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Commentary

Theme: Integrating In Vitro Systems and Physiologically-Based Pharmacokinetics Modeling to Optimize Drug Product Development
Guest Editors: Rodrigo Cristofoletti and Lawrence Yu

Navigating Through Cell-Based *In vitro* Models Available for Prediction of Intestinal Permeability and Metabolism: Are We Ready for 3D?

Yesenia L. Franco[1] · Lais Da Silva[1] · Rodrigo Cristofoletti[1]

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Abstract. Traditionally, *in vitro* studies to quantify the intestinal permeability of drugs have relied on two-dimensional cell culture models using human colorectal carcinoma cell lines, namely Caco-2, HT 29 and T84 cells. Although these models have been commonly used for high-throughput screening of xenobiotics in preclinical studies, they do not fully recapitulate the morphology and functionality of enterocytes found in the human intestine *in vivo*. Efforts to improve the physiological and functional relevance of *in vitro* intestinal models have led to the development of enteroids/intestinal organoids and microphysiological systems. These models leverage advances in three-dimensional cell culture techniques and stem cell technology (in addition to microfluidics for microphysiological systems), to mimic the architecture and microenvironment of the *in vivo* intestine more accurately. In this commentary, we will discuss the advantages and limitations of these established and emerging intestinal models, as well as their current and potential future applications for the pre-clinical assessment of oral therapies.

KEY WORDS cell culture · in vitro models · microdevices · microphysiological system · organoids · permeability

INTRODUCTION

The convenience and non-invasiveness of oral drug delivery makes it one of the most preferred routes of administration by patients; as a result, an estimated 60% of commercially available small molecules are administered orally (1, 2). Despite the advantages of this route of administration in terms of patient adherence, the development of oral formulations is challenged by the complex interplay between drug physicochemical properties and *in vivo* physiological conditions and processes that ultimately govern oral drug bioavailability and disposition (2, 3). Accordingly, experimental models that can adequately predict human intestinal permeability and gut metabolism at early stages of drug development are of paramount importance to streamline the development process.

For at least 30 years, *in vitro* studies to assess intestinal permeability have been performed with immortalized colorectal carcinoma cell lines (i.e., Caco-2, HT-29, and T84 cells) cultured on permeable Transwell inserts (4–7) (Figure Ia). While these *in vitro* models are well established, they fail to recapitulate the architecture, cell heterogeneity, transcriptomics, and microenvironment of the human intestine (8–10). Efforts to improve the functional and physiological relevance of intestinal models and, accordingly, the *in vitro* to *in vivo* translatability of experimental data have led to the development of intestinal enteroids/organoids and intestinal microphysiological systems (11–13) (Figure 1b and c). These models integrate advances in three-dimensional cell culture techniques and stem cell biology (in addition to microfluidics for microphysiological systems), to mimic intestinal cell morphology, cell population heterogeneity,

[1] Center for Pharmacometrics and Systems Pharmacology, Department of Pharmaceutics, College of Pharmacy, University of Florida, Orlando, Florida, USA
[2] To whom correspondence should be addressed. (e–mail: rcristofoletti@cop.ufl.edu)

