DMD Fast Forward. Published on August 20, 2018 as DOI: 10.1124/dmd.118.082784
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Physiologically relevant, humanized intestinal systems to study metabolism and transport of small molecule therapeutics: Mini-review

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Running Title: *in vitro* primary intestinal models of drug disposition

Text pages: 18

Tables: 2

Figures: 1

References: 58

Words in Abstract: 224

Words in Introduction: 1,227

Words in discussions: 3,320

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Abbreviations:

DDI, drug-drug interactions; PK, pharmacokinetics; F_a, fraction of the drug that is absorbed from the lumen into the enterocytes; F_g, fraction of the absorbed drug that is extracted from the gut into the portal vein circulation; F_h, fraction of the drug that is extracted from the liver into the systemic circulation; DMEs, drug metabolizing enzymes; ABCs, ATP-binding cassette proteins; SLCs, solute linked carriers; IVIVE, in vitro in vivo extrapolations; LS180; Caco-2, colon carcinoma cell lines; S9, supernatent fraction collected from tissue homogenized and centrifuged at 9000 g; iPSCs, induced pluripotent stem cells; GI, gastrointestinal; LC-MS/MS, liquid chromatography interfaced with tandem mass spectrometry; CYPs, cytochrome P450; UGTs, Uridine glucuronysyl transferases; HIM, Human intestinal microsomes; ABCB1, ATP binding cassette subfamily B1; ABCC2, ATP Binding Cassette Subfamily C Member 2; ABCC3, ATP Binding Cassette Subfamily C Member 3; ABCG2, ATP-binding cassette sub-family G member 2; ASBT, apical sodium dependent bile acid transporter; OATP2B1, Organic anionic transporter polypeptide 2B1; OCT1, organic cationic transporter 1; OCT3, organic cationic transporter 3; PEPT1, Peptide Transporter 1; OATP1A2, organic anionic transporting polypeptide 1A2; EHC, Entero-hepatic circulation; RT-qPCR, real-time quantitative polymerase chain reaction; Pxr, rat pregnane X-receptor; Rxr, rat retinoid X-receptor; Car, rat constitutive androstane receptor; Ppar, rat peroxisome proliferator-activated receptors; Gsts, rat glutathione transferases; Ugts, rat Uridine glucuronysyl transferases; Pgp, P-glycoprotein; Mrp2, rat multi-drug resistant protein subfamily 2; Ces, rat carboxy esterase; CES, human carboxy esterase; SI; sucrose isomaltase; TEER, trans-epithelial electrical resistance; TER, trans-epithelial resistance;

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hiPS-ELCs, human induced pluripotent epithelial cells; MRP1, multi-drug resistance protein 1; BCRP, breast cancer resistance protein; GI-MPS, gastro-intestinal microphysiological system; IHC, immuno- histochemistry; ZO-1, zonula occludins/tight junctions protein 1; PDMS, polydimethylsiloxane; 3D, 3 dimensional; PBPK, physiologically based pharmacokinetics; WB, western blotting.

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Abstract.

Intestinal disposition of small molecules involves interplay of drug-metabolizing enzymes, transporters, and host-microbiome, which has spurred the development of *in vitro* intestinal models derived from primary tissue sources. Such models have been bio-engineered from intestinal crypts, mucosal extracts, iPSC-derived organoids, and human intestinal tissue. The present mini-review discusses the utility and limitations of these human derived models in support of small molecule drug metabolism and disposition. Enteroids from human intestinal crypts, organoids derived from iPSCs using growth-factors or small molecule compounds, and enterocytes extracted from mucosal scrappings show key absorptive cell morphology; but are limited in quantitative applications due to lack of accessibility to apical compartment, lack of monolayers or low expression of key DMEs, transporters, and nuclear hormone receptors. Despite morphogenesis to epithelial cells, similar challenges have been reported by more advanced technologies that have explored the impact of flow and mechanical stretch on proliferation and differentiation of Caco-2 cells. Most recently, bio-engineered human intestinal epithelial or ileal cells have overcome many of the challenges, as the DME and transporter expression pattern resembles that of native intestinal tissue. Engineering advances may improve such models to support longer term applications and meet end-user needs. Biochemical characterization, transcriptomic, proteomic, and functional end-points of novel intestinal model systems, when referenced to native human tissue, can provide greater confidence and increased utility in drug discovery and development.

