Bioprinted 3D Primary Human Intestinal Tissues Model Aspects of Native Physiology and ADME/Tox Functions

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HIGHLIGHTS

- Bioprinted 3D human intestinal tissues enable complex modeling of ADME/Tox in vitro
- 3D intestinal tissues develop barrier function and polarized transporter expression
- Key cytochrome P450 enzymes are expressed, metabolically active, and inducible
- GI toxicants can trigger barrier disruption and cytotoxicity in 3D intestinal tissues

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Bioprinted 3D Primary Human Intestinal Tissues Model Aspects of Native Physiology and ADME/Tox Functions

Lauran R. Madden,1 Theresa V. Nguyen,2 Salvador Garcia-Mojica,1 Vishal Shah,1 Alex V. Le,1 Andrea Peier,3 Richard Visconti,3 Eric M. Parker,3 Sharon C. Presnell,1 Deborah G. Nguyen,1 and Kelsey N. Retting1,4,*

SUMMARY
The human intestinal mucosa is a critical site for absorption, distribution, metabolism, and excretion (ADME)/Tox studies in drug development and is difficult to recapitulate in vitro. Using bioprinting, we generated three-dimensional (3D) intestinal tissue composed of human primary intestinal epithelial cells and myofibroblasts with architecture and function to model the native intestine. The 3D intestinal tissue demonstrates a polarized epithelium with tight junctions and specialized epithelial cell types and expresses functional and inducible CYP450 enzymes. The 3D intestinal tissues develop physiological barrier function, distinguish between high- and low-permeability compounds, and have functional P-gp and BCRP transporters. Biochemical and histological characterization demonstrate that 3D intestinal tissues can generate an injury response to compound-induced toxicity and inflammation. This model is compatible with existing preclinical assays and may be implemented as an additional bridge to clinical trials by enhancing safety and efficacy prediction in drug development.

INTRODUCTION
Systemic availability, diminished efficacy, and off-target effects remain challenges to the successful clinical prediction of candidate drugs and contribute to attrition in drug development, highlighting the need for better preclinical tools to model intestinal function in vitro (Alqahtani et al., 2013; Jones et al., 2016; Peters et al., 2016). Oral delivery is the most common method for drug administration, and in addition to absorption and first-pass metabolism, the intestine serves directly as a site of off-target toxicity for compounds such as non-steroidal anti-inflammatory drugs (NSAIDs) (Boelsterli et al., 2013) and chemotherapeutic agents (Aprile et al., 2013) and indirectly as a site of drug-drug interactions (Bentz et al., 2013; Peters et al., 2016). Animal models are frequently used to estimate compound bioavailability; however, species differences can lead to disparity in the expression of metabolic enzymes and transporters, resulting in poor clinical prediction (Jones et al., 2016; Musther et al., 2014; Peters et al., 2016). Human intestinal slices can provide correct cellular architecture and physiological complexity; however, limited viability ex vivo (about 24 hr) and incompatibility with cryopreservation restrict use to short-term studies (Li et al., 2016). Predominant in vitro systems used to study intestinal bioavailability and toxicity include intestinal microsomes and two-dimensional (2D) monolayers. Microsomes are a convenient tool for initial assessment of metabolism, but cannot model cellular level outcomes. Physiological conditions lacking in 2D culture may be improved by microfluidic chip models; however, maintenance of cell-cell and cell-matrix interactions and scale-up for high-throughput applications remain challenging (Bhatia and Inger, 2014; Chi et al., 2016). Standard 2D monolayers and more recent gut-on-a-chip systems commonly include cell lines originating from colorectal and duodenal tumor tissue (Alqahtani et al., 2013; Kim et al., 2012) that may exhibit altered metabolic profiles and expression patterns of xenobiotic-metabolizing enzymes and transporters potentially leading to misrepresentation of the native response (Prueksaritanont et al., 1996; Yamaura et al., 2016). The colorectal tumor-derived Caco-2 cell line is the most established model to study permeability and predict intestinal absorption. Known limitations of the Caco-2 model include reduced CYP450 activity (Prueksaritanont et al., 1996; Yamaura et al., 2016), variability with passage number, and inconsistencies between subclones of the cell line (Bentz et al., 2013).

Limitations of common in vitro models have sparked the development of methods utilizing primary human intestinal cells. Although primary monocolonies more closely resemble in vivo tissue compared with cell lines (Kauffman et al., 2013; Takenaka et al., 2016), separation of the epithelium from supportive cells...
may impair function and limit the ability to study complex cell interactions including interplay with interstitial cells or inflammatory responses. The discovery of organoids to expand primary cells (Sato et al., 2011; Yin et al., 2014) or differentiate pluripotent stem cells (Kauffman et al., 2013; Watson et al., 2014) revealed another path to model the intestine in vitro. Organoids derived from all regions of the intestinal tract (Wang et al., 2015) can be applied to intestinal research including organ development, disease modeling, and regenerative medicine (Fatehullah et al., 2016; Sinagoga and Wells, 2015), although they may lack in vivo organ physiology. Notably, the closed lumen of the organoid structure with inward epithelial orientation makes the apical surface relatively inaccessible for absorption, distribution, metabolism, and excretion (ADME) assays. Approaches to create accessible compartments can require a complex bioreactor setup (Schweinlin et al., 2016) and produce monolayers with non-physiological transepithelial electrical resistance (TEER) (Kauffman et al., 2013; VanDussen et al., 2015). Cell-seeded engineered three-dimensional (3D) tubular scaffolds have recently been utilized with hollow lumens to promote a more native-like microenvironment (Chen et al., 2017); however, functional characterization is restricted to the apical compartment by geometry, making most standard ADME/Tox assays that require access to both apical and basal compartments incompatible.

To overcome existing limitations of the current in vitro systems, a proprietary bioprinting platform was utilized to develop a multicellular 3D primary human intestinal tissue model to better mimic native tissue structure and function (Nguyen et al., 2016; King et al., 2017). The fully human primary cell-derived tissue designed with laminar architecture incorporates a polarized intestinal epithelium supported by an interstitial tissue layer allowing compatibility with standard histological and biochemical ADME/Tox readouts. Tissues maintained viability over 2 weeks in culture, developed barrier function, and demonstrated selective permeability. Presence of functional key CYP450 metabolic enzymes and transporters was confirmed with endogenous expression similar to native intestine. Last, tissue injury was evaluated in response to known toxicants indomethacin and tumor necrosis factor alpha (TNF-α) revealing barrier disruption and cytotoxicity at the biochemical and histological levels. The human tissue combined with the reproducibility of the automated 3D bioprinting platform and compatibility with standard assay approaches make this system a practical in vitro tool for ADME/Tox applications across drug development.