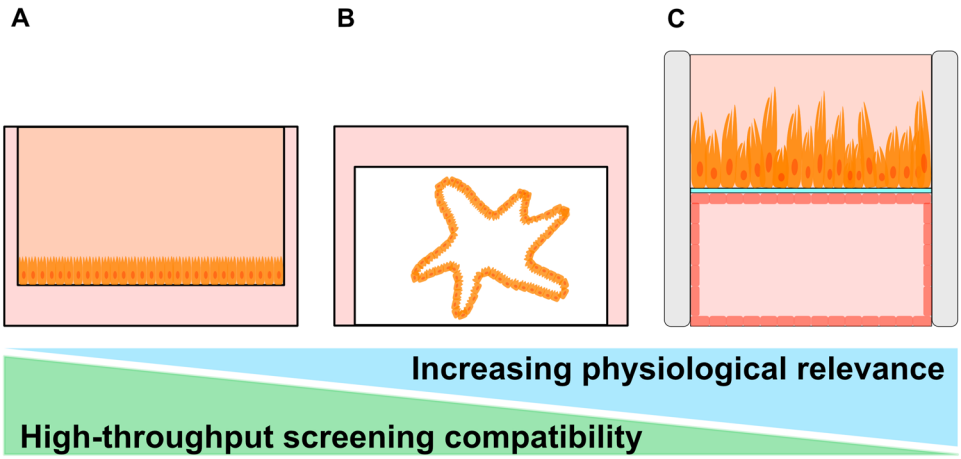

Figure 1. Schematic of **A** Caco-2 cell monolayer cultured on permeable Transwell inserts; **B** multipotent stem cell-derived enteroid embedded in extracellular matrix; and **C** intestinal microphysiological system co-cultured with epithelial cells in the upper channel and endothelial cells in the lower channel. The upper and lower channels are flanked by hollow vacuum channels that expand and contract to mimic peristaltic motions

and expression patterns of metabolizing enzymes and transporters (14, 15).

In subsequent sections, we will discuss the advantages and limitations of these established and emerging intestinal models, as well as their current and potential future applications in the early stages of development of oral drugs.

TWO-DIMENSIONAL CELL CULTURE SYSTEMS

Although Caco-2 cells are colonic in origin, when cultured as a monolayer, they spontaneously differentiate and exhibit a cell morphology resembling that of the columnar epithelial cells found in the small intestine (5, 6, 16, 17). In addition, confluent Caco-2 cell monolayers express brush border hydrolases, well-defined tight junctions, and some transporters present in the human small intestine (5, 7, 16).

Of note, lipophilic drugs that rely mainly on passive membrane diffusion display a good correlation between *in vitro* Caco-2 permeability and *in vivo* intestinal permeability. In contrast, the intestinal permeability of hydrophilic drugs that are mainly absorbed by paracellular or carrier-mediated processes has been mispredicted by Caco-2 models (18). Using data for 19 drugs obtained from a publication by Sun and colleagues (18), we used an epidemiological approach to evaluate the predictive capacity of Caco-2 cells. Briefly, we calculated sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Caco-2 apparent permeability (P_{app}) vs. *in vivo* effective permeability (P_{eff}) using the following equations:

$$\text{Sensitivity} = \frac{\textit{True high permeability}}{\textit{True high permeability} + \textit{False low permeability}} \quad (1)$$

$$\text{Specificity} = \frac{\textit{True low permeability}}{\textit{True low permeability} + \textit{False high permeability}} \quad (2)$$

$$\text{Positive predictive value (PPV)} = \frac{\textit{True high permeability}}{\textit{True high permeability} + \textit{False high permeability}} \quad (3)$$

$$\text{Negative predictive value (NPV)} = \frac{\textit{True low permeability}}{\textit{True low permeability} + \textit{False low permeability}} \quad (4)$$

The results of this analysis are summarized in Table I. While the Caco-2 model was able to adequately classify low permeability compounds, the sensitivity of Caco-2 predictive capacity was 64%, as four out of eleven highly permeable drugs according to P_{eff} were misclassified as having low

Table 1. Predictive Capacity of Caco-2 cells

		In vivo P_{eff}		
		High permeability	Low permeability	
Caco-2 P_{app}	High permeability	**True high permeability 7**	**False high permeability 0**	**7**
	Low permeability	**False low permeability 4**	**True low permeability 8**	**12**
		11	**8**	**19**

Sensitivity = 64 %
Specificity = 100 %
Positive predictive value (PPV) = 100%
Negative predictive value (NPV) = 67 %

permeability with the Caco-2 assay. Two of the misclassified drugs, cephalexin and enalapril, are substrates of the proton-coupled oligopeptide transporter (PEPT1) (19, 20). PEPT1 is not expressed in the colon, and its expression in Caco-2 cells is highly variable and strongly dependent on cell origin (21, 22). Glucose and phenylalanine intestinal permeability were also underestimated, suggesting that Caco-2 cells fail to recapitulate the expression of sodium-dependent and sodium-independent transport systems (23, 24). Furthermore, paracellular transport of drugs across Caco-2 monolayers is generally underestimated. For example, apparent permeability of low molecular weight polyethylene glycols was 100-fold lower in Caco-2 cells than across human intestinal epithelium (25–27).