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Introduction

Predicting pharmacokinetics and DDIs (inhibition and induction) of novel small molecule therapeutics is an important consideration in drug discovery and development. While the liver is known to be a predominant clearance organ, over time it has been increasingly accepted that the intestine also plays a major role in clearance (secretory), metabolism, and DDIs. As with the liver, numerous DMEs (Phase I and II) and transporters (ABCs and SLCs) reside in the gut and are loci of important DDIs as well as major determinants of F_a*F_g and oral bioavailability (Rogers et al., 1987). Thus the gut is not simply an "absorption" organ; it is also regarded as an integral part of the enterohepatic axis and the site of host-microbiome interactions (Li et al., 2016; Malik et al., 2016).

Absorption (F_a) is a combination of solubility and permeability, and can be predicted with confidence in human (Artursson and Karlsson, 1991; Fagerholm, 2007; Wei et al., 2009) and rat (Nilsson et al., 2017). In contrast, discrete measurement of F_g, *in vitro*, has not been reported due to the lack of reliable assays derived from human native tissue intestinal sections. This is less of an issue for CYP3A substrates due to the availability of *in vitro* systems like human intestinal microsomes, S9 fractions, recombinant CYP3As and in silico approaches (Gertz et al., 2011). One can scale CYP3A turnover rate from these systems, using relative protein abundance or relative activity factor to predict human intestinal intrinsic clearance (Paine et al., 1997). However, for non-CYP3A substrates, there are no suitable *in vitro* models that resemble the human intestinal apical environment experienced by novel, orally administered compounds. This is also convoluted by the heterogeneous expression of DMEs and

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transporters along the length of human intestine (discussed later). Orally administered drugs undergo intestinal and hepatic metabolism in tandem, further complicating the separation of F_g from F_h. This has necessitated the development of *in vitro* models to support the study of drug metabolism and DDI in the GI, to derive IVIVE (Jones et al., 2016), and to enable non-clinical screening of compounds.

Early attempts to study disposition of small molecules, *in vitro*, leveraged Ussing chambers (Wallon et al., 2005; Wuyts et al., 2015) with isolated human (endoscopic biopsied) tissue, followed by migration to gut tissue-derived subcellular fractions, and finally the deployment of human gut "surrogate" reagents such as LS180 and Caco-2 cells (Rogers et al., 1987; Yamaura et al., 2016). Only recently has it been possible to consider using physiologically-relevant human intestinal models by analogy with human primary hepatocytes (Khetani *et al*, Bale *et al* in this issue). Of late, several intestinal models bio-engineered from iPSCs, crypts, mucosal extracts, and healthy human intestinal tissue have been reported. While these models were initially developed to meet the needs of epithelial biologists to study GI disorders like UC and CD, recently many of these have been evaluated for expression and function of DMEs and transporters. This mini-review will discuss the state-of-the-art and limitations of such emerging, primary, human-derived intestinal models to study disposition of small molecules. The mini-review concludes with a framework describing end-user needs of drug metabolism scientists, validation end-points, and computational parameters to enable scaling the outputs from pre-clinical discovery to development.

Anatomy of the GI

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The GI tract consists of 4 layers of tissue which, starting from outside to inside, are called: serosa, muscularis, sub-mucosa, and mucosa (Mescher, 2016). The mucosa is the innermost layer enveloping the lumen, and specializes in absorption and secretion. This innermost mucosal layer is lined by an epithelium, which displays distinct morphologies along each of the small and large intestinal sections. The small intestinal segments (duodenum, jejunum, and ileum), are arranged into invaginations (crypts) and finger-like protrusions (villi) that represent units specialized in the absorption of micronutrients (Elliott and Kaestner, 2015). The colon comprises the large intestine and consists of crypts; villi are absent in this region. Six differentiated cell types populate the crypt-villus axis of the intestinal epithelium. The most abundant cells are the enterocytes (i.e., absorptive cells) and goblet cells (i.e., mucus secreting cells). Other, less abundant, cell types include entero-endocrine cells that secrete gastrointestinal hormones, tuft cells thought to play a role in immune activation, M-cells that deliver antigen to lymphoid tissues, and Paneth cells, which localize at the base of the crypts and secrete anti-microbial peptides. With the exception of Paneth cells, which have a lifetime of approximately 2 months in the mouse (Ireland et al., 2005), all differentiated cell types in the human intestinal epithelium are short lived. Enterocytes, goblet cells, and the other secretory lineages are born in the crypts and migrate to the top of the villi over 5-7 days in human, and 3–5 days in mouse after which they are shed into the lumen of the intestine (Marshman et al., 2002; Barker et al., 2007; Clevers, 2013; Clevers and Batlle, 2013). Enterocytes expressed at the brush border or villi of the small intestinal wall, are the major absorptive cells of the intestine, offering an enhanced surface area for absorption for drugs, xenobiotics, and nutrients. In addition to

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absorptive properties, these villi express drug metabolizing enzymes and transporters responsible for carrier-mediated disposition at the epithelial barrier (Pang, 2003; Miyauchi et al., 2016).

