

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

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- 31 API: active pharmaceutical ingredient
- 32 BCS: biopharmaceutical classification system
- 33 CAT: compartmental absorption and transit model
- 34 CYP: cytochrome P450
- 35 DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
- 36 EGF: epithelium growth factor
- 37 FAE: human follicle-associated epithelium
- 38 GST: glutathione S-transferase
- 39 MATE: multidrug and toxic compound extrusion transporters
- 40 MD: molecular dynamics
- 41 MDCK: Madin–Darby canine kidney
- 42 MGS: metagenomic shotgun sequencing
- 43 MM: molecular modeling
- 44 NAT: *N*-acetyltransferase
- 45 OAT: organic anions transporters
- 46 OATP: organic anions transporting polypeptides
- 47 OCT: organic cations transporters
- 48 OCTN: zwitterion transporters
- 49 P-gp: P-glycoprotein
- 50 PAMPA: parallel artificial membrane
- 51 PB-PK: physiology-based pharmacokinetics
- 52 PEPT: peptide transporters
- 53 PVPA: phospholipid vesicle-based permeation assay
- 54 QSAR: quantitative structure–activity relationship
- 55 Ro5: Rule of Five
- 56 SLC: solute carrier family
- 57 SULT: sulfotransferase
- 58 TEER: transepithelial electrical resistance

59 TMD: transmembrane domains

60 UGT: UDP-glucuronosyltransferase

61

## 62 **Highlights:**

63

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

69

70

## 71 **Introduction**

72 The oral route is the most common and practical way to administer drugs to the body; even if  
73 certain problems remain, especially for anticancer agents [1]. Unfortunately, not all drugs are  
74 good candidates for oral administration. The Biopharmaceutical Classification System (BCS)  
75 proposed by Amidon *et al.* in 1995 [2] shows that solubility and permeability can be used to  
76 determine whether a drug is a good candidate for oral administration. Similarly, the Rule of Five  
77 (Ro5; see Glossary for list of abbreviations used in this review) proposed by Lipinski *et al.* in  
78 1997 [3] is also a quick way to assess the suitability of a drug for the oral route. The BCS and  
79 Ro5 are related to chemical properties of drugs. They are ways to predict a good absorption  
80 process, helping to reach a better bioavailability of the drug and often a low interpatient  
81 variability, which means reliability. These positive features are coined in the term drugability,  
82 which reflects the fact that the drug is a good candidate for the oral route. Then, the active drug  
83 must be formulated to obtain an oral dosage form. In the early stages of drug development, *in*  
84 *vitro* and/or *in vivo* models are extensively used to determine the best formulation of the drug  
85 product. Ideally, those models must be easy to implement, relevant, simple, cost effective,  
86 accurate and compatible with high-throughput screening. Some of those features are difficult to  
87 obtain altogether. As a matter of fact, the complexity of the absorption process makes it  
88 impossible for the models to be relevant and to remain simple. These models must also be  
89 suitable to assess the absorption of new formulations such as nanomedicines. Besides predicting  
90 the extent of drugs absorbed, models are also used to explore the very different barriers to cross  
91 and the complex mechanisms of this transport process. Models are also used to study the stability

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

102 **Where are we?**

103

104 ***Physiology and pharmacology of the small intestine***

105

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

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

124  
125 **Impact of drug transporters.** Drug absorption is mainly subject to significant transporters, even if  
126 some drugs can be absorbed by passive diffusion, and to the effects of metabolism. The fate of  
127 these drugs is represented schematically in Figure 1a. The paracellular transport, which is related  
128 to molecules below 3.6 Å or 250 Da, occurs through tight junctions between epithelial cells along  
129 the intestinal mucosa and shows an important intra- and inter-individual variability [4]. However,  
130 it has been previously shown that bigger structures such as nanoparticles can cross the tight  
131 junction structures [5]. Nevertheless, several current drug classes cross the epithelial cells by  
132 transcellular transport, either by passive transcellular transport (only small lipophilic drugs)  
133 and/or by carrier-mediated transport (antivirals, penicillins, statins, etc.) [6].

