Special Section – New Models in Drug Metabolism and Transport

Cryopreserved Human Intestinal Mucosal Epithelium: A Novel In Vitro Experimental System for the Evaluation of Enteric Drug Metabolism, Cytochrome P450 Induction, and Enterotoxicity

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ABSTRACT

We report here a novel in vitro enteric experimental system, cryopreserved human intestinal mucosa (CHIM), for the evaluation of enteric drug metabolism, drug-drug interaction, drug toxicity, and pharmacology. CHIM was isolated from the small intestines of four human donors. The small intestines were first dissected into the duodenum, jejunum, and ileum, followed by collagenase digestion of the intestinal lumen. The isolated mucosa was gently homogenized to yield multiple cellular fragments, which were then cryopreserved in a programmable liquid cell freezer and stored in liquid nitrogen. After thawing and recovery, CHIM retained robust cytochrome P450 (P450) and non-P450 drug-metabolizing enzyme activities and

demonstrated dose-dependent induction of transcription of CYP24A1 (approximately 300-fold) and CYP3A4 (approximately 3-fold) by vitamin D₃ as well as induction of CYP3A4 (approximately 3-fold) by rifampin after 24 hours of treatment. Dose-dependent decreases in cell viability quantified by cellular ATP content were observed for naproxen and acetaminophen, with higher enterotoxicity observed for naproxen, consistent with that observed in humans in vivo. These results suggest that CHIM may be a useful in vitro experimental model for the evaluation of enteric drug properties, including drug metabolism, drug-drug interactions, and drug toxicity.

Introduction

Oral administration is the preferred route of drug delivery due to its convenience and noninvasiveness. Bioavailability of an orally administered drug is a combination of both enteric and hepatic events. Drug entrance into enterocytes is determined by drug permeability across the plasma membrane and/or uptake transport, with intracellular enteric drug concentration further defined by enteric drug metabolism and efflux. The fraction of drug that is delivered to the portal vein upon enteric drug absorption is further subjected to absorption into the liver via passive or transporter-mediated uptake, with the fraction delivered to the systemic circulation determined by both hepatic drug metabolism and biliary excretion. Furthermore, as the first organ encountering an orally administered drug, the small intestine is the target of adverse effects commonly observed in the liver (namely, drug toxicity and drug-drug interactions).

Because the metabolic fate of an orally administered drug is a result of both enteric and hepatic events, accurate definition of both enteric and hepatic drug properties represents an important discipline in drug

development. Currently, estimation of human hepatic drug properties is facilitated by well established in vitro hepatic experimental systems, including human liver microsomes, cDNA-expressed cytochrome P450 (P450) isoforms, and human hepatocytes. Of these in vitro hepatic systems, hepatocytes are considered the “gold standard” due to their complete drug-metabolizing enzyme pathways and cofactors. Successful cryopreservation and culturing of human hepatocytes (Li, 2007) has allowed the development of numerous effective approaches to evaluate key hepatic drug properties, including transporter-mediated drug uptake (Shitara et al., 2003; Badolo et al., 2010), intrinsic hepatic clearance (Di et al., 2012; Ménochet et al., 2012; Baudoin et al., 2013; Peng et al., 2016), metabolite profiling (Bursztyka et al., 2008), metabolic enzyme pathway identification (Yang et al., 2016), inhibitory and inductive drug-drug interactions (Doshi and Li, 2011; Mao et al., 2012), transporter-mediated drug efflux (Kanda et al., 2018), and hepatotoxicity (Li, 2014, 2015; Zhang et al., 2016). Cryopreserved human hepatocytes used in conjunction with cell-free systems such as human liver microsomes, cDNA-derived P450 isoforms, and transporter membrane vesicles have allowed an accurate assessment of hepatic drug properties. Physiologically based pharmacokinetic approaches in combination with various databases and software have been applied successfully

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ABBREVIATIONS: 25-OH-D₃, 25-hydroxyvitamin D₃; AO, aldehyde oxidase; CES, carboxylesterase; CHIM, cryopreserved human intestinal mucosa; ECOD, ethoxycoumarin-O-deethylase; FMO, flavin-containing monooxygenase; LC, liquid chromatography; MAO, monoamine oxidase; MS/MS, tandem mass spectrometry; NAT, N-acetyltransferase; P450, cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcription; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

for the translation of in vitro observations to clinical events (Shaffer et al., 2012; Marsousi et al., 2018).