Transcriptional and proteomic signatures of DMEs and transporters, in human intestinal sections

The mRNA expression and protein content of clinically relevant DMEs (http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.) and transporters (Hillgren et al., 2013) as measured by immuno-quantification, western blotting (WB) or LC-MS/MS peptide quantification has been published previously. (Paine et al., 2006) have reported the relative abundance of P450 isozymes in duodenal and jejunal mucosal scrapings, using western blotting; CYP3A and CYP2C9 were the most abundant P450 isozymes accounting for 80% and 15% of total P450 content, respectively, followed by P450s 2C19>2J2>2D6. By application of RT-PCR and LC-MS/MS (peptide quantification) (Drozdzik et al., 2017) have observed similar trends of P450 expression in biopsies from human jejunum, ileum, and colon. In addition to the CYP3A, CYP2C9/19, and CYP2D6, authors also reported significant expression of UGT1A1/1A3 and UGT2B7 **(Table 1)**. In addition, all detected enzymes confirmed highest expression in jejunum with significant correlations between mRNA and protein levels for most enzymes.

Similar assessment of clinically relevant intestinal transporters (Hillgren et al., 2013) using quantitative LC-MS/MS assays and WB, has been reported (Labroo et al., 1997; Paine et al., 2006; Tucker et al., 2012; Hillgren et al., 2013; Drozdzik et al., 2014; Nakamura et al., 2016; Drozdzik et al., 2017; Akazawa et al., 2018). SLC5A1 (PEPT1)

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has been consistently reported to be the most abundant jejunal transporter, whereas SLC10A2 showed the greatest variability across different GI regions **(Table 1)**. According to (Drozdzik et al., 2014; Drozdzik and Oswald, 2016), OATP1A2 (mRNA and protein) is not detected in any intestinal segment. In contrast, MRP3, OCT3, and, to a lesser extent, MRP2 were more abundant in the colon than in the small intestine, whereas OATP2B1 and OCT1 were uniformly expressed along the entire gut. All drug transporter protein abundances taken from individual reports are summarized in **Table 2.** Overall, region-specific expression of DMEs and drug transporters suggests that humanized systems designed to predict portal availability and intestinal, pharmacokinetic drug-drug interactions should be appropriately evaluated. Keeping in mind this heterologous expression pattern, this mini-review will highlight opportunities offered by each of the systems discussed below, for drug metabolism and transporter scientists.

Enteroids

Some of the earliest human intestinal cultures were obtained from crypts isolated from intestinal tissue. Seminal work by (Clevers, 2013) demonstrated that under optimized media conditions intestinal stem cells can form “mini-intestines” or enteroids which differentiate into small intestinal villus-like or colonic cells consisting of enterocytes, goblet cells, entero-endocrine cells, and Tuft cells (Mahe et al., 2015; Zachos et al., 2016). To date, there are no reports on expression of DMEs and transporters in human crypt-derived enteroids. However, recently (Lu et al., 2017) have explored expression of DMEs in enteroids derived from crypts isolated from wild-type

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and genetically modified murine intestine. This study demonstrated the expression of nuclear receptors like Pxr, Rxr, Car, Rxr and Ppar, Cyp isozymes, Ces, Gsts and Ugts as well as drug transporters *Pgp* (*Abcb1a*), *Mrp2* (*Abcc2*), and *Abcg5/8*. Authors particularly explored functional activity of Ces and Ugt in these enteroids by determining Ces catalyzed conversion of irinotecan to SN-38 and Ugt catalyzed detoxification of SN-38 to SN-38-G (Chen et al., 2013). Enteroids generated from $Ugt1^{F/F}$ mice efficiently generated SN-38 and SN-38-G; irinotecan mediated cytotoxicity was observed at doses ranging from 10 to 100 μM. In contrast, enteroids from $Ugt1^{\Delta IEC}$ mice showed SN-38 mediated cytotoxicity at 1 μM dose of irinotecan, due to depletion of Ugts. Similar trends in functional activity and cytotoxicity were also observed in enteroids generated from $Ugt1^{F/F}$ deficient mice. This murine proof-of-concept report exemplifies a systematic approach to examine drug metabolism and toxicity of small molecules using enteroids. While these enteroids may mimic human physiology by differentiating into key intestinal cell types, the apical compartment is not accessible, limiting the utility of this system to measure net extraction ratios across a typical intestinal epithelial bilayer. Lack of homogenous monolayers on a medium to high throughput device limits the scalability of these enteroids to discovery applications.