134  
135 **Role of transporters in the absorption of drugs.** Transporters are usually classed into two  
136 categories. The first category is the solute carrier (SLC) family, which mediates the uptake of  
137 drugs. The ATP-binding cassette (ABC) family is the second category and gathers efflux  
138 transporters (Figure 1b). Most of the SLC transporters are secondary active transporters, for  
139 which transport is driven by various energy-coupling mechanisms [7]. This category is divided  
140 into the SLCO superfamily (former SLC21), which gathers the organic anions transporting  
141 polypeptides (OATP), the SLC22 superfamily, which gathers organic cation transporters (OCT)  
142 and zwitterion transporters (OCTN), the SLC47 superfamily, which gathers the nucleotide  
143 transporters (ENT and CNT), and peptide transporters (PEPT).

- 144
- 145 • Few members of the SLCO superfamily are found in the intestine. Although OATP2A1  
146 and OATP4A1 are ubiquitous and transport mainly prostaglandins and bile salts,  
147 respectively, only OATP1A2 can have a role in transporting drugs. Situated at the apical  
148 side of the enterocytes, its substrates are bile salts, thyroid hormones and xenobiotics  
(antibiotics, anticancer drugs, antifungals,  $\beta$ -blockers, statins).
  - 149 • In the SLC22 superfamily, all the members share a common structure: 12  $\alpha$ -helical  
150 transmembrane domains (TMDs). This family actively participates in small intestinal  
151 absorption but also in hepatic and renal excretion of drugs. In the intestine, the most  
152 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<sup>+</sup>-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

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

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

207  
208 Obtaining an exhaustive list of substrates for each transporter would be worthwhile, but  
209 laborious. However, transporters seem to be more class-specific rather than drug-specific. As  
210 such, drugs are commonly grouped into classes with similar physicochemical properties, which  
211 renders the screening of hypothetical drug transporters easier (Table 2).

212



213 ***Impact of intracellular and microflora metabolism.*** Drug metabolism takes place in the  
214 intracellular milieu and depends on two classes of enzymes. Phase I enzymes are responsible for  
215 the functionalization of the drugs (i.e., hydroxylation, amination, etc.). Phase II enzymes tend to  
216 conjugate the metabolites by glycosylation, glucuronidation, transmethylation or acetylation and  
217 sulfoconjugation (Figure 1c). Phase I enzymes are cytochromes. Owing to their broad specificity,  
218 high abundance in the intestine and powerful capacity for oxidizing xenobiotics, cytochrome  
219 P450 (CYP) proteins are the most often studied. CYP3A and CYP2C represent the major  
220 intestinal CYPs, accounting for ~80% and ~18%, respectively, of total immunoquantified CYPs  
221 [12]. Phase II metabolism implies UDP-glucuronosyltransferases (UGTs), glutathione S-  
222 transferases (GSTs), *N*-acetyltransferases (NATs) and sulfotransferases (SULTs).

223  
224 UGTs are a superfamily of enzymes that catalyze the glucuronidation of endogenous and  
225 exogenous molecules. Among the 21 different UGT proteins that have been identified in humans,  
226 ten are expressed in the small intestine: UGT1A1, 1A3, 1A4, 1A6, 1A10, 2B4, 2B7, 2B10, 2B11  
227 and 2B15 [13]. Each enzyme encoded by a UGT gene reveals a unique but usually overlapping  
228 substrate specificity, tissue localization and regulation [14]. Human UGT1A1 is the most highly  
229 expressed UGT in the small intestine, with activities even greater than in the liver [15]. UGT1A  
230 enzymes conjugate endogenous and exogenous substrates.

231 GSTs occur in three cellular compartments and can be divided into cytosolic GSTs,  
232 mitochondrial GSTs and microsomal GSTs. The most expressed GSTs in the human enterocytes  
233 are GSTP1, GSTA1 and GSTA2 [16]. GSTA1 and GSTA2 catalyze the conjugation of  
234 glutathione with electrophiles whereas GSTP1 inactivates toxic and carcinogenic compounds by  
235 conjugation of glutathione. Most studies focus on the role of GST in pathogenic, mainly  
236 cancerous, tissue. Although GSTs are found in enterocytes, their behavior in this tissue has been  
237 poorly explored. NAT1 and NAT2 can be found in the intestine. They catalyze the acetyl  
238 conjugation from acetyl-CoA to various arylamine and hydrazine substrates. There is an  
239 increasing interest in SULTs, because they seem to contribute significantly to drug clearance  
240 [17]. They catalyze the transfer of a sulfate group to several pharmacologically important endo-  
241 and xeno-biotics.