RESULTS
Histological Characterization of 3D Human Intestinal Tissue

The Organovo 3D NovoGen Bioprinter system was used to create an in vitro model of the human intestine. The tissue was generated with bilayered architecture, consisting of a human intestinal myofibroblast (IMF) interstitium supporting an epithelial layer containing human intestinal epithelial cells (hIEC) and maintained up to 21 days in culture. Tissues were printed on cell culture inserts (Figure 1A) allowing access to both apical and basolateral surfaces for direct compound testing. Histological analysis at day 17 showed that tissues exhibited polarized columnar epithelial morphology and secondary structure formation (Figures 1B–1I). The epithelial and interstitial tissue compartments are in direct contact with each other but remained distinct, with correct expression of epithelial (CK19) and myofibroblast (vimentin) cell-specific markers (Figure 1C). E-cadherin and ZO-1, key proteins involved in tight junction formation and barrier function, were expressed between epithelial cells of the hIEC layer (Figure 1D). Correct polarization of the hIEC and brush border formation were seen by positive staining for brush border protein villin at the apical surface (Figure 1E). Periodic acid-Schiff (PAS)/Alcian blue staining confirmed an apical brush border and suggested the presence of a subpopulation of goblet cells as well as the excretion of mucus (Figure 1F). Immunohistochemistry for mucin-2 confirmed the presence of goblet cells and mucous secretion (Figure 1G), a feature similar to native tissue and an indication of normal intestinal function. In addition to goblet cells, other specialized cell types present in the intestinal epithelium included lysozyme-positive Paneth cells and chromogranin-expressing enteroendocrine cells (Figures 1H and 1I). Patterning of 3D intestinal tissues was similar to that observed in native intestinal tissue and consistent between multiple donors (Figure S1). Tissue architecture of the 3D intestinal model was also compared with that of standard Caco-2 monolayer culture (Figure S2). Caco-2 monolayers achieved a polarized epithelial phenotype with tight junction formation, although epithelial morphology appeared less columnar and lacked secondary structure formation. Subpopulations of specialized cells and evidence of mucus production were also absent in Caco-2 monolayers (Figure S2). Substitution of hIEC with Caco-2 cells in the 3D bioprinted model produced a bilayered structure with increased thickening of the epithelial layer and cyst formation over time, akin to the cancer origin (Figure S3A). However, this phenotype change did not improve transporter or enzyme gene expression to the level of 3D intestinal tissues fabricated with primary hIECs (Figure S3B).
Thus the standard Caco-2 monolayer was used as the main comparison to bioprinted tissues composed of primary hIECs.

Characterization of Gene Expression

Gene expression analysis was utilized to further evaluate the expression of key intestinal epithelial tissue markers, metabolic enzymes, and transporters in the 3D intestinal tissue and was compared with both native donor intestinal tissue and standard Caco-2 monolayers (Figure 2). Although five native donor tissues were processed, challenges in obtaining high-quality donor material resulted in the utilization of three of five donors for gene expression analysis. To specifically study differential expression in the epithelium and to remove any variance in the total cell number, genes were analyzed relative to the expression of epithelial-specific marker CK19. Heatmap visualization shows close clustering of 3D intestine to the native intestine compared with more disparate Caco-2 monolayers (Figure 2A). In support of histological findings, tight junction marker E-cadherin (CDH1) and brush border marker villin (VIL1) were highly expressed, as well as markers for specialized cell subpopulations including Paneth cells (LYZ), enteroendocrine cells (CHGA), and goblet cells (MUC2) (Figure 2B). Key xenobiotic-activated nuclear receptors involved in drug metabolism and disposition including VDR, PXR (NR1I2), and CXR (NR1I3) were expressed in 3D intestinal tissue at comparable levels to native intestine. Major intestinal Phase I CYP450 metabolic enzymes including CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP2J2 were also detected. Clinically important CYP3A4 was highly expressed in the 3D intestinal model, whereas it was notably not detectable in Caco-2 monolayers (Figure 2B). Key intestinal Phase II metabolic enzymes GSTP1 and UGT1A1 were expressed, as well as regulators of fatty acid metabolism, including DGAT1, MOGAT2, and MTTP. Major efflux transporters P-gp (ABCB1, MDR1) and BCRP (ABCG2), key uptake transporters PEPT1 (SLC15A1) and OATP2B1 (SLCO2B1), and intestinal bile acid-related transporters ASBT (SLC10A2), OSTa (SLC51A), and OSTb (SLC51B) were expressed in 3D intestinal tissues with levels comparable with those in native intestine (Figure 2B). Collectively, gene expression levels in 3D intestinal tissues corresponded to endogenous levels observed in native tissue, whereas Caco-2 monolayers exhibited reduced, overexpressed, or absent expression of several genes suggesting that the bioprinted model more closely resembles native tissue.
Demonstration of Cytochrome P450 Metabolic Function in 3D Intestinal Tissue

Functional assays were performed to evaluate the activity and specificity of CYP3A4 and CYP2C9, two major intestinal Phase I cytochrome P450 metabolic enzymes profiled in gene expression studies (Figure 3). CYP2C9 activity was readily detected in 3D intestinal tissue by luminogenic P450 substrate conversion and could be significantly inhibited by sulfaphenazole (Figure 3A). CYP3A4 activity and specific inhibition by ketoconazole were confirmed by both a luminogenic P450 substrate conversion (Figure 3B) and midazolam metabolite formation (Figures 3D and 3E). Luminogenic substrate conversion occurred at a higher level in 3D intestine compared with Caco-2 monolayers (Figure 3C), and only 3D tissues were induced by rifampicin treatment (Figures 3C and 3E). Rifampicin treatment was associated with significantly higher turnover of CYP3A substrates in treated tissues and significant upregulation of PXR-inducible genes including CYP2C9, CYP3A4, CYP3A5, P-gp, and UGT1A1 (Figures 3E and 3F). Uptake transporters SLC15A1 (PEPT1) and SLC28A1 (OATP2B1) are similarly expressed by native and 3D intestine, but not by Caco-2 monolayers. Data in (B) are expressed as mean ± SD.

Demonstration of Cytochrome P450 Metabolic Function in 3D Intestinal Tissue

The intestine is a selectively permeable barrier, regulating absorption of both nutrients and xenobiotics. TEER was utilized to measure barrier function in 3D intestinal tissues over a 21-day culture period. Measurements demonstrated that the tissues developed and maintained barrier function between days 10 and 21 of culture, exhibiting values within a physiological range (50–100 Ω*cm²) comparable to native human intestine (Srinivasan et al., 2015) (Figure 4A). Representative compounds with high and low permeability were used to further validate 3D intestinal tissue barrier function (Figure 4B, Table S2). Paracellular transport marker lucifer yellow correctly demonstrated low permeability in 3D intestine (P_app = 0.45 ± 1.5 x 10⁻⁶ cm/s), suggesting the...
presence of an intact physical barrier for drug transport. The 3D intestinal tissues correctly distinguished between low-, intermediate-, and high-permeability compounds. Mitoxantrone (Papp = 0.2 ± 0.03 x 10^6 cm/s) is a low-permeability compound with absorption mainly mediated by ABCG2 (BCRP) efflux transporter. Topotecan and digoxin showed Papp values in the intermediate range (3.1 ± 0.63 x 10^6 cm/s and 8.53 ± 2.02 x 10^6 cm/s, respectively), whereas propranolol, a passive transcellular transport reference compound, showed the highest permeability (Papp = 14.8 ± 1.96 x 10^6 cm/s).

In comparison, Caco-2 monolayers demonstrated significantly higher TEER measurements (840 ± 55 Ω·cm²) (Figure 4C). When substituted for hIEC in the 3D bioprinted model, 3D Caco-2 tissues had reduced TEER (254 ± 45 Ω·cm²) compared with their 2D counterparts, a possible effect of cyst-like secondary structure formation, but the values remained above a physiological range. Both 2D and 3D Caco-2 models had low lucifer yellow permeability (Papp = 0.46 ± 0.25 x 10^6 cm/s and 1.05 ± 0.1 x 10^6 cm/s, respectively) confirming barrier formation, whereas IMF tissue alone did not show barrier function (163 ± 29 x 10^6 cm/s) (Figure 4D).