In addition, there are several key characteristics of the human intestine that are difficult to recapitulate with 2-dimensional cell culture techniques. These include physiological and microenvironmental factors such as (1) the tissue-to-tissue interface between enterocytes and endothelial cells, (2) development of heterogenous enterocyte subpopulations, (3) the presence of a mucus barrier lining epithelial cells, (4) vascular perfusion and mechanical strain mimicking fluid flow and peristaltic motions, and (5) long-term co-culture of enterocytes and commensal bacteria (10, 14, 28–30) .

Of note, several of these physiological and microenvironmental factors are relevant when considering oral drug absorption. Goblet cells, an epithelial cell subpopulation found in the intestines, secrete mucus that coats epithelial cells and serves as a barrier against pathogens, digestive enzymes, and xenobiotics (31, 32). Larhed and colleagues reported pronounced declines in diffusion coefficients across pig intestinal mucus for lipophilic compounds relative to hydrophilic compounds (33). This observation has been attributed mainly to interactions or partitioning of lipophilic compounds with lipids present in mucus and interactions with proteins, to a lesser extent (33, 34).

Furthermore, transcriptomic and proteomic fingerprints of colonic immortalized cell lines fail to recapitulate the expression and abundance of metabolic enzymes and transporters in intestinal cells (35–37). The cytochrome P450 (CYP) superfamily is responsible for the majority of phase I reactions, with the CYP3A and CYP2C subfamilies found to be the most abundantly expressed intestinal CYPs. Prueksaritanont et al. (38) reported no measurable cytochrome P450 activity by Caco-2 cells under conditions comparable to those used for conventional transport studies. The expression profiles of transporters, ion channels, and metabolizing enzymes have differed by at least threefold when comparing Caco-2 cells and human duodenum samples (18). A publication by Englund and colleagues comparing the regional mRNA expression of drug transporters from the ATP-binding cassette (ABC) and solute carrier (SLC) families reported differences in the rank order of transporter transcripts between Caco-2 cells and the human intestine. In the duodenum, Breast Cancer Resistance Protein (BCRP) and Peptide Transporter 1 (PEPT1) were the two most prevalent transcripts. In contrast, for Caco-2 cells, the most prevalent transcripts were Multidrug resistance-associated protein 2 (MRP2), organic anion-transporting polypeptide B (OATPB) and P-glycoprotein (P-gp) (22).

Overexpressing cell systems have been used to inform early-stage investigations of intestinal metabolism and transporter-mediated kinetics. While this approach was essential to unveil the dynamic interplay between efflux transporters and metabolic enzymes in the gut (39), *in vitro* to *in vivo* translatability of the results may not be straightforward. Expression and activity of transporters and enzymes may differ between the *in vitro* and *in vivo* systems. Therefore, forward translational approaches are challenged by the limited confidence in the extrapolation between these systems. Reverse translational approaches have been used by different groups to generate empirical scaling factors (40). While this practice clearly improves data characterization, the introduction of empirically derived scaling factors may belie the extrapolative power of subsequent modeling approaches. Increasing the physiological complexity of the *in vitro* system, e.g., recapitulating *in vivo* transcriptomics, could simplify and increase the confidence in the *in vitro* to *in vivo* extrapolation of absorption-related properties at early stages of drug development.

THREE-DIMENSIONAL *IN VITRO* CELL CULTURE MODELS

Attempts to address the limitations of and improve the physiological complexity of traditional two-dimensional, cell-based *in vitro* models has led to the development of *in vitro* “mini intestines” known as enteroids or intestinal organoids, depending on the source of the stem cells used for their generation (13, 41).