242 The human microflora contains obligate anaerobes (i.e., *Bacteriodes*, *Clostridium*,  
243 *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  $\beta$ -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

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

276

### 277 **Current models**

278

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

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

297 Although the permeability of passively absorbed drugs correlates well with human data  
298 [31], this is not as clear for drugs absorbed by active transport. To specifically study the  
299 impact of a transporter, however, several knockout models have been developed in recent  
300 years. The first model was developed by Schinkel *et al.* using mice without P-gp (Mdr1a<sup>-/-</sup>  
301 ) [32]. Subsequently, several other models appeared, such as Bcrp1<sup>-/-</sup> mice [33] and Oct1<sup>-/-</sup>  
302 mice [34]. Despite this attempt, it has been observed that genes of the same family of the  
303 knocked-out gene are overexpressed in a mechanism of compensation, thus obtaining  
304 puzzling results [35]. To conclude, this method is relatively easy to set up, quick and  
305 cheap, which in part explains its notoriety.

- 306
- 307 • Loc-I-Gut<sup>™</sup>. A method to determine intestinal permeability in humans has been developed
- 308 by Lennernäs *et al.* [36]. This system is based on a jejunal perfusion system composed of
- 309 a multichannel tube with two inflatable balloons (Loc-I-Gut<sup>™</sup>). The obtained permeability
- 310 results can be further used as a gold standard to compare different models. However,
- 311 permeability studies using volunteers are limited because of ethical issues and cost.
- 312 Nevertheless, this is perhaps the most realistic and predictive permeability model ever
- 313 developed.
- 314
- 315 • Pharmacokinetic study. Individuals take the drug orally and venous blood is sampled at
- 316 different times post-dose. Although it seems like one of the easiest, most reliable and
- 317 simplest methods to study drug absorption, a pharmacokinetic study can have several
- 318 drawbacks. The observed drug concentrations are extremely variable – inter-individually
- 319 and intra-individually. This can be partially explained by different rates of gastric
- 320 emptying, differences in gut and liver metabolism, differences in transporter expression
- 321 and in elimination. A pharmacokinetic study of population can overcome these
- 322 variabilities but would involve a large number of individuals.

323

324 Although drugs absorbed by passive transport show a good correlation between animals and

325 humans [37], there are huge discrepancies between the different animal models and humans in

326 terms of metabolism, drug transport, flora and of course the surface area of the gastrointestinal

327 tract. For example, while using intestinal perfusion for five passively transported drugs in

328 humans, the apparent permeability was 3.6-times higher than observed in rats but results were

329 similar to those of mice [38]. Moreover, although moderate correlation ( $r^2 > 0.56$ ) was found in

330 the expression levels of transporters in the duodenum of humans and rats, no correlation was

331 found in the expression of metabolizing enzymes between the rat and human intestine [31]. This

332 explains why a reliable scaling from animal models to humans is often absent. Another common

333 limitation of the *in vivo* approach is that it is not suitable for high-throughput screening, it

334 presents a low sensibility and recovery of drugs, which makes analysis by mass spectrometry

335 indispensable. With regard to these advantages and drawbacks, these models might be

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

338  
339 **Ex vivo and in vitro models.** As in the case of the above-mentioned *in vivo* models, everted  
340 intestinal sac techniques are used to determine drug permeability. The intestine is removed from  
341 dead animals and cut into small segments, also making it possible to evaluate the permeability in  
342 different parts of the intestine. These segments are sutured at one end, filled with a drug solution,  
343 sutured at the other end and immersed in an oxygenated medium at 37°C. This model presents  
344 the advantage of a relatively large surface for permeability and the presence of a mucus layer.  
345 However, one limiting parameter of this model is tissue viability [39]. Moreover, no *in vivo*  
346 correlation has been established through this method but results from everted intestinal sac  
347 models have been consistent with *in vivo* findings [40]. As for *in vivo* methods, the major  
348 drawback is the poor screening rate. Nevertheless, these models should be preferred to *in vivo*  
349 models if anesthetic drugs might interfere with the analysis.

