sodium-independent
155 transport. However, its location in enterocytes is still unclear [8,9]. OCT3
156 mediates the uptake of histamine, epinephrine and norepinephrine and cationic
157 drugs together with OCT1 in the intestine.
158 - The OCTN family includes three transporters. OCTN1 and OCTN2 have been
159 found in humans, whereas Octn3 has been found only in mice. OCTN1 and 2
160 uptake organic cations and zwitterions by Na^+-dependent or -independent
161 transport. The most common substrates are oxaliplatin, gabapentin, verapamil,
162 doxorubicin and quinine for OCTN1 and oxaliplatin, ipratropium and tiotropium
163 for OCTN2.

164 • Nucleoside transporters are a major concern in the development of anticancer and
165 antiviral drugs because they transport nucleosides and a large variety of nucleoside-
166 derived drugs. Three transporters of this family are commonly studied in the intestine:
167 CNT1, ENT1 and ENT2. Like the nucleoside CNT1 transporter, located in the apical
168 membranes of polar cells, ENT1 transporters are located predominantly on the apical side,
169 whereas ENT2 is present on the apical and basolateral sides in Caco-2 cells.

170 • PEPT1 encoded by the *SLC15A1* gene is responsible for the influx of di- and tri-peptides
171 in enterocytes. It can also transport peptide-like drugs (i.e., angiotensin-converting
172 enzyme inhibitors, β-lactam antibiotics) and drugs coupled to amino acids (i.e.,
173 valganciclovir or valacyclovir).

174 Once inside the cell the drug must be transported to the basolateral side to reach the blood
175 circulation. In parallel, some transporters can also efflux drugs on the apical side, thus regulating
176 the intracellular concentration of xenobiotics and decreasing the absorption rate. Efflux
177 transporters are ATP-dependent pumps and are responsible for a wide number of drugs and/or
178 metabolite transport. To date, there are seven subfamilies of ABC gathering 51 transporters.
179 Among those transporters, four are responsible for the elimination of drugs from cells into the
180 lumen: P-glycoprotein (P-gp), MDR2/3, MRP2 and breast cancer resistance protein (BCRP).
181 These transporters reduce the uptake of their substrates and are located at the apical side of the
182 enterocyte. By contrast, five transporters are responsible for the efflux of the drugs toward the

blood and the liver: MRP1, MRP3, MRP4, MRP5 and MRP6. They are preferentially located at the basolateral side of the enterocyte.

- The *ABCB1* gene encodes for P-gp, the most well-known efflux pump. It pumps the xenobiotics from the cell back into the lumen. Current recommendations for testing MDR1 during drug development are based on its role in intestinal absorption. Moreover, P-gp has a role in modulating CYP3A4 expression, thus contributing to pharmacological resistance [10].
- The *ABCB4* gene encodes for the MDR2/3 protein. Smith *et al*. reported an increased directional transport of several MDR1 P-gp substrates, such as digoxin, paclitaxel and vinblastine, through cells expressing *ABCB4* [11].
- The *ABCC2* gene encodes the MRP2 efflux protein. MRP2 is mainly located in the liver, in kidney and intestine, supporting a major function in the elimination and detoxification of xenobiotics, and particularly glutathione conjugates.
- BCRP exhibits broad substrate specificity with a considerable substrate overlap with ABCC1 and ABCB1. BCRP is highly expressed in the small intestine, colon, blood–brain barrier, placenta and liver.
- *ABCC1* and *ABCC3* encode for two basolateral efflux transporters. The main roles of these transporters are the efflux of xenobiotic and endogenous metabolites and the transport of inflammatory mediators.
- *ABCC4* and *ABCC5* encode for MRP4 and MRP5, which are ubiquitous efflux transporters. They transport mainly nucleotide analogs such as antivirals and anticancer drugs.
- Not much is known about *ABCC6.* This gene is expressed in the duodenum, colon and liver, but its substrates (endogenous and exogenous) are not known.

Obtaining an exhaustive list of substrates for each transporter would be worthwhile, but laborious. However, transporters seem to be more class-specific rather than drug-specific. As such, drugs are commonly grouped into classes with similar physicochemical properties, which renders the screening of hypothetical drug transporters easier (Table 2).

Impact of intracellular and microflora metabolism. Drug metabolism takes place in the intracellular milieu and depends on two classes of enzymes. Phase I enzymes are responsible for the functionalization of the drugs (i.e., hydroxylation, amination, etc.). Phase II enzymes tend to conjugate the metabolites by glycosylation, glucuronidation, transmethylation or acetylation and sulfoconjugation (Figure 1c). Phase I enzymes are cytochromes. Owing to their broad specificity, high abundance in the intestine and powerful capacity for oxidizing xenobiotics, cytochrome P450 (CYP) proteins are the most often studied. CYP3A and CYP2C represent the major intestinal CYPs, accounting for ~80% and ~18%, respectively, of total immunoquantified CYPs [12]. Phase II metabolism implies UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), *N*-acetyltransferases (NATs) and sulfotransferases (SULTs).

UGTs are a superfamily of enzymes that catalyze the glucuronidation of endogenous and exogenous molecules. Among the 21 different UGT proteins that have been identified in humans, ten are expressed in the small intestine: UGT1A1, 1A3, 1A4, 1A6, 1A10, 2B4, 2B7, 2B10, 2B11 and 2B15 [13]. Each enzyme encoded by a UGT gene reveals a unique but usually overlapping substrate specificity, tissue localization and regulation [14]. Human UGT1A1 is the most highly expressed UGT in the small intestine, with activities even greater than in the liver [15]. UGT1A enzymes conjugate endogenous and exogenous substrates.