Development of enteric experimental approaches like that for the definition of hepatic drug properties would further enhance our ability to develop drugs with optimal clinical properties. We recently demonstrated successful cryopreservation of partially purified human enterocytes isolated via collagenous digestion of the small intestine (Ho et al., 2017). Cryopreserved human enterocytes exhibit various drug-metabolizing enzyme activities including various P450 and non-P450 pathways and have shown promising results when used to evaluate the intestinal clearance drug-drug interaction potential of orally administered drugs (Yan et al., 2017).

During the isolation of enterocytes, we noticed that collagenase digestion could effectively dissociate the mucosal epithelium from the lumen of the small intestine, which we further processed into partially purified enterocytes. We thereby initiated efforts to establish the cryopreserved human intestinal mucosa (CHIM) epithelium as an additional in vitro model of the small intestine. Our expectation is that CHIM, with all of the key intestinal cell types represented and distributed like those in vivo, may represent a physiologically relevant model of the small intestinal mucosa for investigations of enteric drug properties, including drug metabolism, drug-drug interactions, enterotoxicity, and enteric pharmacology.

We report here our initial results showing that the intestinal mucosa from human small intestines can be isolated and cryopreserved as multicellular fragments to retain viability and function. The thawed CHIM exhibited P450 and non-P450 drug-metabolizing enzyme activities, was responsive to the enterotoxicity of acetaminophen and naproxen, and showed robust (approximately 300-fold) induction of CYP2A1 transcription by vitamin D₃ and moderate (approximately 3-fold) induction of CYP3A4 transcription by vitamin D₃ and rifampin. Our results suggest that CHIM may represent a useful in vitro model of the small intestine for the evaluation of enteric drug properties, including drug metabolism, drug-drug interactions, and enterotoxicity.

Materials and Methods

Chemicals

Dextrorphan tartrate, diclofenac sodium salt, 4-hydroxydiclofenac, *S*-mephenytoin, 4-hydroxyquinoline, paclitaxel, and testosterone were purchased from Cayman Chemical (Ann Arbor, MI). 7-Hydroxycoumarin was purchased from Chem Service (West Chester, PA). Benzydamine *N*-oxide, 7-hydroxycoumarin sulfate potassium salt, kynuramine hydrobromide, and *N*-acetyl sulfamethazine were obtained from Santa Cruz Biotechnology (Dallas, TX). 4-Acetamidobenzoic acid, *p*-acetamidophenyl β -D-glucuronide sodium salt, 4-aminobenzoic acid, benzydamine hydrochloride, chlorzoxazone, coumarin, dextromethorphan hydrobromide, 6 β -hydroxytestosterone, 7-hydroxycoumarin β -D-glucuronide sodium salt, 7-ethoxycoumarin, paracetamol sulfate potassium, phenacetin, and sulfamethazine were purchased from Sigma-Aldrich (St. Louis, MO). Carbazeran, 4-hydroxycarbazeran, 6-hydroxychlorzoxazone, 6 α -hydroxypaclitaxel, acetaminophen glutathione disodium salt, midazolam, 1'-hydroxymidazolam, and 4-hydroxy-*S*-mephenytoin were obtained from Toronto Research Chemicals (Toronto, ON, Canada). All other drug-metabolizing enzyme substrates were obtained from Sigma-Aldrich.

Human Intestine

Human small intestines were obtained from the International Institute for the Advancement of Medicine (Exton, PA) as tissues intended but not used for transplantation. The small intestines were collected and stored in University of Wisconsin solution (Preservation Solutions, Inc., Elkhorn, WI) and shipped to our laboratory on wet ice with a cold ischemic time of less than 24 hours.