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

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

380 Another limitation of these cellular models is the absence of a mucus layer and M cells, which  
381 also constitute the intestinal barrier. In this way, co-culture models comprising different cells  
382 have been proposed to represent the heterogeneity of the intestinal epithelium. First, HT-29-H or  
383 HT-29-MTX cells that are mucus-secreting cells have been co-cultivated with Caco-2 cells.  
384 Different methods of culture (cell culture time, Caco-2/HT-29 ratio, culture medium, time of HT-  
385 29 addition, etc.) have been developed, and representative models in terms of mucus layer, P-gp-  
386 mediated efflux expression and paracellular permeability have been obtained [44–46]. Moreover,  
387 these co-culture models make it possible to evaluate absorption enhancers and mucoadhesive  
388 systems on the permeability of drugs [42]. Moreover, Raji cells have been added to Caco-2 cells  
389 to take into account human follicle-associated epithelium (FAE), which represents less than 1%  
390 of the total intestinal surface but shows an impressive propensity to transcytose small inert  
391 particles such as nanoparticles and large molecules from the lumen to the lymphocytes. Finally,  
392 triple co-culture cell models with Caco-2, HT-29 and Raji B cells have been put forward to obtain  
393 a more physiological, functional and reproducible *in vitro* model [54,55]. Antunes *et al.*  
394 demonstrated that this triple co-culture cell model is the most efficient model in predicting insulin  
395 permeability, as compared with permeability values obtained from *ex vivo* experiments [54].  
396 To enhance the relevance of models other modifications have been put forward to improve  
397 correlations between absorbed drugs *in vitro* and *in vivo*. For example, 3D *in vitro* models have

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

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

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

429 Indeed, the lipids in these PAMPA models were mainly commercially available lipids or  
430 extracted from natural tissues. Different lipid compositions are now available: PC-PAMPA  
431 (phosphatidylcholine-PAMPA), DS-PAMPA (a dodecane solution of lecithin mixture-PAMPA),  
432 DOPC-PAMPA [a dodecane solution of highly purified dioleoylphosphatidylcholine (DOPC)-  
433 PAMPA], HDM PAMP (a hexadecane solution of DOPC-PAMPA) and BML-PAMP (a  
434 biomimetic lipid membrane based on a mixture of phosphatidylcholine,  
435 phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, cholesterol, 1,7-octadiene  
436 membrane-PAMPA) [63]. Moreover, with cell models cultivated on the Transwell<sup>®</sup> system, a  
437 filter separates one side containing the test molecule (donor side) from a receiver side (initially  
438 free of the molecule). PAMPA is a high-throughput screening technique, but makes it possible to  
439 study only passive permeability. A comparison of DS-PAMPA with a rat *in situ* close-loop  
440 technique showed an acceptable correlation ( $r^2 = 0.87$ ) for 17 fluoroquinolone drugs [64].  
441 Similarly, at pH 5.5 or 6.5, BML-PAMPA also demonstrated an acceptable correlation ( $r^2 = 0.86$ )  
442 for more than 25 compounds [65]. Moreover, passively transported compounds, other than acidic  
443 compounds, also demonstrate a good permeability on HDM-PAMPA at pH 7.4 [66].  
444 Nevertheless, this model presents limited effectiveness and applicability owing to the absence of  
445 stirring conditions, the presence of solvent and the difficulty to reach sink conditions during  
446 transport studies. Consequently, other artificial models are developed to overcome these  
447 disadvantages. The phospholipid vesicle-based permeation assay (PVPA) is another artificial  
448 membrane model. This model consists of a deposition of liposomes on a filter [67]. Like the  
449 PAMPA model, PVPA can be used to study the transport of passive drugs and, owing to an  
450 excellent correlation with data obtained for the Caco-2 cell model, it represents a valuable  
451 alternative to cell models [68]. As such, even though a good *in vivo* correlation was obtained with  
452 artificial models (PAMPA and PVPA), these models can only be used for drugs passively  
453 transported, compared with cell models that make it possible to study all types of drug transport.  
454 *In silico* approaches (see below) can now simulate pure passive transport, thus limiting the  
455 interest in PAMPAs.