**Transporter Localization and Function in 3D Intestinal Tissue Model**

Intestinal efflux and uptake transporters can be both sites of drug-drug interactions and limiting factors for drug absorption. Immunohistochemical staining confirmed the correct polarized epithelial expression of key efflux transporters at the apical (P-gp [ABCB1], BCRP [ABCG2], MRP2 [ABCC2]) and basolateral (MRP3 [ABCC3]) surfaces in a continuous pattern similar to native intestine (Figures 5A, 5B, and S4). To assess the capability of 3D intestinal tissues to predict active efflux, P-gp and BCRP function were evaluated by measuring bidirectional transport with and without inhibition (Figures 5C and 5D). Under control conditions, asymmetric permeability of P-gp substrate digoxin was observed with an efflux ratio greater than 2. Inhibition of P-gp by zosuquidar (Dantzig et al., 1996) decreased the rate of B to A transport, reducing the efflux ratio to 1.2, to confirm the activity of P-gp. BCRP function was tested by efflux of topotecan (Figure 5D) and mitoxantrone (Figure S5). BCRP/P-gp substrate topotecan and BCRP substrate mitoxantrone were preferentially transported in the B to A direction with efflux ratios of 8.8 and 190, respectively. Furthermore, subsequent inhibition of topotecan transport by the BCRP inhibitor Ko143 (Allen et al., 2002) reduced the efflux ratio to 3.6 and dual inhibition with Ko143 and zosuquidar further decreased the...
efflux ratio to 1.4. Collectively, these results demonstrate that the 3D intestinal tissue model expresses clinically relevant P-gp and BCRP transporters with proper localization and function. Compounds assessed demonstrate greater efflux ratio similarity to other models composed of primary human intestinal cells than to Caco-2 models (Table S3). Histological analysis showed apical P-gp and BCRP expression in patches across Caco-2 monolayers, whereas MRP2 and MRP3 appeared to be overexpressed (Figures 5A, 5B, and S4). Transporter expression patterning was not rescued by substitution of Caco-2 cells into the 3D bioprinted model (Figure S3).

Characterization of 3D Intestinal Tissues as a Model for Gastrointestinal Toxicity

The utility of the 3D intestinal model for compound toxicity applications was evaluated by the NSAID indomethacin, a prostaglandin E2 (PGE2) oxygenase inhibitor and known gastrointestinal (GI) toxicant that can reduce intestinal epithelial barrier function through enterocyte apoptosis and necrosis (Boelsterli et al., 2013). The 3D intestinal tissue showed a dose-dependent decrease in barrier function as measured by TEER in response to 24-hr indomethacin treatment (Figure 6A). Injury to the intestinal cells was detected by lactate dehydrogenase (LDH) release and was significantly increased in the presence of indomethacin doses above 0.25 mM (Figure 6B), consistent with previous reports modeling indomethacin injury in vitro (Tang et al., 1993). All doses of indomethacin demonstrated inhibition of prostaglandin E2 secretion, confirming the known mechanism of activity (Figure 6C). Histological analysis further illustrated dose-dependent disruption of epithelial morphology with decreased E-cadherin expression, consistent with loss in barrier function (Figure 6D). Release of inflammatory cytokines may also damage the intestine, and TNF-α is a major contributor to disease-related inflammation and intestinal toxicity (Neurath, 2014; Peyrin-Biroulet, 2010). To evaluate the susceptibility of the model to pro-inflammatory cytokines, tissues were dosed with TNF-α for 24 hr and evaluated for changes in morphology, LDH release, and gene expression.
expression (Figure 7). TNF-α altered the epithelial morphology and resulted in the dissociation of cells from the interstitial layer accompanied by a significant increase in LDH release, suggesting a cytotoxic response and enterocyte death. Gene expression for several known targets of the TNF-α/nuclear factor (NF)-κB pathway including interleukin (IL)-6, IL-8, CCL2, CXCL10, ICAM1, and TNF-α were upregulated, demonstrating activation of an inflammatory cascade (Figure 7C) in response to tissue injury.

**DISCUSSION**

Current preclinical models are limited in the ability to capture complexities and function of human intestinal tissue, which can lead to poor predictability in drug development (Alqahtani et al., 2013; Aprile et al., 2015; Boelsterli et al., 2013; Jones et al., 2016; Peters et al., 2016). This study incorporated primary cells into a fully human 3D bioprinted intestinal tissue model to recapitulate multiple facets of native intestinal biology and function in vitro. Histological analysis of the 3D intestinal tissues confirmed development and maintenance of a polarized epithelium with columnar epithelial morphology, tight junctions, and apical brush border formation. Stability of tissue architecture and function for greater than 2 weeks in culture suggests that the model may be suitable for extended compound studies.

In this study, the 3D intestinal model was compared with Caco-2 cells cultured as a standard 2D monolayer with results suggesting that the 3D tissues more closely mimic native tissue in gene and protein expression.
patterning and function. The divergence of Caco-2 cells from native tissue, consistent with previous reports (Prueksaritanont et al., 1996; Takenaka et al., 2014), may be due in part to their cancer origin and variability in expression patterns of metabolic enzymes and transporters along the GI tract (Paine et al., 1997; Peters et al., 2016). Both native donor tissue and the 3D intestinal model were derived from the ileum, whereas Caco-2 cells are derived from the colon. Substitution of primary intestinal epithelial cells with Caco-2 cells in the 3D system produced phenotypes more consistent with native tissue, including secondary structure formation, indicating a benefit to the 3D microenvironment that may be in part influenced by cross talk with interstitial cells, but did not rescue aberrant expression patterning.

Histology and gene expression analyses demonstrated the presence of specialized cell subpopulations in 3D intestinal tissues that are notably absent in cell lines including the Caco-2 model. The presence of mucin-2-expressing goblet cells, evidence of mucus production, and lysozyme-expressing Paneth cells suggests the potential for modeling mucosal barrier function and antimicrobial or microbiome function. Chromogranin-positive cells suggest the potential to study enteroendocrine function in the gut, including glucagon-like peptide (GLP)-1 signaling. Cell subpopulations indicate that primary cells in 3D intestinal tissues may be able to undergo directed differentiation by modification of culture conditions similar to maturation observed in organoid cell culture (Sato et al., 2011; Spence et al., 2011; Yin et al., 2014). Incorporation of induced pluripotent stem cells may be considered as an alternative cell source to primary cells, although maturity can resemble a fetal stage phenotype (Sinagoga and Wells, 2015) with inconsistent barrier function over time (Kauffman et al., 2013).

The 3D intestinal tissue demonstrated the expression and function of key enzymes involved in xenobiotic metabolism including CYP3A4, an enzyme known to be absent in Caco-2 models (Yamaura et al., 2016), consistent with our findings. Enzyme activity was confirmed by modulation of midazolam hydroxylation.

Figure 6. Indomethacin Toxicity in 3D Intestinal Tissue
(A) TEER measurements of tissues following 24-hr incubation with ethanol vehicle or varying doses of indomethacin show dose response decrease in TEER with increasing indomethacin.
(B) LDH activity increased with increasing indomethacin dose, suggesting increased cytotoxicity.
(C) Prostaglandin E2 synthesis decreased to similar levels for all indomethacin doses tested, confirming drug activity (n = 6–7, A–C).
(D) Histology of indomethacin-treated tissues shows disruption of the epithelium and distorted nuclear staining at higher doses of indomethacin, accompanied by a reduction in E-cadherin, a marker of barrier function. Level of significance: ****p < 0.0001 by one-way ANOVA. Data in (A–C) are expressed as mean ± SD.
through both inhibition and induction—CYP3A4 activity and PXR-regulated genes were induced in response to rifampicin. The 3D intestinal model demonstrated reproducible midazolam metabolism in tissues separately fabricated from three different donors with interindividual donor variation as expected (Paine et al., 1997). Values were similar to those shown for intestinal slices (van de Kerkhof et al., 2006) and much higher than those reported for 2D systems (Takenaka et al., 2014; Yamaura et al., 2016), suggesting suitability of this model for drug-induced metabolic and transporter studies, which cannot be achieved by previous primary or Caco-2 models (Takenaka et al., 2014; Yamaura et al., 2016).