GSTs occur in three cellular compartments and can be divided into cytosolic GSTs, mitochondrial GSTs and microsomal GSTs. The most expressed GSTs in the human enterocytes are GSTP1, GSTA1 and GSTA2 [16]. GSTA1 and GSTA2 catalyze the conjugation of glutathione with electrophiles whereas GSTP1 inactivates toxic and carcinogenic compounds by conjugation of glutathione. Most studies focus on the role of GST in pathogenic, mainly cancerous, tissue. Although GSTs are found in enterocytes, their behavior in this tissue has been poorly explored. NAT1 and NAT2 can be found in the intestine. They catalyze the acetyl conjugation from acetyl-CoA to various arylamine and hydrazine substrates. There is an increasing interest in SULTs, because they seem to contribute significantly to drug clearance [17]. They catalyze the transfer of a sulfate group to several pharmacologically important endo- and xeno-biotics.

The human microflora contains obligate anaerobes (i.e., *Bacteriodes, Clostridium, Lactobaccillus, Bifidobacterium, Eschericia*), together with a variety of yeasts and other

244 microorganisms, forming a complex ecosystem of thousands of species. About 90% of the
245 intestinal microbiota is composed of the *Bacteroides* and *Firmicutes* (i.e., *Clostridium*, *Bacillus*)
246 families. The microbiota can metabolize drugs, thus decreasing or favoring the absorption of
247 drugs and/or their metabolites. Although most of these mechanisms remain unknown, this
248 knowledge is crucial for new drug development. For example, some studies have revealed that
249 the microflora mediates the reduction [18] and hydrolysis [19] of drugs, but also the removal of
250 succinate groups, dihydroxylation, (de)acetylation, proteolysis, (de)conjugation and *N*-
251 demethylation [20], thus activating prodrugs such as lovastatin or inactivating drugs such as
252 digoxin [21,22]. In parallel, flora can influence cell behavior, for example by increasing the
253 activity of CYP phase II enzymes in the gut or even in the liver [23,24]. To illustrate, in germ-
254 free mice, mRNA of Cyp3a has decreased by ~87% compared with control mice with normal
255 flora, GSTs from 32% to 66% and SULTs from 52% to 68%, thus leading to a significant
256 decrease in drug metabolism [25]. It is clear that flora can significantly contribute to drug
257 metabolism in beneficial and deleterious ways. Some bacteria can also reduce or induce drug-
258 related toxicity. For example, bacteria expressing β-glucuronidase (i.e., *Escherichia coli*) increase
259 significantly the number of ulcers in mice receiving nonsteroidal anti-inflammatory drugs
260 (NSAIDs) [26,27] and enhance the formation of toxic compounds in humans undergoing
261 chemotherapy [28]. The development of new-generation sequencing techniques to replace the
262 classical culture techniques, which failed at identifying most of the species, makes it possible to
263 study the minor species that can also have a role in drug absorption and metabolism. Currently,
264 microbiotic flora are explored by targeting the highly conserved 16S ribosomal RNA gene
265 sequences or metagenomic shotgun sequencing (MGS) [29]. Combining the modern, highly
266 sensitive analytical approaches with 16S rRNA and metagenomic data makes it possible to detect
267 and quantify the metabolites that are derived or modified from the gut microbiota and to identify
268 the species involved.
269 At this stage, it is easy to understand that drug absorption depends on several steps: reaching the
270 apical side of the enterocyte, transport by several different proteins and metabolism by many
271 more of enzymes before reaching the liver. Such a complex pathway involves risks relating to
272 drug–drug interactions or even food–drug interactions, for example when the transporters or
273 enzymes are saturated, thus rendering the accurate prediction of drug absorption impossible

among individuals. Drug permeability could also be affected by genetic polymorphisms among previous genes and disease states.

Current models

In vivo *models*. *In vivo* models are of great interest because currently these are the only models to potentially contain all the physiological parameters. Among these models, intestinal perfusion is the most common experiment used to study the *in vivo* drug permeability and intestinal metabolism in different regions of the intestine.

- Animal intestinal perfusion. Briefly, intestinal perfusion consists of: (i) exposing the small intestine and ligating the part of interest for perfusion; (ii) rinsing the cannulated segment; (iii) perfusing the solution of interest and collecting the perfusate. Figure 2 illustrates the *in vivo* perfusion system. In this system, the mesenteric vein is cannulated and blood is collected. During this experiment, special care should be taken to maintain an intact blood supply. Finally, the animal must be euthanized. Another variation is the closed loop model. In this model, the gut remains in the animal and each extremity of the part of interest is ligatured. The drug is then injected in the isolated part. At the end of the experiment, drug concentration is measured inside the gut and the absorption rate is deducted from the initial concentration. It presents several advantages over the previous method, such as the exploration of the effects of inhibitors or drug interactions, and can be used to study drugs that are poorly permeable. Because the variation in the quantity is extremely insignificant, this method requires an analytical method with a very low limit of quantification.

  Although the permeability of passively absorbed drugs correlates well with human data [31], this is not as clear for drugs absorbed by active transport. To specifically study the impact of a transporter, however, several knockout models have been developed in recent years. The first model was developed by Schinkel *et al*. using mice without P-gp ($Mdr1a^{-/-}$) [32]. Subsequently, several other models appeared, such as $Bcrp1^{-/-}$ mice [33] and $Oct1^{-/-}$ mice [34]. Despite this attempt, it has been observed that genes of the same family of the knocked-out gene are overexpressed in a mechanism of compensation, thus obtaining puzzling results [35]. To conclude, this method is relatively easy to set up, quick and cheap, which in part explains its notoriety.

- Loc-I-Gut™. A method to determine intestinal permeability in humans has been developed by Lennernäs *et al.* [36]. This system is based on a jejunal perfusion system composed of a multichannel tube with two inflatable balloons (Loc-I-Gut™). The obtained permeability results can be further used as a gold standard to compare different models. However, permeability studies using volunteers are limited because of ethical issues and cost. Nevertheless, this is perhaps the most realistic and predictive permeability model ever developed.