456

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



460 diffusion systems, such as the Ussing chamber [69] or a multicompartment model (membrane  
461 bioreactor) to simulate flow-mediated transport through the biological membrane [70]. For  
462 dynamic artificial membrane models, an impregnated membrane with a lipid mixture is inserted  
463 into a diffusion cell connected to a donor and receiver compartments, where liquid circulation is  
464 maintained using a peristaltic pump. This dynamic artificial membrane demonstrated an excellent  
465 correlation ( $r^2 = 0.95$ ) with permeability data in humans for highly absorbed hydrophobic drugs  
466 [71]. Of course, such systems are not useful when studying actively transported drugs.  
467 The Ussing chamber technique was also used with excised human or animal intestinal segments.  
468 Similarly, there are two diffusion compartments between the intestinal segments. The diffusion  
469 chambers can be filled with a physiological buffer solution (Krebs-ringer-bicarbonate buffer),  
470 which can also contain glucose, glutamate, fumarate, or with a simulated intestinal fluid (into the  
471 donor chamber). The system is kept at 37°C and the solution is constantly gassed with oxygen  
472 and carbon dioxide to maintain tissue viability and to create a fluid movement [69]. This model  
473 provides a good prediction for intestinal drug permeability, interaction with efflux transporters  
474 and drug metabolism [72]. Moreover, using this model, permeability could be determined at  
475 different parts of the intestine (jejunum, ileum and colon). Indeed, depending on the part of the  
476 intestine used and the origin tissue, *in vivo* correlation might be different. Comparison from *in*  
477 *vivo* and excised rat jejunal segments showed a high correlation ( $r^2 = 0.95$ ) for drugs transported  
478 by passive diffusion with high or low permeability, whereas drugs transported via carriers  
479 displayed certain differences [73].  
480 However, this model reveals several drawbacks: it shows a relatively low throughput and  
481 recovery, and it requires several human or animal biopsies that: (i) are difficult to obtain (for  
482 human samples); (ii) present important intraindividual or interspecies variabilities (for animal  
483 versus human) thus contributing to a poor reproducibility [74]; (iii) have a viability beyond 2 h;  
484 and (iv) decrease the functionality of their active transporters. Unfortunately, there is no  
485 comparison between these models and the Loc-I-Gut™ model. Although they seem easier to set  
486 up, present fewer ethical questions and can be used to test more drug than the *in vivo* methods,  
487 they lose several physiological aspects partially (i.e., mucus, active transport, flux and  
488 microbiotic flora) or totally (i.e., chyme and blood environment and peristalsis). Nevertheless,  
489 despite these issues, the model is, undoubtedly, after the Loc-I-Gut™ approach, the current most  
490 reliable model to simultaneously study passive and active drug absorption in animals and in

491 humans.

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

503

504 **Where are we going?**

505

506 ***In silico models***

507

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

520

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

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

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

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

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

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

557  
558 **QSAR approach.** QSAR methods attempt to establish quantitative relationships between the  
559 structure of a molecule and its activity. Briefly, QSAR uses a library of molecules with well-  
560 known structures and activities and relates a biological effect on a new molecule with unknown  
561 biological effects. QSAR methods might be useful tools to predict passive drug absorption or  
562 interaction with regions of interest in influx or efflux proteins involved in active drug absorption  
563 [79]. The interest in QSAR methods is growing thanks to the European Registration, Evaluation,  
564 Authorization and Restriction of Chemicals (REACH) protocol, which strongly incites to use  
565 QSAR methods rather than living models to evaluate chemical toxicity.

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

572 Ensemble learning methods are powerful tools for SAR approaches owing to their unique  
573 advantages in dealing with small sample sizes, high dimensionality and complex data structures  
574 [81]. Ensemble learning methods are particularly adapted to model drug permeability when the  
575 sample size is small or when the relationships between predictors and the dependent variables are  
576 not clear. Briefly, ensemble learning is based on the computer choice of the most suitable  
577 algorithms to solve a complex problem. In this case, ensemble learning helps choose the best  
578 algorithms to relate an activity to a complex structure. Ensemble learning methods have not yet  
579 been applied to absorption prediction modeling [82].

580  
581 **PB-PK modeling.** The above methods sequentially describe the transport of drugs. Whole kinetic  
582 studies are still unpredictable when using the previously described *in silico* methods. By contrast,  
583 PB-PK enable the simultaneous study of drug absorption and metabolism using realistic  
584 physiological models. These models aim at predicting the target tissue dose(s) for different  
585 exposure situations and to evaluate the disposition of drugs within the body. The first step is to

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

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

605

## 606 **In vitro models**

607

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

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

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

637  
638 **3D models: organ-on-chip models.** Several 3D models have been developed, such as organ-on-  
639 chip devices or organoids. Organoids are structure-like organs that present several drawbacks,  
640 which limits their use for drug absorption modeling. Given that they are cells grown in a 3D  
641 matrix, it is difficult to observe them, to inject the drug into the lumen without altering the  
642 membrane or even to quantify the drug in the matrix. The most promising system is the  
643 microengineered biomimetic systems, which can be used to culture key functional units of human  
644 organs. Microengineered biomimetic systems make it possible to mimic epithelium–endothelium  
645 interfaces, along with complex organ-specific physiological microenvironments in a simple and  
646 well-controlled environment (Figure 3). For example, organ-on-chip models can reproduce gut  
647 3D tissue architecture with the chyme and the blood flux [91].