A differentiating factor of this 3D model over other 3D primary cell systems including organoids and more recent cell-seeded tubular scaffolds (Chen et al., 2017) is the laminar architecture that provides both apical and basal access and compatibility with standard barrier function and directional transport assays. Physiological barrier function was successfully demonstrated by TEER (74.4 ± 24.5 Ω•cm²) in 3D intestinal tissues at day 10 and maintained through day 21 of culture. Furthermore, tissues could successfully differentiate between low-, intermediate-, and high-permeability substrates such as paracellular marker lucifer yellow and transcellular marker propranolol. With the advantage of physiological TEER values, models with hiECs may yield a better correlation with in vivo permeability compared with Caco-2 monolayers with artificially high measurements (840 ± 55 Ω•cm²), which may be due in part to observed differences in E-cadherin expression (Srinivasan et al., 2015). Barrier measurements in the 3D intestinal model were consistent with reported monolayer cultures of adult intestinal epithelial cells (70–120 Ω•cm²) (Takenaka et al., 2016). These monolayer models, however, are typically limited to <11 days in culture, can suffer from low CYP expression similar to Caco-2 cells, and have not yet been assessed for toxicity or inflammatory response (Kauffman et al., 2013; Takenaka et al., 2014, 2016).

Intestinal efflux and influx transporters are key mediators of absorption for low-permeability drugs. Clinically relevant P-gp and BCRP efflux transporters, which can significantly affect the net fraction of compound absorbed (Bentz et al., 2013; Peters et al., 2016), were correctly expressed in the apical epithelium and

Figure 7. TNF-α-Induced Toxicity in 3D Intestinal Tissue
(A) Tissues treated with TNF-α for 24 hr showed increased epithelial disorganization compared with controls.
(B) Increased LDH activity correlated with changes in cell morphology following TNF-α treatment (n = 5–6).
(C) A subset of genes related to inflammation, TNF-α, IL-6, IL-8, CCL2, and ICAM, were upregulated in response to TNF-α treatment (n = 3). Level of significance: ***p < 0.001 by t test in (B), **p < 0.01, ***p < 0.001, and ****p < 0.0001 by two-way ANOVA in (C). Data in (B) and (C) are expressed as mean ± SD.
functional in response to known substrates digoxin and topotecan, respectively. The level of efflux in the 3D intestinal tissue model was more similar to that previously observed in 2D human intestinal cell monolayers than with Caco-2 monolayers (Kauffman et al., 2013; Li et al., 2008; Takenaka et al., 2014). Expression of functional transporters suggests that this system could be applied to assess the relative contributions of efflux transporters to drug disposition, or could be used as a potential model for increased absorption by targeting uptake transporters such as PEPT1 and OATP2B1. The dual presence of transporters and enzymes in the 3D intestinal tissue model suggests that it could be used to shed light on complex drug-drug interactions, such as those seen with overlapping P-gp/CYP3A4 substrates (Kim et al., 1999). Furthermore, high expression of enzymes involved in fatty acid metabolism in the 3D intestinal tissue indicates a potential application for evaluating compounds targeting these enzymes to combat obesity (Shi and Cheng, 2009).

GI toxicity is a common clinical adverse event that cannot be accurately predicted or characterized with current in vitro or in vivo models (Aprile et al., 2015; Boelsterli et al., 2013; Peyrin-Biroulet, 2010). The NSAID indomethacin demonstrated a toxicity response in 3D intestinal tissue with a dose-dependent decrease in barrier function by TEER and corresponding epithelial cell disruption, correlating with previously reported in vitro (Tang et al., 1993; Tomisato et al., 2001) and in vivo outcomes (Boelsterli et al., 2013). The 3D intestinal tissue also responded to the toxic inflammatory stimulus TNF-α, resulting in epithelial cell death and upregulation of inflammatory genes, consistent with human data (Christophi et al., 2012) and previous Caco-2 studies (Cui et al., 2010; Treede et al., 2009). These data combined with long-term viability suggest that the 3D intestinal model may be applied to characterize other known classes of compounds that have off-target toxicity in the intestine, such as chemotherapeutics (Aprile et al., 2015), to evaluate inflammatory pathways, and to model chronic diseases, such as inflammatory bowel disease (IBD), Crohn disease, and colitis (Aprile et al., 2015; Boelsterli et al., 2013; Peyrin-Biroulet, 2010). Future applications could increase complexity by incorporating additional cell types such as immune cells or cancer cells, and by utilizing healthy and diseased donor material (Fatehullah et al., 2016; VanDussen et al., 2015), to better understand the contribution of each cell type to the disease phenotype and to better characterize candidate modulators for therapeutic intervention on disease-relevant backgrounds.

In conclusion, we provide evidence for a fully human 3D intestinal tissue model composed of primary cells with increased complexity and function compared with standard in vitro models. The 3D tissue recapitulates major intestinal features and physiological functions and is designed to provide a flexible platform compatible with barrier function, permeability, metabolism, transport, and toxicity studies.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Transparent Methods, five figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.isci.2018.03.015.

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AUTHOR CONTRIBUTIONS
Conceptualization, L.R.M., T.V.N., A.P., R.V., E. M.P., S.C.P., D.G.N., K.N.R.; methodology, L.R.M, S.G.-M., V.S., D.G.N., K.N.R.; formal analysis, L.R.M., S.G.-M., V.S., A.V.L., K.N.R.; investigation, L.R.M., S.G.-M., V.S., A.V.L.; Resources, S.C.P.; writing: original draft, L.R.M., K.N.R.; writing: review and editing, L.R.M., T.V.N., V.S., A.P., R.V., E.M.P., S.C.P., D.G.N., K.N.R.; visualization, L.R.M., S.G.-M., A.V.L.; supervision, L.R.M., S.C.P., D.G.N., K.N.R.; project administration, L.R.M., T.V.N., A.P., R.V., E.M.P., S.C.P., D.G.N., K.N.R.; funding acquisition, T.V.N., A.P., R.V., E.M.P., S.C.P., D.G.N., K.N.R.