Enteroids

Enteroids are three-dimensional spheroid-like structures generated from donor intestinal crypts that contain Leucine-rich repeat-containing G-protein coupled receptor 5-positive (Lgr5+) stem cells (41, 42). To grow enteroids, intestinal crypts are embedded in extracellular matrix (ECM) and initially exposed to growth-factor enriched media, selected to emulate the niche in the intestinal crypts. Structurally, the apical surfaces of the intestinal cells are inside the organoid, while the basolateral surfaces are in contact with the ECM and medium (41). After several days of culture in enriched media, growth factors are withdrawn,

and enteroids differentiate to form a villous-like epithelium containing mature enterocytes, enteroendocrine cells, goblet cells, and Paneth cells (41–44). The prevalence of absorptive and secretory lineages is controlled by modulating Wnt and Notch signaling (45). It has been reported that enteroids retain segment-specific phenotypic characteristics, donor-specific gene expression profiles, and disease state-related phenotypic and epigenetic characteristics (46–49). As a result, enteroids have emerged as suitable models to investigate normal intestinal physiology and the pathophysiology of intestinal conditions including inflammatory bowel disease (IBD) (50), human norovirus (51), and adenovirus (52). Even though enteroids have been widely used to map out mechanisms of intestinal diseases and toxicity, this *in vitro* model is rarely used to study intestinal drug permeability. This is mainly due to the fact that the apical membrane is located inside the enteroid. Therefore, microinjections are needed to place the drug in contact with the apical membrane. Alternative methods have emerged as promising alternatives (13). For example, Scott and colleagues cultured human intestinal crypts as monolayers on thin coats of type I collagen or laminin (53). In addition, enteroid dissociation followed by re-seeding on coated plates seems to be a useful approach to bypass the pragmatic limitation of using enteroids to screen drug permeability and metabolism as well as to improve the biorelevance of the cell-based system (51, 52). More research is needed to investigate whether increasing the complexity of the *in vitro* system will indeed increase its biopredictive performance.

Intestinal Organoids

Intestinal organoids are derived from induced pluripotent stem cells (iPSCs). Like enteroids, iPSC-derived intestinal organoids are grown in an extracellular support matrix in the presence of growth factor enriched media, and the resulting intestinal organoids have a similar morphology as enteroids (54). It has been suggested that iPSC-derived organoids more closely resemble the fetal intestine than the adult intestine (41, 43); however Jung and colleagues reported that STAT3 signaling induced by interleukin 2 (IL-2) caused *in vitro* maturation of iPSC-derived intestinal organoids to more closely resemble the adult intestinal epithelium (55). Similarly to enteroids, intestinal organoids have been used to model intestinal diseases. For example, this *in vitro* system has been used to explore the underlying mechanisms of cystic fibrosis (CF) (56), intestinal infections by SARS CoV-2 (57), and IBD (58).

Since iPSC-derived intestinal organoids retain intestinal cell heterogeneity and functionality, it emerges as a promising alternative to biopsy tissue-based enteroids to study intestinal metabolism and permeability (59). Of note, dissociating iPSC-derived organoids and re-seeding on coated plates resulted in an *in vitro* system amenable to interrogate intestinal metabolism of CYP substrates (60).

Intestinal Microphysiological Systems

Intestinal microphysiological systems integrate advances in microfluidics, three-dimensional cell culture, and, more recently, stem cell biology to recapitulate small intestine morphology, cell heterogeneity, and transporter/enzyme expression patterns (10, 14, 15, 61, 62).

Structurally, most intestinal microphysiological systems are microdevices consisting of an upper and lower microchannel, separated by a thin, porous, ECM-coated membrane. Each microchannel has a dedicated inlet port that is used to introduce cells, cell culture medium study drugs, etc., as well as an outlet port to remove effluents from each respective channel. In some configurations, the upper and lower channels are flanked by hollow vacuum chambers that mechanically expand and contract the sidewalls, mimicking peristaltic motions (10, 14, 15). Immortalized intestinal epithelial cells such as Caco-2, primary cells obtained from biopsy or induced pluripotent stem cell (iPSC)-derived organoids are seeded on the top channel, over the ECM-coated porous membrane, and endothelial cells, such as human intestinal microvascular endothelial cells (HIMECs), are seeded in the lower channel (Fig. 2). Following an incubation period to ensure cell adhesion, the cells are continuously perfused with cell culture medium; once monolayers are confluent, cells can be exposed to cyclic mechanical strain, to mimic the fluid flow and peristaltic motion of the intestine, respectively (10, 14, 15).