- Pharmacokinetic study. Individuals take the drug orally and venous blood is sampled at different times post-dose. Although it seems like one of the easiest, most reliable and simplest methods to study drug absorption, a pharmacokinetic study can have several drawbacks. The observed drug concentrations are extremely variable – inter-individually and intra-individually. This can be partially explained by different rates of gastric emptying, differences in gut and liver metabolism, differences in transporter expression and in elimination. A pharmacokinetic study of population can overcome these variabilities but would involve a large number of individuals.

Although drugs absorbed by passive transport show a good correlation between animals and humans [37], there are huge discrepancies between the different animal models and humans in terms of metabolism, drug transport, flora and of course the surface area of the gastrointestinal tract. For example, while using intestinal perfusion for five passively transported drugs in humans, the apparent permeability was 3.6-times higher than observed in rats but results were similar to those of mice [38]. Moreover, although moderate correlation ($r^2 > 0.56$) was found in the expression levels of transporters in the duodenum of humans and rats, no correlation was found in the expression of metabolizing enzymes between the rat and human intestine [31]. This explains why a reliable scaling from animal models to humans is often absent. Another common limitation of the *in vivo* approach is that it is not suitable for high-throughput screening, it presents a low sensibility and recovery of drugs, which makes analysis by mass spectrometry indispensable. With regard to these advantages and drawbacks, these models might be

preferentially used to study actively transported drugs. Drugs that are absorbed passively can be studied using less expensive and simpler models, such as *ex vivo* and *in vitro* models.

Ex vivo *and* in vitro *models.* As in the case of the above-mentioned *in vivo* models, everted intestinal sac techniques are used to determine drug permeability. The intestine is removed from dead animals and cut into small segments, also making it possible to evaluate the permeability in different parts of the intestine. These segments are sutured at one end, filled with a drug solution, sutured at the other end and immersed in an oxygenated medium at 37°C. This model presents the advantage of a relatively large surface for permeability and the presence of a mucus layer. However, one limiting parameter of this model is tissue viability [39]. Moreover, no *in vivo* correlation has been established through this method but results from everted intestinal sac models have been consistent with *in vivo* findings [40]. As for *in vivo* methods, the major drawback is the poor screening rate. Nevertheless, these models should be preferred to *in vivo* models if anesthetic drugs might interfere with the analysis.

Cellular models have been widely used to study drug permeability in the small intestine. Although most cellular models are simple monocellular layers, complex models have also been developed, for example in including a liver-like compartment composed of microsomes [30]. First, Caco-2 cells represent the reference model in the prediction of drug permeability and are routinely used for studying enterocyte transepithelial drug transport for the passive transcellular route, paracellular route, carrier-mediated route and transcytosis [41]. Caco-2 cell lines, derived from a human colorectal carcinoma, are cultivated on semipermeable filters (Transwell® system) for 21–23 days. After differentiation, the cells form a polarized monolayer with apical and basolateral sides displaying a brush border, microvilli and tight junctions, and expressing P-gp and several relevant efflux transporters and enzymes [42]. Subclones of Caco-2 cells (TC-7) are also used and have different levels of transporters and enzyme expressions, closer to human levels than classic Caco-2, are also used [43]. Although a good correlation between *in vitro* permeability (P_{app}) of drugs and their *in vivo* bioavailability was found [44,45], the Caco-2 cell model is not perfect. Indeed, paracellular transport is lower than the *in vivo* permeability [41]. Consequently, alternative cells were put forward [IEC-18 (rat small intestine cells) and 2/4/A1 (fetal rat intestine cells), which display higher paracellular permeability]; however these cells contain few carrier-mediated transport systems compared with Caco-2 cells [46,47]. Moreover,

Caco-2 cells express a low amount of CYP3A enzymes, which are major metabolizing enzymes for many drugs. In this way, CYP3A4-transfected Caco-2 cells with higher levels of CYP3A4 are developed [48]. Moreover, other disadvantages of this model include the long differentiation period, the wide variation with passage number of cells and the inter- and intra-laboratory variability. As a result, two other cell models are also used for permeability transport: Madin–Darby canine kidney (MDCK) I and II derive from canine kidney cells; and Lewis lung carcinoma-porcine kidney 1 (LLCPK1) derived from pig kidney epithelial cell line. MDCK cells exhibit a shorter culture time (3–5 days) and lower transepithelial electrical resistance (TEER) values compared with Caco-2 cells (MDCK values are much closer to the *in vivo* TEER of the small intestine). The MDCK model also presents polarized cells, with brush border and tight junctions, but this model expresses some transporters, such as P-gp, with lower levels compared with Caco-2 cells [49]. Nevertheless, MDCK cells transfected with the human *MDR1* gene (MDCK II) have been developed to express P-gp.

Another limitation of these cellular models is the absence of a mucus layer and M cells, which also constitute the intestinal barrier. In this way, co-culture models comprising different cells have been proposed to represent the heterogeneity of the intestinal epithelium. First, HT-29-H or HT-29-MTX cells that are mucus-secreting cells have been co-cultivated with Caco-2 cells. Different methods of culture (cell culture time, Caco-2/HT-29 ratio, culture medium, time of HT-29 addition, etc.) have been developed, and representative models in terms of mucus layer, P-gp-mediated efflux expression and paracellular permeability have been obtained [44–46]. Moreover, these co-culture models make it possible to evaluate absorption enhancers and mucoadhesive systems on the permeability of drugs [42]. Moreover, Raji cells have been added to Caco-2 cells to take into account human follicle-associated epithelium (FAE), which represents less than 1% of the total intestinal surface but shows an impressive propensity to transcytose small inert particles such as nanoparticles and large molecules from the lumen to the lymphocytes. Finally, triple co-culture cell models with Caco-2, HT-29 and Raji B cells have been put forward to obtain a more physiological, functional and reproducible *in vitro* model [54,55]. Antunes *et al.* demonstrated that this triple co-culture cell model is the most efficient model in predicting insulin permeability, as compared with permeability values obtained from *ex vivo* experiments [54].