DECLARATION OF INTERESTS
L.R.M., S.G.-M., V.S., A.V.L., S.C.P., D.G.N., and K.N.R. are currently or have been employed by and may be shareholders of Organovo, Inc. T.V.N., A.P., R.V., and E.M.P. are currently or have been employed by and may be shareholders of Merck.
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Supplemental Information

Bioprinted 3D Primary Human Intestinal Tissues Model Aspects of Native Physiology and ADME/Tox Functions

Lauran R. Madden, Theresa V. Nguyen, Salvador Garcia-Mojica, Vishal Shah, Alex V. Le, Andrea Peier, Richard Visconti, Eric M. Parker, Sharon C. Presnell, Deborah G. Nguyen, and Kelsey N. Retting
Figure S1, related to Figure 1. Comparative histology of native intestine and two donors of 3D intestinal tissue. H&E shows that both native intestine and 3D intestine have a bi-layered structure containing CK19 expressing epithelial cells and vimentin stained myofibroblasts. Native intestine and both donors of 3D intestinal tissue express E-Cadherin continuously within the epithelium, indicating tight junction formation.
**Figure S2, related to Figure 1. Caco-2 histology.** Day 21 monolayers of Caco-2 cells were stained for general and specialized cell subtype epithelial markers. Caco-2 express CK19, E-Cadherin, and villin across the monolayer. No staining was observed for chromogranin, lysozyme, or mucin-2.
Figure S3, related to Figure 1 and Figure 5. 3D Bioprinted Caco-2 tissues form a thick bi-layered structure. CK19 expressing Caco-2 cells expand over time while vimentin positive IMF remain constant (a). Villin staining shows disorientation of epithelial cells and the formation of cyst-like structures over time. Low amounts of P-gp are present at both days 7 and 14. While BCRP expression increased over time, much is found in the cyst-like structures compared to normal apical expression. (b) Gene expression of several key transporters and CYP3A4 is reduced or absent in 2D and 3D Caco-2 tissues compared to 3D intestinal tissue fabricated with primary human intestinal epithelial cells (n=3). nd= not determined. Data are represented as ± STD.
Figure S4, related to Figure 5. MRP2 and MRP3 transporter expression. Native intestine, 3D intestinal tissue, and Caco-2 monolayers were compared for expression of MRP2 (ABCC2) and MRP3 (ABCC3). Similar levels of MRP staining were observed between native intestine and 3D intestine with higher levels seen in Caco-2 monolayers.
Figure S5, related to Figure 5. BCRP efflux of mitoxantrone. Low permeability A to B of mitoxantrone, a BCRP substrate, was observed with much higher permeability in the B to A direction, efflux ratio = 190. In the presence of BCRP inhibitor Ko143, Mitoxantrone permeability B to A decreased and efflux ratio reduced to 145. Note: samples for A to B were near or below limit of detection. (n=4) Level of significance: ***P < 0.001 by two-way ANOVA. Data are represented as ± STD.
Table S1, related to Figure 3. Interindividual variability in midazolam metabolism of 3D intestine. Data are represented as ± STD.

|             | Untreated pmol/min/mg | Ketoconazole pmol/min/mg | % untreated | Rifampicin pmol/min/mg | % untreated |
|-------------|-----------------------|--------------------------|-------------|------------------------|-------------|
| Donor 1     | 19.3 ± 1.9            | 2.7 ± 0.2                | 13.9 ± 1.0  | 26.2 ± 3.9             | 136 ± 20    |
| Donor 2     | 2.85 ± 0.59           | 0.81 ± 0.2               | 28.4 ± 7.5  | 10.1 ± 2.0             | 355 ± 69    |
| Donor 3     | 3.6 ± 3.6             | 0.67 ± 0.1               | 18.6 ± 3.4  | 10.1 ± 0.9             | 281 ± 25    |
**Table S2, related to Figure 4.** Comparison of human absorbed fraction (Fa) to $P_{\text{app}}$ ($\times 10^{-6}$ cm/s) of 3D intestine and 2D monolayers. Lucifer yellow (n=28), mitoxantrone (n=4), topotecan (n=4), digoxin (n=6), propranolol (n=5). Data are represented as ± STD for 3D intestine.

| Compound    | Fa (%) | $P_{\text{app}}$ ($\times 10^{-6}$ cm/s) | Pathway                  | Ref. for 2D hIEC and Caco-2 values |
|-------------|--------|------------------------------------------|--------------------------|-----------------------------------|
|             |        | 3D intestine | 2D hIEC | 2D Caco-2 |                               |
| Lucifer yellow | n/a    | 0.45 ± 1.5 | < 1     | < 1       | Paracellular                  | Bentz et al. (2013) and Kauffman et al. (2013) |
| Mitoxantrone    | n/a    | 0.2 ± 0.03 | ~0.2    | ~0.1      | Transcellular / BCRP, P-gp    | Takenaka et al. (2014) |
| Topotecan      | 30     | 3.1 ± 0.63 | 0.78 ± 0.14 | 0.35 ± 0.06; 3.67 ± 0.02 | Transcellular / BCRP, P-gp    | Takenaka et al. (2014) and Li et al. (2008) |
| Digoxin        | 75     | 8.53 ± 2.02 | 3.5 ± 0.2 | 0.96 ± 0.03 | Transcellular / P-gp          | Takenaka et al. (2014) and Takenaka et al. (2016) |
| Propranolol    | 93     | 14.8 ± 1.96 | 22 ± 2  | 41 ± 3    | Transcellular                 | Takenaka et al. (2016) |
Table S3, related to Figure 5. Comparison of compound transport in 3D intestine and 2D monolayers.

| Compound     | Efflux Ratio \((B \rightarrow A)/(A \rightarrow B)\) | Pathway                | Ref. for 2D hIEC and Caco-2 values                              |
|--------------|-----------------------------------------------|------------------------|---------------------------------------------------------------|
|              | 3D intestine | 2D hIEC | 2D Caco-2 |                                                                 |
| Digoxin      | 2.1         | 2.2; 4-8| 3.1; 6.1 - 102 | Transcellular / P-gp | Takenaka et al. (2014); Kauffman et al. (2013); Benzt et al. (2013) |
| Topotecan    | 8.8         | n/a    | >2        | Transcellular / BCRP, P-gp                                   | Li et al (2008)                                              |
| Mitoxantrone | 190         | 38.2   | 4         | Transcellular / BCRP, P-gp                                   | Takenaka et al. (2014)                                       |
## Transparent Methods