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

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

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

679 [91]. To conclude, gut-on-chip microdevices offer a more physiological model than classic static  
680 models but are also more complex to set up.

681

### 682 **Concluding remarks**

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

709



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713

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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 (m <sup>2</sup> ) [96]			[98] <sup>a</sup>	
Duodenum	0.25–0.30	0.09	5.7–6.8	~40 min	FAL: 16 ±3 LAL: 154 ±39	10 <sup>1</sup> –10 <sup>4</sup> Aerobes and facultative anaerobes
Jejunum	3	60	6.6–7.0	2–3 h	FAL: 15 ±2 LAL: 108 ±5	10 <sup>4</sup> –10 <sup>8</sup> Facultative anaerobes and aerobes
Ileum	3	60	7.0–7.3	3–4 h	FAL: 29 ±8 LAL: 447 ±47	10 <sup>4</sup> –10 <sup>8</sup> 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 <sup>10</sup> –10 <sup>12</sup> Facultative aerobes to strict anaerobic bacteria (mainly <i>Clostridia</i> )

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996 <sup>a</sup>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**

<b>Nomenclature</b>	<b>Gene name</b>	<b>Common abbreviation</b>	<b>Function</b>	<b>Intestinal localization</b>	<b>Substrates</b>	<b>Inhibitors</b>
Organic anions transporting polypeptide 1A2	<i>SLCO1A2</i>	OATP1A2	Uptake of bile acids, thyroid hormones and PGE2	Apical	Antibiotics, anticancer drugs, antifungals, $\beta$ -blockers, statins	Naringin [100]
Organic cation transporter 1	<i>SLC22A1</i>	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	<i>SLC22A3</i>	OCT3	Uptake of organic cations	Not clear	Amantadine, atropine, epinephrine, histamine, metformin, norepinephrine and cationic drugs	Quinine
Organic cation/carnitine transporter 1	<i>SLC22A4</i>	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	<i>SLC22A5</i>	OCTN2	Uptake of organic cations and zwitterions	Apical	Verapamil, pyrilamine, L-carnitine, oxaliplatin, ipratropium, tiotropium, organic cations and zwitterions	
Concentrative nucleotide transporter 1	<i>SLC28A1</i>	CNT1	Uptake of nucleosides $\pm$ Na <sup>+</sup>	Apical	Nucleotides Nucleotides analogs	
Equilibrative nucleotide transporter 1	<i>SLC29A1</i>	ENT1	Uptake/Exchange of nucleotides	Apical	Nucleotides Nucleotides analogs	KF24345, NBM
Equilibrative nucleotide transporter 2	<i>SLC29A2</i>	ENT2	Uptake/Exchange of nucleotides	Apical/basolateral	Nucleotides Nucleotides analogs	KF24345, NBM
Peptide transporter 1	<i>SLC15A1</i>	PEPT1	Uptake of an tripeptide $\pm$ 2H <sup>+</sup>	Apical	Di and tripeptides Peptide like drugs Drugs coupled to amino acids, cephalosporins,	Lys[Z(NO <sub>2</sub> )] Pr [103] 4 AMBA [104] Glycylsarcosine
P-glycoprotein	<i>ABCB1</i>	MDR1, P-gp	Efflux of hydrophobic amphipathic or cationic molecules steroids	Apical	>20 penicillins xenobiotics drugs (anticancer, digoxin...)	Valspodar [106]

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

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

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1004 **Table 3 Characteristics of monoculture models**

<b>Cells lines</b>		Caco-2/TC-7 [43]	2/4/A1 [47]	IEC-18 [48]	MDCK [108]
<b>Origin</b>		Derived human colon cells	Rat fetal intestinal epithelial cells	Rat fetal intestinal epithelial cells	Dog kidney epithelial cells
<b>Morphologies</b>		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
<b>Paracellular transport (TEER values)</b>		Underpredicted (higher values of TEER)	Close to <i>in vivo</i> (TEER values close to <i>in vivo</i> )	Close to <i>in vivo</i> (TEER values close to <i>in vivo</i> )	Close to <i>in vivo</i> (TEER values close to <i>in vivo</i> )
<b>Passive transcellular transport</b>	<b><i>Transport of drug with low permeability</i></b>	Underpredicted	Close to <i>in vivo</i>	Close to <i>in vivo</i>	Underpredicted but higher than Caco-2 cells

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

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