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Enterocytes

Recently, (Ho et al., 2017; Yan et al., 2017) have reported the isolation and cryo-preservation of human intestinal enterocytes. In this model, enterocytes were produced by digesting the intestinal lumen, followed by differential centrifugation; enterocytes were cryo-preserved for long term storage. Functional activities of all major P450

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isozymes, UGT, SULT, and CES2 were measured in this system by quantification of metabolites of specific probe substrates, using LC-MS/MS. Quantifiable activities were observed for CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP3A4, CYPJ2, CES2, UGT, and SULT, but not for CYP1A2, CYP2A6, CYP2B6, and CYP2D6; significant donor-to-donor variability was observed in the activities of P450 isozymes. (Yan et al., 2017) subsequently characterized this system (MetMax™), as an *in vitro* model to assess intestinal clearance, by normalizing the functional activity of probe CYP substrates in MetMax™ enterocytes to that in hepatocytes. As expected, this normalization demonstrated that the functional activity of CYP3A4 was lower in enterocytes than in hepatocytes; however, the activity of UGTs (raloxifene hydroxylase) was 3-fold greater in enterocytes than in hepatocytes, which was unexpected. Authors also reported 9-fold lower activity of CES2 in enterocytes compared to that in hepatocytes and no turnover of salbutamol (a predominant SULT1A1 substrate). MetMax™ enterocytes when treated with elacridar, (CYP3A/P-gp inhibitor), suppressed metabolism of loperamide, a (CYP3A4/P-gp substrate), suggesting that P-gp may not be functional in this system. Overall, this system is useful to determine the metabolism of predominant CYP3A substrates; absence of P-gp and BCRP, and discrepancies in functional activities of DMEs expressed in this system, limits its utility to study non-CYP3A mediated drug disposition. Additional optimization and transcriptional as well as proteomic characterization may enable quantitative scaling of CYP3A and other isozymes that are expressed in MetMax™ enterocytes.

iPSCs

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Growth-factors-directed differentiation of human pluripotent stem cells to functional enterocytes, was first reported by (Spence et al., 2011). Since then several efforts have been undertaken to characterize iPSC derived intestinal systems for biology and ADME studies. (Iwao et al., 2014) differentiated human iPSCs to functional enterocytes using epidermal growth factors, followed by maturation of the enterocytes using low serum concentration (2% FBS). As determined using mRNA quantification, these mature enterocytes expressed SLC15A1/PEPT1 and CYP3A, with expression levels being 9-30 fold higher in differentiated vs non-differentiated cells; however, the expression levels were reported to be lower than those observed in adult intestine. Subsequently, (Takenaka et al., 2014) reported optimized monolayer of enterocytes differentiated from iPSCs, to support longer term cultures. Quantitative RT-PCR confirmed mRNAof CYP3A5, UGT1A3, and UGT1A6; however CYP3A4, CYP2D6, CYP2C19, and CYP2C9 were poorly expressed in this system. This system has expressed all major intestinal drug transporters OCT1, OATP2B1, MCT1, CNT3, ENT1, ENT2, ENT3, P-gp, BCRP, MRP1, MRP2, and MRP3 and the expression levels were similar to or ~10-fold higher than that in human small intestine. Functional and inhibitable activity of P-gp (digoxin/verapamil) and BCRP (mitoxantrone/Ko143) was also confirmed in this system. (Takenaka et al., 2014) have shown that the monolayers in this system were “leaky” in comparison to Caco-2 monolayers and suggested that the TEER in this system is closer to human intestinal TEER, with the caveat that TEER values in Caco-2 could be a function of cell source and experimental conditions; permeability measures of paracellular model compounds was higher than that observed in PAMPA and Caco-2 monolayer. Thus while this system formed enterocyte monolayers that may enable HT

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screening, and expressed P-gp, BCRP, CYP3A5, UGT1A1, and UGT1A6, it may have limited applications in drug disposition due to lower expression levels of predominant DMEs, including CYP3A.