*Materials used in experimental procedures*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-cytokeratin 19 antibody [EP1580Y] | Abcam | ab52625; RRID AB_2281020 |
| Anti-cytokeratin 19 antibody [A53-B/A2] | Abcam | ab7754; RRID AB_306048 |
| Vimentin antibody (V9) | SCBT | sc-6260; RRID AB_628437 |
| Chr-A antibody (C-20) | SCBT | sc-1488; RRID AB_2276319 |
| Anti-E-Cadherin antibody [EP700Y] | Abcam | ab40772; RRID AB_731493 |
| Anti-Villin antibody [SP145] | Abcam | ab130751; RRID AB_11159755 |
| Mucin 2 Antibody (H-300) | SCBT | sc-15334; RRID AB_2146667 |
| Anti-Lysozyme antibody [EPR2994(2)] | Abcam | ab108508; RRID AB_10861277 |
| Anti-P Glycoprotein antibody [EPR10364-57] | Abcam | ab170904; RRID AB_2687930 |
| Anti-BCRP/ABCG2 antibody [BXP-21] | Abcam | ab3380; RRID AB_303758 |
| Anti—MRP2 antibody [M2 III-6] | Abcam | ab3373; RRID AB_303751 |
| Anti-MRP3 antibody [M3II-9] | Abcam | ab3375; RRID AB_303753 |
| **Oligonucleotides** |        |            |
| 18s rRNA | Applied Biosystems | Assay ID: Hs99999901_s1 |
| ABCB1 | Applied Biosystems | Assay ID: Hs00184500_m1 |
| ABCC2 | Applied Biosystems | Assay ID: Hs00166123_m1 |
| ABCC3 | Applied Biosystems | Assay ID: Hs00978473_m1 |
| ABCG2 | Applied Biosystems | Assay ID: Hs01053790_m1 |
| CCL2 | Applied Biosystems | Assay ID: Hs00234140_m1 |
| CDH1 | Applied Biosystems | Assay ID: Hs01023894_m1 |
| CDH17 | Applied Biosystems | Assay ID: Hs00900408_m1 |
| CDX2 | Applied Biosystems | Assay ID: Hs01076080_m1 |
| CES2 | Applied Biosystems | Assay ID: Hs01077945_m1 |
| CHGA | Applied Biosystems | Assay ID: Hs00900375_m1 |
| Gene       | Manufacturer   | Assay ID                      |
|------------|----------------|-------------------------------|
| CK19       | Applied Biosystems | Assay ID: Hs00761767_s1       |
| CXCL8      | Applied Biosystems | Assay ID: Hs00174103_m1       |
| CXCL10     | Applied Biosystems | Assay ID: Hs00171042_m1       |
| CYP2C19    | Applied Biosystems | Assay ID: Hs00426380_m1       |
| CYP2C8     | Applied Biosystems | Assay ID: Hs02383390_s1       |
| CYP2C9     | Applied Biosystems | Assay ID: Hs02383631_s1       |
| CYP2D6     | Applied Biosystems | Assay ID: Hs02576167_m1       |
| CYP2J2     | Applied Biosystems | Assay ID: Hs00356035_m1       |
| CYP2S1     | Applied Biosystems | Assay ID: Hs00258076_m1       |
| CYP3A4     | Applied Biosystems | Assay ID: Hs00604506_m1       |
| CYP3A5     | Applied Biosystems | Assay ID: Hs00241417_m1       |
| DGAT1      | Applied Biosystems | Assay ID: Hs01017541_m1       |
| GAPDH      | Applied Biosystems | Assay ID: 4326317E            |
| GSTP1      | Applied Biosystems | Assay ID: Hs00943350_g1       |
| ICAM1      | Applied Biosystems | Assay ID: Hs00164932_m1       |
| IL-6       | Applied Biosystems | Assay ID: Hs00174131_m1       |
| IL-8       | Applied Biosystems | Assay ID: Hs00991010_m1       |
| LGR5       | Applied Biosystems | Assay ID: Hs00969422_m1       |
| LYZ        | Applied Biosystems | Assay ID: Hs00426232_m1       |
| MOGAT2     | Applied Biosystems | Assay ID: Hs00228268_m1       |
| MTTP       | Applied Biosystems | Assay ID: Hs00165177_m1       |
| MUC2       | Applied Biosystems | Assay ID: Hs003005103_g1      |
| NR1I2      | Applied Biosystems | Assay ID: Hs01114267_m1       |
| NR1I3      | Applied Biosystems | Assay ID: Hs00901571_m1       |
| COX-2      | Applied Biosystems | Assay ID: Hs00153133_m1       |
| SLC10A2    | Applied Biosystems | Assay ID: Hs01001557_m1       |
| SLC15A1    | Applied Biosystems | Assay ID: Hs00192639_m1       |
| Gene   | Assay Source   | Assay ID                        |
|--------|----------------|--------------------------------|
| SLC16A1| Applied Biosystems | Hs01560299_m1                   |
| SLC28A1| Applied Biosystems | Hs00984403_m1                   |
| SLC51A | Applied Biosystems | Hs00380895_m1                   |
| SLC51B | Applied Biosystems | Hs01057182_m1                   |
| SLC7A5 | Applied Biosystems | Hs01001183_m1                   |
| SLC7A8 | Applied Biosystems | Hs00794796_m1                   |
| SLCO2B1| Applied Biosystems | Hs01030343_m1                   |
| TNF    | Applied Biosystems | Hs01113624_g1                   |
| UGT1A1 | Applied Biosystems | Hs02511055_s1                   |
| VDR    | Applied Biosystems | Hs00172113_m1                   |
| VIL1   | Applied Biosystems | Hs01031724_m1                   |

**Chemicals, Peptides, and Recombinant Proteins**

| Chemical                  | Manufacturer     | Catalogue Number |
|---------------------------|------------------|------------------|
| Digoxin                   | Sigma Aldrich    | 04599-50MG       |
| Zosuquidar                | Sigma Aldrich    | SML1044-5MG      |
| Lucifer yellow            | Sigma Aldrich    | L0144            |
| Mitoxantrone              | Cayman chemicals | 14842            |
| Topotecan                 | Cayman chemicals | 14129            |
| Ko143                     | Cayman chemicals | 15215            |
| Propranolol               | Cayman chemicals | 17291            |
| Ketoconazole              | Sigma Aldrich    | K1003-100MG      |
| Sulfaphenazole            | Cayman chemicals | 14844            |
| Rifampicin                | Sigma Aldrich    | R8883-150MG      |
| Midazolam                 | Cayman chemicals | 19391            |
| Indomethacin              | Cayman chemicals | 70270            |
| TNF α                     | R&D Systems      | 210-TA-005/CF    |

**Critical Commercial Assays**

| Assay Kit                        | Manufacturer     | Catalogue Number |
|----------------------------------|------------------|------------------|
| RNeasy Mini Kit                  | Qiagen           | 74106            |
| SuperScript III First-Strand Synthesis SuperMix for qRT-PCR | ThermoFisher  | 11752050         |
| TaqMan Universal Master Mix II with UNG | ThermoFisher  | 44400038         |
| TaqMan Fast Virus 1-Step Master mix | ThermoFisher  | 4444434          |
| P450-Glo CYP2C9 Assay            | Promega          | V8791            |
| P450-Glo CYP3A4 Assay – Luciferin IPA | Promega        | V9001            |
| Pierce BCA Protein Assay kit     | Thermo Fisher Scientific | 23227            |
| Lactate dehydrogenase assay kit  | Abcam            | 102526           |
| Prostaglandin E2 ELISA          | Cayman chemicals | 514010           |
**Experimental Models: Cell Lines**

| Cell Line                              | Supplier       | Catalog #       |
|----------------------------------------|----------------|-----------------|
| Caco-2 cell line                       | Sigma          | Cat # 86010202-1VL; RRID CVCL_0025 |
| Human intestinal myofibroblasts (IMF)  | Lonza          |                 |

| Other Tools                           | Supplier       | Item #          |
|---------------------------------------|----------------|-----------------|
| NovoGen Bioprinter                    | Organovo       | n/a             |
| EVOM2 Epithelial Voltohmmeter         | World Precision Instruments | # EVOM2 |
| STX2 chopstick electrodes             | World Precision Instruments | # STX2 |

**Intestinal cell isolation and cell culture**

We acknowledge the use of tissues procured by the National Disease Research Interchange (NDRI) with support from NIH grant 2 U42 OD011158 and protocol approved by NDRI and Organovo Inc. Native intestine was processed from five donors, three of which resulted in primary intestinal epithelial cell isolation (1: 18 / M; 2(native donor A): 29 / M; 3: 23 / M; 4(native donor B): 36 / M; 5(native donor C): 57 / M). Adult human intestinal myofibroblast (IMF) cells were sourced from Lonza (age/sex not available). The Caco-2 cell line (derived from a primary colonic tumor of a 72 year old male) was used at passage 46 as a comparison to primary human cells.