Intestinal Mucosa Isolation and Cryopreservation

Isolation of mucosa from human intestines was performed via enzymatic digestion of the intestinal lumen based on procedures previously reported for porcine intestines (Bader et al., 2000; Hansen et al., 2000). The following lengths (postpyloric sphincter) were used to aid the dissection of the various regions of the small intestine for enterocyte isolation: duodenum, 26 cm; jejunum, 2.5 m; and ileum, 3.5 m. The

**Collagenase
Digestion**

**Gentle
Homogenization**

Cryopreservation

Fig. 1. Scheme for the preparation of CHIM. (Top) Intestinal mucosal epithelium was isolated from the lumen of a human small intestine by collagenase digestion. (Middle) The freshly isolated mucosal epithelia consisted mainly of intestinal villi (bright-field microscopy). (Bottom) The epithelia were gently homogenized to smaller fragments for cryopreservation (phase-contrast microscopy). The size of the fragments was approximately 100 μ M, which formed a visually homogeneous suspension after brisk pipetting up and down three times with a P1000 micropipette set at a volume of 700 μ l and could be delivered with a P200 micropipette as 50- to 200- μ l aliquots for experimentation. Original magnification, $\times 40$ in (Middle); $\times 200$ in (Bottom).

intestines were recovered with a warm ischemic time of less than 15 minutes and were shipped to our laboratory on wet ice in University of Wisconsin preservation solution, with a cold ischemic time of less than 24 hours. Upon receipt of the small intestines, adipose tissue associated with intestines was removed by dissection. For three of the four human small intestines used in this study, three (donors 1, 2, and 4) were

TABLE 1

A summary of the P450 isoform-selective substrates and their respective metabolites quantified for CHIM

Isoform	Substrate	Substrate Concentration μM	Metabolites Quantified
CYP1A1	7-Ethoxyresorufin	20	Resorufin
CYP1A2	Phenacetin	100	Acetaminophen
CYP2A6	Coumarin	50	7-HC, 7-HC-sulfate, 7-HC-glucuronide
CYP2B6	Bupropion	500	Hydroxybupropion
CYP2C8	Paclitaxel	20	6 α -Hydroxypaclitaxel
CYP2C9	Diclofenac	25	4-Hydroxydiclofenac
CYP2C19	S-Mephenytoin	250	4-Hydroxy S-mephenytoin
CYP2D6	Dextromethorphan	15	Dextrorphan
CYP2E1	Chlorzoxazone	250	6-Hydroxychlorzoxazone
CYP3A4/5-1	Midazolam	20	1-Hydroxymidazolam
CYP3A4/5-2	Testosterone	200	6 β -Hydroxytestosterone
CYP2J2	Astemizole	50	O-Demethyl astemizole

HC, hydroxycoumarin.

dissected into duodenum, jejunum, and ileum portions. For donor 3, the entire small intestine from the duodenum to ileum was used for the isolation. For each of the intestinal sections, the lumen was washed rapidly with cold calcium and magnesium-free Hanks' balanced salt solution to remove intestinal contents, followed by digestion with an isotonic buffer containing 0.25 mg/ml type I collagenase (Sigma-Aldrich). The intestinal mucosal epithelia released from the intestinal lumen were partially purified by differential centrifugation (100g, 20 minutes). The isolated intestinal mucosal epithelia were resuspended in an approximately 20 $\times$ volume of Hepatocyte/Enterocyte Incubation Medium (In Vitro ADMET Laboratories, Columbia, MD) and gently homogenized with a loose-fitting Dounce homogenizer (VWR, Philadelphia, PA) to create a relatively homogenous suspension of small multicellular fragments. The intestinal mucosa fragments were collected by centrifugation and resuspended in a proprietary cryopreservation medium; they were then cryopreserved using a programmable liquid nitrogen cell freezer and stored in the vapor phase of liquid nitrogen maintained at $< -150^{\circ}\text{C}$. A schematic representation of the preparation of CHIM is shown in Fig. 1.