Pharmacokinetic enhancement in differentiated enterocytes by treatment with epidermal growth factors and small-molecule enhancers Y-27632, dorsomorphin, 2-(2-amino-3-methoxyphenyl)4H-1-benzopyran-4-one (PD98059), 5-aza-2′-deoxycitidine, 3-(6-methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide (A-83-01) was reported by (Iwao et al., 2015). Quantitative RT-PCR confirmed the mRNA levels of CYP3A, CYP2C, CYP2D and PEPT1 genes. Similar efforts were reported by (Kodama et al., 2016) using analogous small molecule regulators: mitogen-activated protein kinase (MAPK), DNA methyltransferase (DNMT), and (TGF)-β inhibitors. Authors reported that these regulators increased the expression of CYP3A and functional activities of CYP1A1/2, CYP2B6, CYP2C9, CYP2C19, CYP3A4/5, UGT, and SULTs. Interestingly, CYP3A4 mRNA expression level was induced by treatment with 1α,25-dihydroxyvitamin D_3 but not by treatment with rifampin, suggesting that PXR pathway (and its downstream enzymes and transporters like P-gp) were not functional in this enterocyte model, which is a major limitation of this model. Subsequent efforts by (Negoro et al., 2016) has generated PXR inducible CYP3A from mature human epithelial cell lines, by overlaying matrigel on the differentiated iPSC-derived enterocytes. The iPSC derived mature enterocytes using matrigel overlay, successfully expressed nuclear receptors, PXR, GR and VDR, as confirmed by treatment with rifampin, phenobarbital, 1α,25-dihydroxyvitamin D_3, and dexamethosone. Treatment with dexamethasone, phenobarbital, rifampicin, or 1α,25-dihydroxyvitamin D3 resulted

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in ~5.8-fold, ~13.4-fold, ~9.8-fold, or ~95.0-fold induction of CYP3A4 expression relative to that in the untreated controls, respectively. The levels of intestinal nuclear receptors *PXR*, *GR*, *FXR*, *SHP*, and *VDR* were also reported to be higher than in Caco-2 and LS180 cells. Similar improvement in expression of PXR-inducible CYP3A was reported by (Ozawa et al., 2015), where enterocytes were derived from human iPSCs using epithelial growth factors: EGF, SB431542, and Wnt3A, and by extending the differentiation period. This treatment improved TEER and also resulted in increased gene expression of CYP3A4 and PEPT1. Most recently, (Onozato et al., 2018) have differentiated iPSCs using the small-molecule compounds described previously [A-83-01, PD98059, and 5-aza-2′-deoxycytidine (A/PD/5-aza) or A/PD/5-aza plus DAPT], followed by seeding them on EZSPHERE plates to generate uniform spheroids. Treatment with these small-molecule compounds expressed CYP3A4, PEPT1, OATP2B1, MRP2, BCRP, and P-gp; mRNA activities of CYPs 2C9, 2C19, and 2D6 were also detected in this system but expression of ASBT (as measured via mRNA) was lower than that in human adult intestine. Modest induction (mRNA and enzyme activity) of CYP3A4 was observed when treated with rifampicin and with 1, 25-dihydroxyvitamin D_3. Authors have reported morphological characterization of microvilli as well as immunofluorescence staining of villin, and occludin, confirming the formation of tight junctions and a functional barrier; permeability measurements across the bilayer have not been reported. Additional characterization of permeability and optimization of inducible PXR pathway may enhance its utility to study intestinal disposition of drugs. In conclusions, small-molecule compounds and growth factors-directed differentiation of human iPSCs can yield mature enterocytes expressing some of the DMEs or

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transporters; additional optimization could maximize utility of such models in studying intestinal drug disposition.

Microfluidics-based platforms

Apart from small molecule cocktails, impact of fluid flow and mechanical stretch on cell differentiation, has been recently explored. (Kim et al., 2012) have investigated the effect of flow on proliferation and differentiation of Caco-2 cells. The authors reported that mechanical stretch differentiated Caco-2 cells into absorptive enterocytes and morphogenesis to form intestinal villi. In addition, CYP3A expression increased during villous formation but plateaued thereafter. Quantitation using the P450-Glo CYP3A4 assay demonstrated a greater activity in this system compared to that in Caco-2 transwells. This activity was lower than that reported for human small intestinal microsomal fractions from human epithelial preparation (erythromycin N-demethylase activity: 0.30 to 0.76 nmol/min/mg microsomal protein) by (Zhang et al., 1999). This low CYP3A activity is probably due to the intrinsically low expression of CYP3A in Caco-2 cells. Functional activity and abundance of key intestinal transporters (P-gp, BCRP, PEPT1, ASBT) has not been reported in this system limiting its use for drug disposition studies.