Human intestinal epithelial cells (hIEC) were isolated from and cultured according to reported methods (Sato et al., 2011, Lahar et al., 2011). Briefly, ileal tissue was received on ice, aseptically cleaned with ice cold PBS, then cut into three inch segments. Segments were opened and mucosectomy was performed, then tissues were minced to about five by five millimeter chunks. Tissue pieces were washed vigorously in PBS by vortexing, then epithelial cells were dissociated by incubation in PBS with EDTA and DTT. Cells were dissociated from the tissue by repeat vortexing with PBS changes between each vortex. Epithelial cells were plated in growth factor reduced Matrigel with intestinal growth media comprised of Advanced DMEM/F12 with supplements. Adult human intestinal myofibroblast (IMF) cells and Caco-2 cells were cultured according to manufacturer’s instructions.

**3D bioprinting and tissue culture**

3D intestinal tissues comprised of an interstitium and epithelium were manufactured by Organovo (San Diego, CA) using a proprietary bioprinting platform and standard protocols previously described (Nguyen et al., 2016, King et al., 2017). In brief, cultured cells were resuspended in thermo-responsive Novogel® Bio-ink and bioprinted onto membranes of standard 24-well 3 μm Transwell® culture permeable supports using a NovoGen® Bioprinter (Organovo Inc., San Diego, CA) with the interstitial and epithelial compartments comprised of IMF and hIEC, respectively or IMF and Caco-2 cells, respectively. Tissues were then cultured in 3D intestinal media containing Advanced DMEM/F12 with supplements at 37°C. Tissues were maintained in 3D intestinal media (Organovo Inc., San Diego, CA) for up to 21 days with media exchanges every day and were washed apically with PBS every other day. After printing, intestinal tissues were cultured for ten days prior to initiation of studies to allow barrier formation. Caco-2 monolayer studies were conducted according to previously reported studies (Bentz et al., 2013).
Briefly, cells were seeded at 30,000 cells/cm² per well onto standard 24-well Transwell® permeable supports and cultured at air-liquid interface in DMEM with L-glutamine + 10% FBS with media changes every 48 hours. Monolayers were grown for 21 days at 37°C then qualified for use by TEER (785 ± 56 Ω*cm²).

Histology

Tissues were fixed in 2% paraformaldehyde solution (10mM calcium chloride, 50 mM sucrose in PBS) processed, and paraffin embedded by standard techniques. Sections were stained by hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) / Alcian blue (pH 2.5) using standard protocols. Immunofluorescent staining using a panel of antibodies (Key resource table) to specific cell types or components was performed following deparaffinization, heat-mediated antigen retrieval, and permeabilization. Signal was detected using the appropriate fluorescently conjugated secondary antibodies. Staining methods and antibodies were validated on normal human small intestine control tissue prior to use on bioprinted tissues.

Gene Expression

RNA was extracted by RNeasy Mini Kit and analyzed by real-time quantitative polymerase chain reaction (RT-PCR; qPCR) using TaqMan gene expression assays (see table). TaqMan Array analysis (Figure 2) was carried out using the ViiA 7 System with 200ng of cDNA, synthesized using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR, per channel quantified with a NanoDrop spectrophotometer. TaqMan Array gene expression was analyzed using ExpressionSuite Software and shown as relative expression \((2^{-\Delta Ct})\) calculated using comparative Ct method to endogenous control 18S \((\Delta Ct = Ct_{target} - Ct_{18S})\) and analyzed as a ratio to amount of a gene present compared to epithelial-specific cytokeratin 19 \((\Delta Ct_{CK19} = Ct_{CK19} - Ct_{18S})\): Relative quantity = \((2^{-\Delta Ct})/(2^{-\Delta Ct_{CK19}})\). A heatmap of the TaqMan Array data was generated with Spotfire (TIBCO Software Inc.). The values displayed were calculated by taking the log of the Relative quantity = \((2^{-\Delta Ct})/(2^{-\Delta Ct_{CK19}})\) (calculation methods shown above), the MIN was -4.90 and MAX was 0.49. The samples were clustered by unweighted pair group method (UPGMA) with a correlation distance measurement and average value ordering weight. The gene categories were clustered by UPGMA with a Euclidean distance measurement and average value ordering weight. Individual gene expression data for 3D intestinal tissues and Caco-2 are expressed as an average of three biological replicates and plotted with GraphPad Prism. Quantitative PCR reactions were conducted with TaqMan Universal Master Mix II with UNG, under the following conditions: 50°C for 2 minutes; 95°C for 10 minutes; and 40 cycles of amplification at 95°C for 15 seconds followed by 60°C for 1 minute. Single-tube assays (Figure 3f, 7c, S3b) were carried out using the StepOnePlus System with 20 ng of DNase treated RNA quantified with a NanoDrop spectrophotometer. Fast RT-PCR reactions were conducted with Fast 1-Step mix under the following conditions: reverse transcription at 50°C for 5 minutes; RT inactivation/denaturation at 95°C for 20 seconds; and 45 cycles of amplification at 95°C for 3 seconds followed by 60°C for 30 seconds. Single tube assay gene expression data (Figure 3f, 7c, S3c) was normalized to GAPDH endogenous control and calculated according to the \(\Delta\Delta Ct\) method. Data is
graphed as a fold change of treated relative to untreated to obtain the effects of treatment induction on gene expression. Gene targets were first normalized to endogenous control GAPDH (ΔCt = Ct_{target} - Ct_{GAPDH}) and then calculated relative to treatment (ΔΔCt = ΔCt_{treated} - ΔCt_{untreated}). Final data is represented as $2^{\Delta\Delta C_t}$ for fold-change and graphed with GraphPad Prism. Data is expressed as the average of 3-4 grouped biological replicates plus or minus the standard deviation; each biological replicate is represented by the average of 2 technical replicates.

**TEER measurements**

The EVOM2 Epithelial Voltohmmeter with STX2 chopstick electrodes was used to measure construct transepithelial electrical resistance (TEER). Instrument was calibrated with a 1000 Ω resistor prior to measurements and an empty transwell used as a blank. Constructs were equilibrated to room temperature and switched to basal media for readings. The blank transwell and all samples were measured three times. Samples and blanks were measured in triplicate, then each averaged and used in the following calculations:

$$R_{Tissue}(\Omega) = R_{Tissue} - R_{Blank}$$

$$TEER_{Reported} = R_{Tissue}(\Omega) \times M_{Area}(cm^2)$$

Where $M_{Area} = 0.33 \ cm^2$

**Permeability and transport**

For permeability assays, compound was prepared at 10-100 µM in Advanced DMEM/F12 media then 100 µL of compound was added to the apical side and 600 µL of blank media was added to the basal side. Linearity of flux was established by assessing Rhodamine 123 transport at 20 minute intervals, however, single time points were used for test compounds as most compounds could not be detected at short time intervals. After 60 minutes (Lucifer yellow and mitoxantrone) or 120 minutes (digoxin, propranolol, topotecan), the basal side was sampled for analysis. Digoxin and propranolol concentrations were determined by mass spectrometry and Lucifer yellow, topotecan, and mitoxantrone concentrations were determined by fluorescence using a BMG POLARstar. For transport assays, tissues were pre-incubated with vehicle or inhibitor for 30 minutes prior to assay. Compounds for transport were assayed from both apical to basal (A to B) and basal to apical (B to A) transport. Efflux ratio was calculated as the ratio of (B to A)/(A to B).

**Metabolic activity assays**

CYP2C9 and CYP3A4 activities were measured by P450-Glo luminescent assays per manufacturer’s instructions. Briefly, tissues were washed with Advanced DMEM/F12 media plus vehicle or inhibitor (ketoconazole for CYP3A4, sulfaphenazole for CYP2C9) for 30 minutes. Tissues were incubated with P450-Glo substrates in the presence of vehicle or inhibitor for one hour then supernatant was transferred to white assay plates and incubated with luminescent detection reagent. Luminescence was
detected using a BMG POLARstar. Tissues were lysed with RIPA buffer and protein quantified using Pierce BCA Protein Assay kit.