To enhance the relevance of models other modifications have been put forward to improve correlations between absorbed drugs *in vitro* and *in vivo*. For example, 3D *in vitro* models have

been proposed to reproduce intestinal villi [56] and epithelial–stromal interactions [57]. Moreover, studies have been performed with simulated gastrointestinal fluid apposed on the apical side to mimic luminal conditions in the gastrointestinal tract, but no effect of simulated media was demonstrated as compared with a classical medium [58,59]. The main characteristics of each model are summarized in Table 3. From these data, two cellular models seem of great interest owing to their similarity with human permeability. First, the Caco-2 cell clone TC-7 improves significantly the initial Caco-2 model in terms of transporter expression and metabolism, but still shows important inter- and intra-laboratory variations. Second, the 2/4/A1 model demonstrates the potential to make reliable permeability predictions – even more reliable than Caco-2 TC-7 cells. Unfortunately, there are scarce data available concerning this model. Moreover, Caco-2 TC-7 cells are easier to grow than 2/4/A1 cells, which require an overexpression of the antiapoptotic protein Bcl-2 to be maintained in culture. Moreover, passive transport is more suitable for IEC-18 cells than Caco-2 cells, whereas no carrier transport can be determined in IEC-18 in comparison to Caco-2 cells [46]. To date, no *in vivo* correlation with the IEC-18 cell model has been published.

Consequently, although all of these cellular models show good or moderate correlations with human passively absorbed drug permeability, correlations with actively transported drugs are variable and mainly low. This lower active transport obtained with the Caco-2 cell models could be explained either by the underexpression of carrier-mediated transporters in Caco-2 cells when compared to *in vivo* or by the saturation of the carriers [60]. However, even if *in vivo* correlation is slow in the Caco-2 cells, this is an interesting model to determine the drug transport mechanism, and identifying the relevant carrier used and active transport mechanism need to be extensively studied [61]. With regard to these advantages and drawbacks, these models might be used to study several passively transported drugs and to predict, in some but not all cases, carrier-mediated transport of drugs.

Finally, because drug permeability can also be related to passive diffusion, one static model, parallel artificial membrane (PAMPA), was used to study this transport. This model involves adding a mixture of phospholipids and organic solvent onto a porous hydrophobic filter support to form a lipid membrane. There are different types of PAMPA models based on the nature of the filter, lipids and transport media used [62]. Several factors influence PAMPA permeability performance, such as incubation temperature, pH conditions and lipid membrane composition.

Indeed, the lipids in these PAMPA models were mainly commercially available lipids or extracted from natural tissues. Different lipid compositions are now available: PC-PAMPA (phosphatidylcholine-PAMPA), DS-PAMPA (a dodecane solution of lecithin mixture-PAMPA), DOPC-PAMPA [a dodecane solution of highly purified dioleyoylphosphatidylcholine (DOPC)-PAMPA], HDM PAMP (a hexadecane solution of DOPC-PAMPA) and BML-PAMP (a biomimetic lipid membrane based on a mixture of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, cholesterol, 1,7-octadiene membrane-PAMPA) [63]. Moreover, with cell models cultivated on the Transwell® system, a filter separates one side containing the test molecule (donor side) from a receiver side (initially free of the molecule). PAMPA is a high-throughput screening technique, but makes it possible to study only passive permeability. A comparison of DS-PAMPA with a rat *in situ* close-loop technique showed an acceptable correlation (r^2 = 0.87) for 17 fluoroquinolone drugs [64]. Similarly, at pH 5.5 or 6.5, BML-PAMPA also demonstrated an acceptable correlation (r^2 = 0.86) for more than 25 compounds [65]. Moreover, passively transported compounds, other than acidic compounds, also demonstrate a good permeability on HDM-PAMPA at pH 7.4 [66].

Nevertheless, this model presents limited effectiveness and applicability owing to the absence of stirring conditions, the presence of solvent and the difficulty to reach sink conditions during transport studies. Consequently, other artificial models are developed to overcome these disadvantages. The phospholipid vesicle-based permeation assay (PVPA) is another artificial membrane model. This model consists of a deposition of liposomes on a filter [67]. Like the PAMPA model, PVPA can be used to study the transport of passive drugs and, owing to an excellent correlation with data obtained for the Caco-2 cell model, it represents a valuable alternative to cell models [68]. As such, even though a good *in vivo* correlation was obtained with artificial models (PAMPA and PVPA), these models can only be used for drugs passively transported, compared with cell models that make it possible to study all types of drug transport. *In silico* approaches (see below) can now simulate pure passive transport, thus limiting the interest in PAMPAs.

Dynamic models. Dynamic models have been put forward to avoid the limitations of static models and potentially enhance correlation with *in vivo* studies. As such, Caco-2 cells have been cultivated on permeable filters, as previously described. Subsequently, filters were mounted into

diffusion systems, such as the Ussing chamber [69] or a multicompartment model (membrane bioreactor) to simulate flow-mediated transport through the biological membrane [70]. For dynamic artificial membrane models, an impregnated membrane with a lipid mixture is inserted into a diffusion cell connected to a donor and receiver compartments, where liquid circulation is maintained using a peristaltic pump. This dynamic artificial membrane demonstrated an excellent correlation ($r^2 = 0.95$) with permeability data in humans for highly absorbed hydrophobic drugs [71]. Of course, such systems are not useful when studying actively transported drugs.