Similar efforts to build a microfluidic platform have been reported (Chen et al., 2017) where human gut-liver tissue interactions were evaluated by an integrated multi-organ platform consisting of Caco-2 derived intestinal cultures differentiated into enterocytes, goblet cells, and dendritic cells. This system maintained barrier integrity over two weeks, as measured by TEER and was comparable to previously reported

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TEER values for Caco-2 and HT29-MXT co-cultures in trans-well assays, as well as primary intestinal monolayers (VanDussen et al., 2015). The same integrated gut-liver system has also been applied by (Tsamandouras et al., 2017) to quantitatively measure the entero-hepatic disposition of cytochrome P450 substrates. Kinetics of diclofenac and hydrocortisone were investigated followed by mechanistic modeling to determine the in-vitro-to-in-vivo-correlation of this system. However both of these systems have been derived from Caco-2 cell lines.

A significant development in studying ADME using primary-cell derived intestinal models was recently reported by (Ayehunie et al., 2018). This is a micro-tissue system where ileal epithelial cells and fibroblasts were expanded in a monolayer culture and seeded into single-well cell culture inserts or into a high throughput 96-well membrane bottom plate for up to 42 days at the air liquid interface. The epithelial cells differentiated into Paneth cells, Tuft cells, and Muc-2 positive Goblet cells, and enterocytes with villi-like brush borders structures, as concluded by the authors. This system produces an average TEER value of 152.5 ± 39 $\Omega*cm^2$ in 96-well cultures and is able to functionally differentiate flux of high vs low permeability compounds. IHC and functional activity measured by concentration dependent efflux of digoxin±verapamil confirmed P-gp expression on the apical surface. RNA levels of CYP3A4, CYP3A5, CYP2C9, and GSTs and key efflux transporters including P-gp/MDR1, MRP1, MRP-2, and BCRP were also confirmed using RT-PCR. P-gp/MDR1 was shown to be upregulated by >10-fold when co-cultured with fibroblasts. The mRNA levels level of GSTs was comparable to that of native intestinal tissue.

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This model is an important development, as it simulates several structural, transporter, and metabolic features of human ileum and can be used to measure permeability, CYP450 mediated metabolism, and P-gp/BCRP mediated efflux of small molecules. A limitation of this system is the absence of SULTs and UGTs 1A1 and 2B7. Nevertheless, metabolism of CYP3A and CYP2C9 substrates can be predicted pending further validation of this model (discussed later). Further investigation of this system for functional activity of the clinically relevant P450s and transporters could enhance its application in drug disposition.

Most recently (Madden et al., 2018) have reported the generation of human primary intestinal epithelial cells by “bioprinting” primary isolated epithelial cells. The intestinal tissue formed through this technique demonstrated a polarized epithelium with tight junctions. PXR, CXR, CYP3A, CYP2C9, CYP2C19, CYP2D6, and CYP2J2, and phase II enzymes GSTP1 and UGT1A1 are expressed in this system. The initial expression data is promising; the activity of CYP3A across three donors tested in this study ranged from 2.85-19.3 pmol/min/mg of protein which is lower than the previously published activity of CYP3A in human intestinal mucosal scrappings of 8.8-150 pmol/min/mg of microsomal protein (Paine et al., 2006). PXR-inducible CYP3A activity was confirmed by midazolam hydroxylation; this functional activity was comparable to that measured previously in intestinal tissue slices. Transcriptomic profiling also demonstrated the presence of P-gp, BCRP, PEPT1, OATP2B1, and ASBT. The authors have concluded that the expression of these transporters was similar to that in native tissue preparations. Functional activity of P-gp and BCRP was determined by measuring the bidirectional transport of digoxin (ER 8.8) and topotecan (ER 190), which

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was inhibitable by zosuqridar. As such, absolute efflux ratios generated in an *in vitro* system generated at single concentrations can be best used to rank order compounds. Scaling functional activity from in vitro models to humans requires the magnitude of expression of the transporters, and kinetics (V_{max} and K_m) of probe substrates. Overall, this system may become a suitable tool to study intestinal disposition of substrates of major drug metabolizing enzymes and transporters, once characterized fully (as discussed later).

In summary, this mini-review has discussed opportunities and limitations of enteroids, enterocytes, iPSC derived enterocytes, bio-engineered Caco-2 models, and human primary cell derived models in studying in vitro intestinal drug disposition. Future models should be designed by leveraging the information gained from existing models and addressing gaps in the engineering and biochemical attributes experienced by the current ones.