Recovery of CHIM

CHIM vials were removed from liquid nitrogen storage and thawed in a 37 $^{\circ}\text{C}$ water bath for approximately 2 minutes. The contents of each individual vial were decanted into a 50-ml conical tube containing Cryopreserved Enterocyte Recovery Medium (In Vitro ADMET Laboratories) that was prewarmed in a 37 $^{\circ}\text{C}$ water bath. The thawed CHIM was recovered by centrifugation at 100g for 10 minutes at room temperature. After centrifugation, the supernatant was removed by decanting. A volume of 5 ml of 4 $^{\circ}\text{C}$ Hepatocyte/Enterocyte Incubation Medium (In Vitro ADMET Laboratories) was added to the intact pellet of enterocytes at the bottom of the conical tube, followed by brisk repipetting five times with a P1000 micropipette to create an even suspension of the intestinal mucosal fragments.

Incubation of CHIM with Drug-Metabolizing Enzyme Substrates

Substrates, concentrations, and the metabolites quantified for the multiple drug metabolism pathways evaluated are shown in Tables 1 and 2 for P450 isoforms

and non-P450 drug-metabolizing enzymes, respectively. Incubations of CHIM and metabolism substrates were performed in a cell culture incubator maintained at 37 $^{\circ}\text{C}$ with a humidified atmosphere of 5% CO_2 . A 50- μl volume of drug-metabolizing enzyme substrates at 2 $\times$ the final desired concentrations was added to the designated wells of a 96-well plate (reaction plate). The reaction plate was placed in a cell culture incubator for 15 minutes to prewarm the substrate solutions to 37 $^{\circ}\text{C}$, followed by the addition of CHIM at a volume of 50 μl /well to initiate the reaction.

The reaction plates were then incubated at 37 $^{\circ}\text{C}$ for 30 minutes. All incubations were performed in triplicate. Metabolism was terminated in each well by the addition of 200 μl acetonitrile containing 250 nM of the internal standard tolbutamide. The incubated samples were stored at -80°C for subsequent liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis.

Evaluation of Gene Transcription

Gene transcription was quantified based on real-time reverse transcription (RT) polymerase chain reaction (PCR) using the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions for isolation of total RNA from the hepatocytes. At first, RT was performed with approximately 200 ng isolated RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). Subsequently, quantitative PCR analysis was performed on RT reactions using gene-specific primer/probe sets and TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific). The relative quantity of the target gene was compared with that of the reference transcription of glucose 6-phosphate dehydrogenase as determined by the $\Delta\Delta$ threshold cycle method and as previously described (Fahmi et al., 2010). The primer sequences used are presented in Table 3.

Enterotoxicity Evaluation

In vitro cytotoxicity evaluation with CHIM was performed in 96-well plates. After recovery from cryopreservation as described above, the CHIM pellet was resuspended in 5 ml Hepatocyte Incubation Medium (In Vitro ADMET

TABLE 2

A summary of the non-P450 pathway-selective substrates and their respective metabolites quantified in CHIM

DME Pathway	Substrate	Substrate Concentration μM	Metabolites Quantified
ECOD	7-Ethoxycoumarin	100	7-HC, 7-HC-sulfate, 7-HC-glucuronide
UGT	7-HC	100	7-HC-glucuronide
SULT	7-HC	100	7-HC-sulfate
FMO	Benzydamine HCl	250	Benzydamine-N-oxide
MAO	Kynuramine HBr	160	4-Hydroxyquinoline
AO	Carbazeran	20	4-Hydroxycabazeran
NAT1	4-Aminobenzoic acid	200	N-Acetyl-p-aminobenzoic acid
NAT2	Sulfamethazine	100	N-Acetyl-sulfamethazine
CES2	Irinotecan	50	SN38

DME, Drug Metabolizing Enzymes; HBr, hydrobromide; HC, hydroxycoumarin; HCl, hydrochloride; SN38, 7-ethyl-10-hydroxycambdothecin.