Caco-2 Based Microphysiological Systems

The earliest reported intestinal microphysiological systems were developed using Caco-2 cells. Kim et al. (63, 64) demonstrated the importance of shear stress, fluid flow, and cyclic mechanical strain to recapitulate *in vivo*-like morphology and functionality of intestinal cells (63, 64). Application of physiologically relevant shear stress (0.02 dyne cm^{-2}) as a result of a fluid flow equivalent to 30 μL/h led to the rapid development of columnar, polarized epithelial cells with basal nuclei. This was contrasted to static cell culture with the Transwell method, where epithelial cells were described as "flattened and almost squamous in form." (63) Decreasing the fluid flow rate to 10 μL/h led to the growth of cells resembling those of the Transwell system, while increasing the fluid flow rate to 100 μL/h did not cause an increase in cell height beyond what was observed after exposure to 30 μL/h (63).

The importance of cyclic mechanical cues was assessed by comparing the apparent permeability coefficient (P_{app}) in static Transwell system, microphysiological system under

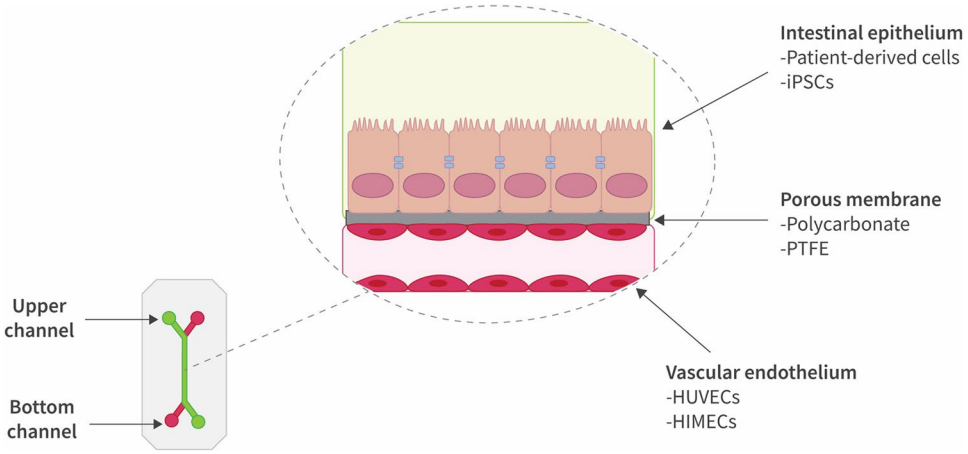

Figure 2. Common characteristics of an intestine-on-a-chip microdevice. Briefly, the chips comprise two microchannels separated by a porous membrane to structurally mimic the in vivo barrier between the intestinal lumen and connected vasculature. Intestinal epithelial cells are seeded on the upper channel and vascular endothelial cells on the lower channel. PTFE = polytetrafluoroethylene; HUVECs = human umbilical vein endothelial cells; HIMECs = human intestinal microvascular cells

fluid flow and microphysiological system under fluid flow and mechanical strain. Cells cultured under both mechanical strain (10% strain; 0.15 Hz) and fluid flow (30 µL/h) had a greater than 4-fold increase in paracellular permeability (63).

In addition to the morphological and microenvironmental resemblance of the "gut-on-a-chip" to the *in vivo* intestine, this system also demonstrated a progressive increase in CYP3A4 enzyme activity, which correlated with villi differentiation. In contrast, Caco-2 cells cultured on a static Transwell system did not exhibit significant CYP3A4 activity (64). Even though culturing Caco-2 cells in microdevices seems to increase the physiological complexity of the system, contradictory results have been published. On the one hand, Pocock et al. (65) demonstrated that P_{app} values of caffeine and atenolol obtained from an Caco-2-based gut-chip model were higher than those obtained from Transwell studies and closer to the respective human P_{eff} values. On the other hand, Kulthong and colleagues reported that P_{app} coefficients obtained in a Caco-2-based gut-chip showed poorer predictive capacity than the traditional Transwell system for three model drugs (60).

It has been hypothesized that replacing Caco-2 cells by enteroids or intestinal organoids may improve the physiological relevance and biopredictive performance of gut-chips.