Engineering needs should consider a thermoplastic, optically clear, low non-specific binding material; in addition, a bilayer design with access to the apical (top) and basolateral (bottom) chambers should be considered to enable direct, frequent and easy sampling without perturbing the system; a bilayer design may also enable the introduction of specialized cell types like immune cells and macrophages, to measure disposition of novel therapeutics under disease state. Similarly, extraction of monolayers and media from top and bottom channels would allow for terminal readouts like RT-PCR and proteomics. It may also be desirable to have TEER sensors, oxygen control, and medium-to-HT capabilities. Biochemical characterization should consider discrete development of cell lines from different intestinal sections of primary human

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tissue (healthy or diseased). Technological advances like the introduction of flow and stretch may help duplicate the biology (Vedula et al., 2017; Bein et al., 2018). Longer term applications of physiologically relevant intestinal systems derived from native tissue or iPS-derived cells should be subject to quality controls to provide consistent differentiation and monolayer formation. Several systems are developed by optimizing media conditions resulting in proprietary media which should be readily available, for scalability. Good viability (>75%) of cryopreserved enterocytes as well as human iPSC derived enterocytes should be considered prior to experimentation (Ho et al., 2017). Continued optimization of primary human intestinal derived cultures to yield a uniform monolayer with >90% viability of cryopreserved enterocytes is recommended.

Almost always, enterocyte cultures are/will be generated from multiple donors (8-10 per experiment) and from both genders. As a result, donor-to-donor variability of ADME endpoints should be characterized prior to establishing cell lines for long term usage. Intra- and inter-lot variability should be monitored after selection of lots that show good reproducibility; this initial quality control can ensure consistent delivery of routine, long standing cultures. In order to measure DMEs and transporter activities, cells are typically incubated with probe substrates/inhibitors in culture media (~150,000 cells/well of 96w plate) with incubation times ranging from 30 mins to 3 h (Ho et al., 2017; Yan et al., 2017); in case of low turnover compounds, these incubation times may be >24 h, to measure the initial slope of elimination. Thus method optimization should consider cell viability during prolonged incubation time. Reproducibility of a system can be ensured by studying at least 3 replicates and statistical analysis to define the variance and robustness of a system. Multiple quality controls including RT-PCR, image-based read-

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outs and baseline levels of metabolic functions should be implemented to assess good physiological behavior of the systems.

Validation criteria.

Expression of intestinal DMEs and transporters requires differentiation of primary intestinal cells to enterocytes. Therefore, while not a necessity for an ADME scientist, differentiation of the human primary cells into absorptive enterocytes should be confirmed by IHC of epithelial markers namely sucrose-isomaltase, alkaline phosphatase, or villin. Once the epithelial markers are established, tight junctions of epithelial barrier should be confirmed using claudin staining as this ultimately leads to increased confidence in measurement of TEER (Mrsny et al., 2008; Landy et al., 2016). Finally reproducible measurements of transcriptional, proteomic, and functional signatures of clinically relevant DMEs and transporters **(Tables 1 and 2)**, in a humanized system enables the choice of appropriate platform to meet end-user needs. Under the best case scenario, transcriptional and proteomic signatures would be similar to those observed in human intestinal segments published by (Drozdzik et al., 2017) and (Drozdzik et al., 2014).

Design, scaling strategies and IVIVE:

While scientists strive to derive a system that recapitulates physiological abundance of DMEs and transporters, it should not be expected that these in vitro primary cell derived systems will produce a quantitative output that is representative of the intestinal permeability, metabolism or transport, and is translatable in vivo; additional

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scaling may be required. Central to this translation is the use of computational methodologies to integrate the operating characteristics of in vitro system (e.g. perfusion flow rate, number of cells in incubation volume, non-specific binding, population and relative ratio of different cell types, the ratio of the tissue (cellular) volume to the volume of the surrounding medium, and residence time of chemical entities of interest within the system)(Tsamandouras et al., 2017a; Tsamandouras et al., 2017b). In addition to integration of system parameters, robustly characterized proteomic, transcriptomic, and functional activities coupled with PBPK approaches could enable appropriate scaling of the in vitro system.

In conclusions, we have reviewed the opportunities and limitations to study drug disposition, using recently published bio-engineered models developed from Caco-2, iPSCs, mucosal scrappings, and human primary intestinal crypts. No single model meets all the needs of drug metabolism scientists, due to limitations of each of the systems described in this mini-review. Models described in this mini-review duplicate some of the enzymes and transporters expressed in human enterocytes, and may need additional optimization for physiological relevance as well as an improved throughout (medium to high). Engineering and biochemical attributes of future human derived models should be designed, optimized and scaled considering long-term, end-user needs and by evaluating end-points discussed in **Figure 1**.