TABLE 3
Primers used for real-time RT-PCR quantification of transcription

Target Gene	Direction	Primer Sequence
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAA
	Reverse	AATGAAGGGGTCATTGATGG
CYP3A4	Forward	TTTGTCTTACCATAAGGGCTTT
	Reverse	CACAGGCTGTTGACCATCAT
CYP24A1	Forward	GGTGACATCTACGGCGTACAC
	Reverse	CTTGAGACCCCTTTCCAGAG

GAPDH represents the housekeeping gene used for normalization of the transcription of CYP3A4 and CYP24A1. GAPDH, glucose 6-phosphate dehydrogenase.

Laboratories). The CHIM suspension was added to each well of the 96-well plates, followed by the addition of 50 μ l of the toxicants (acetaminophen and naproxen) at 2 $\times$ the final desired concentration. The CHIM cultures were then incubated in a CO₂ cell culture incubator kept at 37°C in a highly humidified atmosphere of 5% CO₂. After 24 hours of incubation, cell viability was determined based on cellular ATP content as previously described. Results are presented as relative viability, which is the ratio of the cellular contents of treated cultures to that of solvent control cultures.

Quantification of Protein Concentration

Because CHIM consists of multiple cell aggregates, cellular contents were quantified as protein concentrations. Determination of protein concentration was performed using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) per the manufacturer's instructions.

LC-MS/MS Analysis

Upon thawing, the samples were centrifuged at 3500 rpm for 5 minutes. An aliquot of 100 μ l supernatant from each sample was transferred to a 96-well plate and was diluted with 200 μ l deionized water with mixing before LC-MS/MS analysis. CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 (midazolam 1'-hydroxylation), CYP3A4 (testosterone 6 β -hydroxylation), ethoxycoumarin-O-deethylase (ECOD), UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), flavin-containing monooxygenase (FMO), monoamine oxidase (MAO), aldehyde oxidase (AO), and N-acetyltransferase (NAT) 2 metabolites, as well as acetaminophen metabolism, were quantified by using an API 5000 mass spectrometer with an electrospray ionization source (AB SCIEX, Framingham, MA) connected to a Waters Acquity ultra-performance liquid chromatography system (Waters Corporation, Milford, MA) using LC-MS/MS multiple reaction monitoring mode, monitoring the mass transitions (parent to daughter ion) as previously described (Ho et al., 2017). An Agilent Zorbax Eclipse Plus C18 column (4.6 $\times$ 75 mm i.d., 3.5 μ m; Agilent Technologies, Santa Clara, CA) at a flow rate of 0.7 ml/min was used for the chromatography separation. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient for the positive ion mode operation was programed as follows: 0–1 minute, increased B from 5% to 90%; 1–1.5 minutes, kept B at 90%; 1.5–1.75 minutes, decreased B to 5%; run time, 3 minutes. The gradient program for the negative ion mode was as follows: 0–2.5 minutes, increased B from 30% to 95%; 2.5–3.0 minutes, kept B at 95%; 3–3.2 minutes, decreased B to 30%; run

time, 4 minutes. For conjugates, the gradients and run time may be adjusted for better separation. Data acquisition and data processing were performed with Analyst 1.6.2 software (AB SCIEX).

Data Analysis

Data are presented as the mean $\pm$ S.D. of triplicate incubations derived using Microsoft Excel 6.0 software (Microsoft, Redmond, WA). Statistical analysis was performed using the *t* test with Microsoft Excel 6.0 software, with *P* values < 0.05 considered statistically significant. Specific activity (in picomoles per minute per milligram protein) of each drug-metabolizing enzyme pathway was determined by dividing the total metabolite formed by the incubation time and normalized to protein content. GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA) was used to determine EC₅₀ values for the cytotoxicity of acetaminophen and naproxen.