The Ussing chamber technique was also used with excised human or animal intestinal segments. Similarly, there are two diffusion compartments between the intestinal segments. The diffusion chambers can be filled with a physiological buffer solution (Krebs-ringer-bicarbonate buffer), which can also contain glucose, glutamate, fumarate, or with a simulated intestinal fluid (into the donor chamber). The system is kept at 37°C and the solution is constantly gassed with oxygen and carbon dioxide to maintain tissue viability and to create a fluid movement [69]. This model provides a good prediction for intestinal drug permeability, interaction with efflux transporters and drug metabolism [72]. Moreover, using this model, permeability could be determined at different parts of the intestine (jejunum, ileum and colon). Indeed, depending on the part of the intestine used and the origin tissue, *in vivo* correlation might be different. Comparison from *in vivo* and excised rat jejunal segments showed a high correlation ($r^2 = 0.95$) for drugs transported by passive diffusion with high or low permeability, whereas drugs transported via carriers displayed certain differences [73].

However, this model reveals several drawbacks: it shows a relatively low throughput and recovery, and it requires several human or animal biopsies that: (i) are difficult to obtain (for human samples); (ii) present important intraindividual or interspecies variabilities (for animal versus human) thus contributing to a poor reproducibility [74]; (iii) have a viability beyond 2 h; and (iv) decrease the functionality of their active transporters. Unfortunately, there is no comparison between these models and the Loc-I-Gut™ model. Although they seem easier to set up, present fewer ethical questions and can be used to test more drug than the *in vivo* methods, they lose several physiological aspects partially (i.e., mucus, active transport, flux and microbiotic flora) or totally (i.e., chyme and blood environment and peristalsis). Nevertheless, despite these issues, the model is, undoubtedly, after the Loc-I-Gut™ approach, the current most reliable model to simultaneously study passive and active drug absorption in animals and in

humans.

Another interesting dynamic model is the TIM gastrointestinal model™ (TNO). The TIM system, originally developed for food digestion research, is a kind of ancestor of the body-on-a-chip approach [75]. It reveals several compartments mimicking the stomach, the small intestine and the large intestine. Moreover, some other important physiological parameters are either integrated (such as body temperature, peristaltic movements) or can be parameterized (such as acidity, enzymes, bile salt, etc.). These interesting features make the TIM system very interesting for studying gastric digestion and absorption. Nevertheless, the intestinal barrier is made of a semipermeable membrane, which makes it impossible to reproduce *in vivo* processes such as active transport and intestinal metabolism. Other drawbacks remain: the model is large and complex in its use, despite the development of a simplified tiny-TIM (which merges the duodenum, jejunum and ileum into a single compartment).

Where are we going?

In silico *models*

In silico approaches are extremely appealing mainly because they require less living material, consumable and personal material than classical approaches. However, they also require heavy computational resources for the different simulations, which are directly related to the accuracy of the desired model. Among *in silico* models, two classes emerge. The first group of mechanistic models focuses on interactions between drugs and their receptor, transporter or direct environment, through molecular modeling (MM) or QSAR approaches. A second group of models, the physiology-based pharmacokinetics (PB-PK) models, integrates the behavior of a drug in different physiological compartments and pharmacokinetics modeling. At this stage, one can easily understand that the choice of the model depends on the objective of the study – mechanistic models are more suitable for exploration at a small scale (i.e., passive diffusion or active transport, drug–drug interactions, etc.) and PB-PK models are more suitable for explorations on a bigger scale (behavior of the drug in tissues, organs or systems).

Molecular dynamics. MM is a tool that describes the position of the particles in a system using classical physics. Briefly, MM considers a molecule to be a complex structure made of atoms, (considered as balls) and bonds (considered as springs). To obtain an accurate model, the

parameter determination must be based on results obtained from experiments or calculated by high-level quantum methods. High-level quantum methods refer to quantic chemistry, which is more accurate than MM, but is completely unsuitable for big structures (>10 000 atoms) such as proteins and membrane bilayers.

MM helps find the most stable structure from a given 3D structure (mainly obtained by crystallography) – the calculation time depends on the size and the number of the studied molecules and requires powerful computer hardware. These methods give a realistic, but static, model. The evolution of the system can be estimated using molecular dynamics (MD) for timescales from 100 ns to 1 µs. This is of major interest while studying the behavior of drugs toward membranes or proteins (i.e., transporters or cytochromes).

To study proteins, the first step is to obtain their experimental X-ray crystallography. Subsequently, MM simulations provide more-realistic conformations (i.e., the protein in aqueous solution). Finally, MD simulations provide atomistic insights of dynamic processes. More precisely, MD can help determine or confirm binding sites, the different conformations of transporters and the effect of phosphorylation or ATP on the protein conformations, further predicting the effect of a change in the amino acid sequence [76].

Drug membrane crossing depends on many parameters including (i) size, (ii) charge and (iii) lipophilicity of the molecule. Even if membrane crossing can be evaluated by parameters such as logP or logD, an atomistic description is required to fully deal with the mechanisms of action. MD helps determine the orientation and locations of drugs and even their metabolites in the membrane [77]. A common important issue is the composition of the membrane bilayer. Most studies have considered the membrane to be a single phospholipid membrane bilayer (mainly 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPC). Previous studies have demonstrated *in vitro* and *in silico* that this assumption is far away from reality. The reason is twofold: the membrane bilayer contains different lipids (such as triglycerides, sphingomyelin, cholesterol, etc.) in various percentages (depending on the cell type and side) but it is also because of the presence of proteins embedded in the membrane that can interact with the drug of interest [78]. In conclusion, MD simulations are currently capable of predicting the behavior of drugs in simple lipid membranes. Nevertheless, the number of publications exploring the functioning of transporters using a MD approach is considerably increasing. The number and type of membrane components remain limiting for making more-realistic predictions. Thanks to a perpetual improvement of calculation

power, new complete models should appear, paving the way toward a highly predictive *in silico* pharmacology.