Enteroids/Intestinal Organoid-Based Microphysiological Systems

Despite the resemblance of Caco-2 derived "gut-on-a-chip" systems to intestinal epithelial cells, the need for an intestinal model that could more accurately capture the genetic profile and homeostasis of intestinal cells led to the development of stem cell-based microphysiological models (14).

Work by Kasendra and colleagues (10, 14) and Workman and colleagues (15) demonstrated that culturing of organoid-derived epithelial cells from human duodenal biopsies (multipotent stem cells) or iPSCs, in the presence of continuous fluid flow, led to the development of polarized epithelial cells with well-defined tight junctions and villi-like structures. This was qualitatively demonstrated by protein staining of E-cadherin, polarized zona occludens-1 (ZO-1), and villin, in addition to visualization of villus-like extensions into the lumen of the epithelial microchannel via scanning electron or confocal microscopy (10, 14, 15). Kasendra and colleagues reported that the presence of endothelial cells in the lower channel prompted faster confluency of epithelial cells located in the upper channel, demonstrating the physiological relevance of reproducing cell-to-cell communication in *in vitro* models (14).

Intestinal cell heterogeneity in stem cell-based intestinal microphysiological systems was attested by the presence of markers including Mucin 2 (MUC2), a marker for goblet cells; lysozyme, a marker for Paneth cells; and chromogranin A, a marker for enteroendocrine cells (10, 14, 15). Functional analyses revealed that strong intestinal barriers were formed over 12 days of culture, brush border enzymes were capable of hydrolyzing sucrose into glucose and confirmed the secretion of high concentrations of MUC2 by goblet cells (14).

A comparison of the global RNA expression profiles of duodenal enteroid microphysiological systems and human duodenal tissue revealed a high level of similarities in the expression of several genes involved in drug metabolism. Subsequent assessment of the expression and localization of intestinal drug transporters revealed similar expression profiles of efflux and uptake drug transporters between *in vitro* and *in vivo* systems as well as localization of P-gp, BCRP, and PEPT1 on the apical membrane of epithelial cells (10). Furthermore, enteroid-derived gut-chips better resembled human duodenum phenotype than the respective enteroid itself (14).

The application of intestinal microphysiological systems to prospect intestinal permeability and metabolism is in its infancy. Additional research designed to assess the predictive performance of stem cell-based intestinal

microphysiological systems to recapitulate intestinal permeability and gut wall metabolism of selected model drugs covering a broad range of physicochemical properties is needed to assess the potential of this promising model to inform early stages of drug development under biopharmaceutics and clinical pharmacology perspectives.

Disease Models

Intestinal microphysiological systems are powerful research tools that faithfully recapitulate not only the human intestinal physiology but also its pathophysiology (66, 67). Disease-based intestine-chips have been developed by different groups to map out the underlying mechanisms of gastrointestinal diseases as well as to generate a reductionist model that is amenable to support the investigation of drug effect during screening phases (14, 66). For example, biopsy-derived colon organoids cultured in a microfluidic device successfully recapitulated human intestine mucus production and physiology (thickness and bilayer structure) *in vitro*, advancing the investigation of changes in mucus homeostasis in cystic fibrosis patients (68, 69). Furthermore, a complex human "gut inflammation-on-a-chip" model has been developed to study intestinal barrier dysfunction (70). Such disease-based intestinal microphysiological systems have an uncharted potential to inform ADME prediction at early stages of drug development. Conceptually, translating *in vitro* results obtained in human cell-based system to *in vivo* would be more straightforward than applying interspecies extrapolation techniques; however, further research is needed to navigate these uncharted waters and optimally exploit the potential of these pathophysiologically relevant *in vitro* systems.

CONCLUSION

Even though traditional two-dimensional *in vitro* cell-based systems are compatible with high throughput screening philosophy, they have limitations to recapitulate key gastrointestinal (patho)physiology and variability. Integrating intestinal organoids/enteroids and microfluidics emerged as a promising alternative to improve the physiological relevance and predictive capacity of *in vitro* systems to ultimately better inform early stages of drug development. However, further research is urged to thoroughly investigate the biopredictive performance of such systems and identify best practices to streamline model development and reduce the currently high associated costs.

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Declarations

Conflict of Interest The authors declare no conflicts of interest.

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