CYP3A4 inhibition and induction were assessed by metabolism of midazolam. For induction studies, tissues were pre-incubated with 20 uM rifampicin for 72 hours followed by 30 minutes in either blank vehicle or inhibitor before initiating treatment with midazolam. To test CYP3A4 activity, tissues were incubated with 10 µM midazolam then supernatant was assayed for both parent drug and the 1-OH metabolite by mass spectrometry. Tissues were then lysed with RIPA buffer and protein quantified or lysed for RNA extraction.

**Mass spectrometry**

Detection of midazolam, digoxin, and propranolol were performed by mass spectrometry. Analyst was used to determine peak areas and generate standard curves for each compound. Sample values were quantified using the standard curve and normalized to the internal reference compound.

**Midazolam.** Stock solutions of 20 µM of midazolam and 10 µM of α-hydroxy midazolam were prepared in 50/50 methanol:blank media, combined at 1:1 serial dilutions were prepared to generate a standard curve. Each 50 µL standard or sample was diluted with 50 µL of midazolam-d4 (1 µg/mL) prepared in methanol then 10 µL was injected for determination of metabolite formation (1-OH midazolam). Each sample was subsequently diluted with 100 µL of methanol and re-injected for determination of midazolam concentration. LC–MS/MS analyses were performed using an Agilent 1200 HPLC system coupled to a PE Sciex API 4000 Qtrap tandem mass spectrometer with a Turbo Ionspray. The analytical column was an Agilent Zorbax XDB C8, 50 mm × 2.1 mm, 3.5 um and was kept at ambient temperature. Mobile phase A consisted of water with 0.1% formic acid and mobile phase B consisted of acetonitrile with 0.1% formic acid. The total run time was 4 min with an initial gradient of 5% mobile phase B held for 1 minute, followed by an increase to 95% B over 2 minutes. Mobile phase B was held at 95% B for one minute followed by re-equilibration to initial conditions for 2 minutes. The flow rate was set at 0.6 mL/min. The Ionspray needle was maintained at 5.0 kV. The turbo gas temperature was 500 °C and the CAD gas flow setting was medium. Curtain gas, GS1 and GS2 were 10, 20, and 0, respectively. The declustering potentials (DP) and the entrance potential (EP) was 81 and 10 V for midazolam, respectively. The mass spectrometer was operated in MRM mode with collision energy (CE) of 39 V and a collision cell exit potential (CXP) of 16. The transitions (precursor to product) monitored were m/z 326→291 for midazolam, 342→203 for 1-OH midazolam, and m/z 330→295 for midazolam-d4 (IS) in positive mode. The dwell time was 150 ms for all analytes and the internal standard. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

**Digoxin.** A stock solution of 10 µM of digoxin was prepared in 50/50 methanol:blank media. Each stock was combined in a ratio of 1:1 and serial dilutions were prepared to generate a standard curve. Twenty-five µL of each standard or sample was mixed with 200 µL of the internal standard, digitoxin (1 µg/mL)
prepared in methanol. 20 µL was injected for determination of digoxin concentration. LC–MS/MS analyses were performed using an Agilent 1200 HPLC system coupled to a PE Sciex API 4000 Qtrap tandem mass spectrometer with a Turbo Ionspray. The analytical column was a Phenomenex Synergi Max RP, 50 mm × 2.1 mm, 4 um and was kept at ambient temperature. Mobile phase A consisted of water with 5 mM ammonium formate (pH 3.4) and mobile phase B consisted of acetonitrile with 0.1% formic acid. The total run time was 5 min with an initial gradient of 5% mobile phase B at a flow rate of 0.750 mL/min. The initial conditions were held for 1 minute, followed by an increase to 99% B and 1 mL/min over 1.5 minutes. Mobile phase B was held at 99% B for one minute followed by re-equilibration to initial conditions for 2 minutes. The Ionspray needle was maintained at 5.0 kV. The turbo gas temperature was 325 °C and the CAD gas flow setting was medium. Curtain gas, GS1 and GS2 were 10, 20, and 0, respectively. The declustering potentials (DP), entrance potential (EP) and a collision exit potential (CXP) was 76, 10 and 14 V, respectively. The mass spectrometer was operated in MRM mode with collision energy (CE) of 20 V and 19 V for digoxin and digitoxin, respectively. The transitions (precursor to product) monitored was m/z 798→651 for digoxin, and m/z 782→365 for digitoxin (IS) in positive mode. The dwell time was set to 150 ms. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

**Propranolol.** A stock solution of 20 µM of propranolol was prepared in 50/50 methanol:blank media. Each stock was combined in a ratio of 1:1 and serial dilutions were prepared to generate a standard curve. Ten µL of each standard or sample was mixed with 200 µL of the internal standard, metaprolol (1 µg/mL) prepared in methanol. 5 µL was injected for determination of propranolol concentration. LC–MS/MS analyses were performed using an Agilent 1200 HPLC system coupled to a PE Sciex API 4000 Qtrap tandem mass spectrometer with a Turbo Ionspray. The analytical column was a Phenomenex Synergi Max RP, 50 mm × 2.1 mm, 4 um and was kept at ambient temperature. Mobile phase A consisted of water with 5 mM ammonium formate (pH 3.4) and mobile phase B consisted of acetonitrile with 0.1% formic acid. The total run time was 5.5 min with an initial gradient of 2% mobile phase B. The initial conditions were held for 1 minute, followed by an increase to 95% B over 0.5 minutes. Mobile phase B was held at 95% B for 1.5 minute followed by re-equilibration to initial conditions for 2.5 minutes. The flow rate was set to 0.5 mL/minute. The Ionspray needle was maintained at 5.0 kV. The turbo gas temperature was 350 °C and the CAD gas flow setting was medium. Curtain gas, GS1 and GS2 were 10, 20, and 0, respectively. The declustering potentials (DP), entrance potential (EP) and a collision exit potential (CXP) was 60, 10 and 18 V, respectively. The mass spectrometer was operated in MRM mode with collision energy (CE) of 25 V for digoxin and digitoxin. The transitions (precursor to product) monitored was m/z 260→183 for propranolol, and m/z 268→191 for metoprolol (IS) in positive mode. The dwell time was set to 150 ms. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

**Toxicity testing**

Bioprinted intestinal tissues were cultured as described until day ten. Baseline TEER of each sample was measured prior to treatment with indomethacin or TNFα. Tissues were treated with vehicle or test compound (indomethacin at 0.1-1 mM or 100 ng/mL TNFα) for 24 hours, then study media was collected and replaced with blank culture media before repeat TEER measurements. Tissues were collected for histology. Spent media collected from control or indomethacin treated tissues was
measured using a colorimetric assay for lactate dehydrogenase (LDH) per manufacturer’s instructions. Prostaglandin E\(_2\) was measured in spent media collected from control or indomethacin treated tissues by ELISA per manufacturer’s instructions. Tissues treated with TNF\(\alpha\) were collected for RNA isolation and gene expression analysis.

*Statistical analysis*

All data was analyzed using GraphPad Prism 6 software by t-test, one-way Anova, or two-way Anova as described in the text or figure legend. Data are presented as mean +/- standard deviation. The number of biological replicates, ‘n’, is noted in the figure or table legends.