QSAR approach. QSAR methods attempt to establish quantitative relationships between the structure of a molecule and its activity. Briefly, QSAR uses a library of molecules with well-known structures and activities and relates a biological effect on a new molecule with unknown biological effects. QSAR methods might be useful tools to predict passive drug absorption or interaction with regions of interest in influx or efflux proteins involved in active drug absorption [79]. The interest in QSAR methods is growing thanks to the European Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) protocol, which strongly incites to use QSAR methods rather than living models to evaluate chemical toxicity.

As for MM, QSAR approaches are inadequate to deal with highly complex molecules (mainly because of low predictive power owing to a poor library and because of the difficulty to associate a combination of several pharmacophores with an effect). Although recent developments in QSAR approaches make it possible to study noncovalent field (3D-QSAR) and the ensemble of ligand configuration (4D-QSAR) and further even to put forward rough toxicity predictions [80], these approaches suffer from a lack of parameters that describe drug–receptor interactions.

Ensemble learning methods are powerful tools for SAR approaches owing to their unique advantages in dealing with small sample sizes, high dimensionality and complex data structures [81]. Ensemble learning methods are particularly adapted to model drug permeability when the sample size is small or when the relationships between predictors and the dependent variables are not clear. Briefly, ensemble learning is based on the computer choice of the most suitable algorithms to solve a complex problem. In this case, ensemble learning helps choose the best algorithms to relate an activity to a complex structure. Ensemble learning methods have not yet been applied to absorption prediction modeling [82].

PB-PK modeling. The above methods sequentially describe the transport of drugs. Whole kinetic studies are still unpredictable when using the previously described *in silico* methods. By contrast, PB-PK enable the simultaneous study of drug absorption and metabolism using realistic physiological models. These models aim at predicting the target tissue dose(s) for different exposure situations and to evaluate the disposition of drugs within the body. The first step is to

obtain the animal PB-PK of the drug. Briefly, after dose administration, drug concentrations are measured in each organ of interest. Then, the PB-PK approach models the whole body as a closed compartment with several subcompartments representing an organ (i.e., the gut, the liver, etc.) or a tissue. All these subcompartments are connected with mathematical equations such as rate constants, clearance, among others. Simulations provide the equivalent human model using mathematical techniques, parameterized with known physiological features of the organs in animals and in humans (blood flow, organ mass, enzymes activity, etc.). Finally, PB-PK simulations provide physiological and pharmacokinetics insights into the behavior of a drug. More precisely, PB-PK can help to determine or confirm accumulation sites and rates, and can contribute to the study of efficacy and toxicity.

A model widely used to study drug absorption is the compartmental absorption and transit (CAT) model. The CAT model views the gastrointestinal tract to be a series of compartments ruled by mathematical absorption equations. A more complete model (ACAT) has been proposed by Agoram *et al.* [83]. The major feature is the addition of the hepatic first pass metabolism to the CAT model. In literature, there are several physiologically based models that consider other covariates developed to predict oral drug absorption. An exhaustive list has been summarized by Huang *et al*. [84]. To conclude, although the CAT model can estimate accurately the rate of drug absorption and is easily coupled with compartmental pharmacokinetics models, it seems limited to passively transported drugs.

In vitro *models*

Culture of human digestive epithelium. As previously seen, huge discrepancies can be observed in drug permeability between cultured cells and intestinal cells. Similarly, data obtained from animal models do not adequately describe permeability or absorption in humans. The culture of a human digestive epithelium can help overcome the limitations of these models. Recently Barker *et al*. isolated stem cells in the human digestive epithelium [85]. These cells express a specific G-protein-coupled receptor called Lgr5. In the intestinal crypts, stem cells and Paneth cells are in close contact and collaborate actively. For example, Paneth cells can secrete the epithelial growth factor (EGF) and WNT3A (a protein involved in embryogenesis and oncogenesis) [86]. Exposing these $Lgr5^{+}$ cells to defined culture conditions results in perpetual stemcellness. To date, there are

no consensual guidelines on how to culture these cells, but most methods used the following growth factors: WNT-3A, R-Spondin and Noggin. WNT-3A and R-Spondin are ligands of LRP5/6-Frizzled and Lgr4/5, respectively, both of which activate the Wnt–catenin pathway [87]. Briefly, the Wnt pathway consists in an accumulation of β-catenin in the cytoplasm, which finally enters the nucleus to act as a transcriptional factor to promote stemcellness. Noggin is the inhibitor of the BMP receptor, which is involved in cell differentiation. Moreover, this inhibition tends to lead to the appearance of crypt-like structures along the flanks of the villi [88]. Isolated, stem cells have the capacity to self-renew and differentiate into several specialized intestinal cells. From a single $Lgr5^+$ cell, Sato *et al*. established a long-term culture (>1.5 years) of intestinal epithelium [89]. Consequently, human stem cell gut organoids can be obtained when cultured in Matrigel® with subtle changes in the culture conditions. Moreover, all the cells present naturally in the gut can be found in this organoid model: Paneth cells, enterocytes, enteroendocrine cells and goblet cells [90].

Such epithelium could provide a highly relevant model to study permeability. This model might also be useful for drug screening and tissue regeneration. Adding the chyme flux and blood flux could contribute to recreating physiological conditions. Currently, there are several limitations: (i) models are cultured 3D in Matrigel®, making drug transport studies difficult; (ii) stem cells are commonly obtained from patients admitted to the surgical department for a bowel disease, thus limiting interpretation; (iii) the model is complex and expensive to set up; and (iv) there is little control over the morphogenesis and composition of the epithelium.