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Declaration of conflicts of interest

The authors hereby declare no conflicts of interest.

Authorship Contributions:

Wrote or contributed to the writing of the manuscript: Sawant-Basak, Rodrigues, Lech, Doyonnas, Kasaian, Prasad, Tsamandouras.

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Figure legends:

Figure 1. Proposed characterization and optimization of humanized intestinal *in vitro* models, for drug disposition studies

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Tables

Table 1. Proteomic abundance of clinically relevant drug metabolizing enzymes (Paine et al., 1997; Paine et al., 2006; Drozdzik et al., 2017)

	pmol/mg of protein		
DME	jejunum	ileum	HIM
CYP3A4	6.26-9.84	8.03-12.9	8.8-150
CYP3A5	ND	ND	4.9-25
CYP2C19	<0.122-0.15	<0.122-0.961	<0.6-3.9
CYP2D6	<0.14-1.18	<0.139-1.16	<0.2-1.4
CYP2J2	<4.15-5.28	2.71-5.55	<0.2-3.1
CYP2C9	ND	ND	2.9-27
CYP4A11	<0.126-0.246	<0.122-0.129	ND
CYP51A1	0.722-1.33	0.7-1.07	3.6-7.7
UGT1A1	<0.109-4.57	<0.111-5.05	ND
UGT1A6	0.574-1.76	<0.513-1.54	ND
UGT2B7	<2.11-5.29	<2.13-5.59	ND
UGT2B17	4.91-50.7	7.63-33.7	ND

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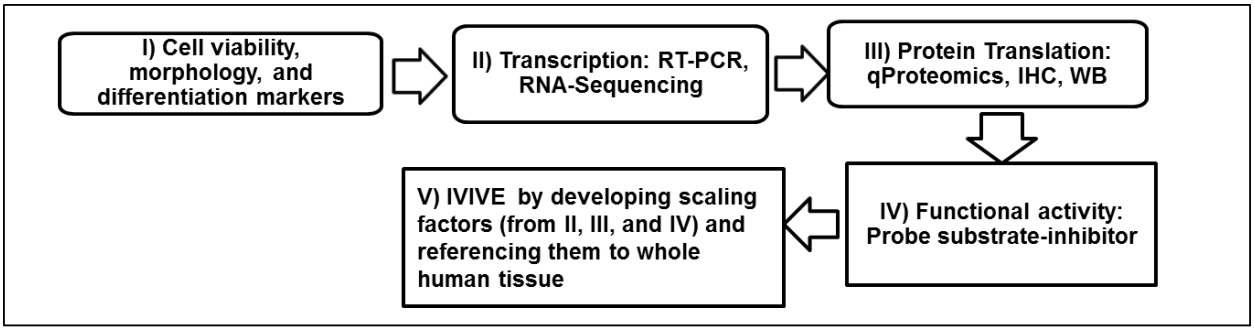
Table 2. Proteomic abundance of clinically relevant drug transporters (Drozdzik et al., 2014; Burt et al., 2016; Drozdzik and Oswald, 2016; Miyauchi et al., 2016)

	pmol/mg of protein		
Transporter	jejunum	ileum	colon
ABCB1 (Pgp)	0.4-3.49	2.43-7.42	
ABCC2 (MRP2)	0.28-1.39	<0.205-1.16	0.15-0.37
ABCC3 (MRP3)	<0.213-0.58	<0.129-0.303	0.95-1.77
ABCG2 (BCRP)	0.34-6.26	3f-13	1.53-2.11
SLC10A2 (ASBT/IBAT)	0.01	0.18	0.04-0.16
SLC15A1 (PEPT1)	3.57-13.1	1.86-19.5	traces
SLCO2B1(OATP2B1)	0.4	0.4	0.19-0.31
SLC22A1(OCT1)	0.65		0.48-0.73
SLC22A3(OCT3)	0.0112-0.55	<0.19	0.47-0.73
SLC51A/B(OST-alpha/beta	0.557-4.63	0.556-9.56	0.11-0.14
MRP4	0.323-0.555	0.613-0.487	NA
MRP1	0.206-0.503	0.395-0.467	NA
MRP5	0.111-0.33	<0.0629-0.124	NA
MRP6	0.0896-0.37	<0.0796-0.25	NA
OAT2	0.110-0.33	0.12-0.28	NA
OAT3	0.0988-0.22	<0.168	NA
OATP1B3	0.058-0.27	<0.0507-0.217	NA
PEPT2	0.107-0.54	<0.0881-0.385	NA

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Figure 1.



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