3D models: organ-on-chip models. Several 3D models have been developed, such as organ-on-chip devices or organoids. Organoids are structure-like organs that present several drawbacks, which limits their use for drug absorption modeling. Given that they are cells grown in a 3D matrix, it is difficult to observe them, to inject the drug into the lumen without altering the membrane or even to quantify the drug in the matrix. The most promising system is the microengineered biomimetic systems, which can be used to culture key functional units of human organs. Microengineered biomimetic systems make it possible to mimic epithelium–endothelium interfaces, along with complex organ-specific physiological microenvironments in a simple and well-controlled environment (Figure 3). For example, organ-on-chip models can reproduce gut 3D tissue architecture with the chyme and the blood flux [91].

Microfluidic systems can generate controlled concentration gradients to be integrated with cultured intestinal cells. These biomimetic microdevices can mimic physiological gradients of drugs, oxygen, growth factors and hormones in the gut. Such models can offer more-predictive models to study drug transport. For example, using Caco-2 cells, Kim *et al.* demonstrated that cell genetic profiles evolved toward a more reliable model; preliminary studies have shown that such flux led the Caco-2 epithelium to form villi-like structures [92]. More interestingly, these authors have added bacteria on the cells, thus improving the model.

Some studies have already used a microsystem approach to evaluate cell permeability [93]. These models are mainly made of glass or transparent polymer (i.e., PDMS, polycarbonate and polyester), contain microchannels, are easy to sterilize and composed of a membrane similar to the Transwell® membrane. Cell viability is maintained on these membranes, with different culture medium and flow rates. Similar methods have been used to integrate polarized epithelium with living vascular endothelium in organ-on-chip devices that reproduce tissue interfaces in organs (mainly the lung, eye, breast and brain) [94]. Using this approach, some studies showed that it is possible to generate *in-vivo*-like epithelial or endothelial tissues and to study their interactions [95].

One of the limitations of microsystem approaches is the necessity to use different culture media, especially if more-accurate models must be developed (human epithelium and endothelium). Moreover, their use is further restricted because they require skills in microfluidics and biomaterials. Another drawback is the difficulty to perform classical cell culture on these devices. For example, it is extremely challenging to harvest or passage cells. Nevertheless, these models reveal several advantages: reproducibility, high throughput and control over physiological factors such as flow rates. Moreover, different tissues can be used, such as Caco-2 cells, but also HUVECs to study the permeability to the blood or even the intestinal stem cells to reproduce a human intestinal epithelium. Such systems are not restricted to a specific organ but have the possibility to integrate several organs, such as the gut and liver, to study multiorgan interactions. This is an interesting new challenge that could promote more-advanced and -accurate predictions relating to drug permeability and even drug absorption. This also reveals a great interest in personalized medicine: an intestinal biopsy grown in such a system could predict the required dose to be administrated, overcoming a part of the intraindividual variability in drug response. For more information on organ-on-chip models, we refer the reader to the work of Skardal *et al.*

[91]. To conclude, gut-on-chip microdevices offer a more physiological model than classic static models but are also more complex to set up.

Concluding remarks

Modeling complex processes such as drug absorption is a great challenge. In fact, the more relevant the model the more difficult it is to implement and to validate. The trend of new model development in pharmacology can be divided into two main categories. First, there are the very simple, high-throughput *in silico* models that can screen the active pharmaceutical ingredient (API) candidates in the early stages of development. Second, there are the more sophisticated *in vitro* or *ex vivo* models, which can predict (with a high accuracy) the bioavailability of the API and study the mechanisms of absorption and the impact of biological parameters. *In silico* models will continue to grow because mathematical processing of data becomes faster every day. Modeling in 3D is now performed routinely and can help design drugs able to reach a higher bioavailability and to diffuse as requested in the organism. However, to take into account the relative impact of many biological parameters on the fate of an API in the human body, sophisticated models are necessary. *In vivo* models are still the only models able to predict active uptake. Nevertheless, for some poorly absorbed API, animal models, formerly used as the gold standard, offer poor predictions of permeability compared with the human Loc-I-Gut™. For *in vitro* models, although organs-on-chips are very promising, classical 2D models (cell cultures) will remain the most widely used tools for many years owing to their low cost and ease. Modeling is always a problem of choice. It is impossible to find a model that corresponds perfectly to the reality. In our field of pharmacology and pharmacokinetics the task is even more difficult because reality is versatile and depends on the genetics of the subjects. Modeling, therefore, is choosing what might be taken into account to predict a phenomenon while leaving other parameters aside. It is important to keep this in mind while interpreting the results of the experiments and to understand that models only give what they are designed for. By definition, models can give false-positive or false-negative results. As such, it is wise to associate different models to obtain a better prediction of the fate of the API. We are convinced that, in the coming decades, 3D models will be increasingly implemented and will be followed by the rise of highly predictive *in silico* models.

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970 **Figure 1** Fate of drugs in contact with intestinal wall. **(a)** Main pathways for drug absorption. **(b)**
971 Main transporters involved in drug absorption. **(c)** Main enzymatic paths involved during drug
972 absorption.

973 **Figure 2** *In vivo* perfusion: example of the rat.

974 **Figure 3** Proposal for a controlled microenvironment to measure drug permeability in a gut-on-
975 chip platform.

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977 **Table 1 Comparison of the physiological parameters in different parts of the gut**

Region	**Average length (m)**	**Absorbing surface**	**pH** [97]	**Residence time** [97]	**Mucus thickness (µm)**	**Bacterial cells (cells/ml)** [98]

	[96]	**area (m²)** [96]			[98][a]	
Duodenum	0.25–0.30	0.09	5.7–6.8	~40 min	FAL: 16 ±3 LAL: 154 ±39	10^{1}–10^{4} Aerobes and facultative anaerobes
Jejunum	3	60	6.6–7.0	2–3 h	FAL: 15 ±2 LAL: 108 ±5	10^{4}–10^{8} Facultative anaerobes and aerobes
Ileum	3	60	7.0–7.3	3–4 h	FAL: 29 ±8 LAL: 447 ±47	10^{4}–10^{8} Facultative anaerobes and aerobes
Colon	1.5	0.3	5.7 (caecum) to 6.6	16.6–19.0 h	FAL: 116 ±51 LAL: 714 ±109	10^{10}–10^{12} Facultative aerobes to strict anaerobic bacteria (mainly *Clostridia*)

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996 [a]Study performed in rats.
997 Abbreviations: LAL, loosely adherent layer; FAL, firmly attached layer.
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1000 **Table 2 Influx and efflux transporters of the gut**

Nomenclature	**Gene name**	**Common abbreviation**	**Function**	**Intestinal localization**	**Substrates**	**Inhibitors**
Organic anions transporting polypeptide 1A2	*SLCO1A2*	OATP1A2	Uptake of bile acids, thyroid hormones and PGE2	Apical	Antibiotics, anticancer drugs, antifungals, β-blockers, statins	Naringin [100]
Organic cation transporter 1	*SLC22A1*	OCT1	Uptake of organic cations	Not clear	Metformin, quinidine, dopamine, choline, PGE2, acyclovir, N1-methylnicotinamide and type 1 cations	Clonidine [101]
Organic cation transporter 3	*SLC22A3*	OCT3	Uptake of organic cations	Not clear	Amantadine, atropine, epinephrine, histamine, metformin, norepinephrine and cationic drugs	Quinine
Organic cation/carnitine transporter 1	*SLC22A4*	OCTN1	Uptake of organic cations and zwitterions, L-carnitine	Apical	Verapamil, pyrilamine, oxaliplatin, gabapentin, doxorubicin, quinine, organic cations and zwitterions	-

Organic cation/carnitine transporter 2	*SLC22A5*	OCTN2	Uptake of organic cations and zwitterions	Apical	Verapamil, pyrilamine, L carnitine, oxaliplatin, ipratropium, tiotropium, organic cations and zwitterions	
Concentrative nucleotide transporter 1	*SLC28A1*	CNT1	Uptake of 1 nucleoside / 1 Na^+	Apical	Nucleotides Nucleotides analogs	
Equilibrative nucleotide transporter 1	*SLC29A1*	ENT1	Uptake/Efflux of nucleotides	Apical	Nucleotides Nucleotides analogs	KF24345, NBM
Equilibrative nucleotide transporter 2	*SLC29A2*	ENT2	Uptake/Efflux of nucleotides	Apical/basolateral	Nucleotides Nucleotides analogs	KF24345, NBM
Peptide transporter 1	*SLC15A1*	PEPT1	Uptake of di and tripeptides / $2H^+$	Apical	Di and tripeptides Peptide like drugs Drugs coupled to amino acids, cephalosporins, penicillins xenobiotics drugs	Lys[Z(NO2)]Pr [103] 4AMBA [104] Glycylsarcosine Valspodar [106]
P glycoprotein	*ABCB1*	MDR1, Pgp	Efflux of hydrophobic amphipathic or cationic molecules steroid	Apical	>200 (anticancer drugs, digoxin…)	

			hormones, bile salts			
MDR2/3	*ABCB4*	PGY3	Efflux of phosphatidylcholine	Apical	Ivermectine, daunorubicin, digoxin, paclitaxel, vinblastine	-
MRP2	*ABCC2*	cMOAT	Efflux of bile salts	Apical	Organic anions, glutathione and conjugates, anticancer drugs (methotrexate), etoposide, sartans, bromosulfophthalein, nucleotide analogs	MK-1 (specif group)
BCRP	*ABCG2*	BCRP	Efflux of porphyrins, flavonoids, estrones and bile acids	Apical	Overlap with P-gp substrates	Ko143 [107]
MRP1	*ABCC1*	MRP1	Transport of hydrophobic drugs, estrogens and prostaglandins	basolateral	Antivirals, anticancer drugs, quinolones, glucuronide conjugate	MK571
MRP3	*ABCC3*	MRP3	Transport of glutathione	Basolateral	Organic anions, anticancer drugs, glucuronide conjugate	MK571

			conjugates			
MRP4	*ABCC4*	MRP4	Nucleotides, prostaglandins	Basolateral	Cephalosporins, antivirals, anticancer drugs	MK571
MRP5	*ABCC5*	MRP5	Cyclic nucleotides, folates	Basolateral	Statins, antivirals, anticancer drugs	MK571
MRP6	*ABCC6*	MRP6	Not clear	Basolateral	?	MK571

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1004 **Table 3 Characteristics of monoculture models**

Cells lines		Caco-2/TC-7 [43]	2/4/A1 [47]	IEC-18 [48]	MDCK [108]
Origin		Derived human colon cells	Rat fetal intestinal epithelial cells	Rat fetal intestinal epithelial cells	Dog kidney epithelial cells
Morphologies		Polarized monolayers with tight junction, brush border and apical microvilli	Polarized monolayers with tight junction, brush border and few microvilli	Polarized monolayers with tight junction, brush border and apical microvilli	Polarized monolayers with tight junction, brush border and apical microvilli
Paracellular transport (TEER values)		Underpredicted (higher values of TEER)	Close to *in vivo* (TEER values close to *in vivo*)	Close to *in vivo* (TEER values close to *in vivo*)	Close to *in vivo* (TEER values close to *in vivo*)
Passive transcellular transport	***Transport of drug with low permeability***	Underpredicted	Close to *in vivo*	Close to *in vivo*	Underpredicted but higher than Caco-2 cells

	Transport of drug with high permeability	Close to *in vivo*	Close to *in vivo*	Close to *in vivo*	Close to *in vivo*
Active transcellular transport	***Carrier and efflux transporter (e.g., P-gp, MRP-1, BCRP)***	High and variable	Absence	Absence	Low
Culture time		3 weeks	3–4 days	3 weeks	3–4 days

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