Results

Donor Demographics

CHIM prepared from the small intestines of four individual human donors was used in this study. The donor demographics are presented in Table 4.

Morphology, Size, Viability, and Yield of Cryopreserved CHIM

The isolated epithelia consisted mainly of individual villi. These relatively large mucosal fragments were gently homogenized to form smaller, multicellular fragments, which were then cryopreserved. Figure 1 shows the morphology of the freshly isolated villi and the multicellular fragments of the thawed CHIM. Because CHIM comprises multicellular aggregates, the cell concentration could not be determined and protein content was used for data quantification. The protein content of each vial of CHIM (1 ml total volume) was as follows: 1) donor 1: duodenum (2.5 mg), jejunum (1.8 mg), and ileum (3.9 mg); 2) donor 2: duodenum (1.8 mg), jejunum (4.0 mg), and ileum (3.3 mg); 3) donor 3: duodenum, jejunum, and ileum combined (1.6 mg); and 4) donor 4: duodenum (1.7 mg), jejunum (1.2 mg), and ileum (1.2 mg).

Drug-Metabolizing Enzyme Activities

P450 Isoform Activities. CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP2J2 activities quantified in CHIM using isoform-selective substrates (Table 1) are shown in Table 5. The highest activity was observed for CYP3A4 measured as testosterone 6 β -hydroxylation. CYP2C9 and CYP2C19 activities were higher than that observed for CYP2A6, CYP2B6, CYP2C8, CYP2D6, and CYP2E1. The relative distribution of P450 isoform activities is shown in Tables 6 and 7. The activity-based P450 "pie" using testosterone 6 β -hydroxylation for CYP3A4 activity for CHIM from the duodenum, jejunum, and ileum of donor 1 is shown in Fig. 2.

TABLE 4
Demographic information for donors of the small intestines used in the preparation of CHIM for this study

Characteristic	Donor 1	Donor 2	Donor 3	Donor 4
Sex	Male	Male	Male	Male
Age (yr)	20	48	59	20
Race	Caucasian	Caucasian	Hispanic	Caucasian
Cause of death	CVA, anoxia	Anoxia	CVA second to ICH	Asphyxiation
Body mass index (kg/m ²)	30.62	21.04	30.27	26.34
Smoking status	No	No	Yes	No
Alcohol use	No	No	No	No
Substance abuse	Yes	No	No	No

CVA, cardiovascular arrest; ICH, intracranial hemorrhage.

TABLE 5

P450 isoform-specific activities of CHIM isolated from the duodenum, jejunum, and ileum of donors 1, 2, and 4 and from the small intestine (from the duodenum to ileum) of donor 3

Isoform	Activity									
	Donor 1			Donor 2			Donor 3	Donor 4		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Combined	Duodenum	Jejunum	Ileum
<i>pmol/min per milligram protein</i>										
CYP1A1	1.46	1.23	3.27	1.58	0.89	1.18	2.10	0.65	0.19	0.14
CYP1A2	5.10	4.99	8.60	12.77	3.54	0.91	2.82	2.06	5.95	2.88
CYP2A6	0.01	0.01	0.01	0.02	0.01	0.03	BQL	BQL	BQL	BQL
CYP2B6	0.97	1.11	1.84	1.26	0.42	0.36	1.30	0.60	1.01	0.57
CYP2C8	0.08	0.13	0.16	0.08	0.03	0.03	0.09	0.08	0.13	0.18
CYP2C9	3.51	13.60	14.93	2.67	1.86	0.24	0.29	0.26	0.38	0.43
CYP2C19	4.79	3.39	1.43	12.85	1.71	BQL	0.18	0.20	0.95	0.83
CYP2D6	0.18	0.16	BQL	0.28	0.05	BQL	BQL	0.02	BQL	0.04
CYP2E1	0.36	0.37	0.66	0.03	0.57	0.66	0.31	BQL	BQL	0.05
CYP3A4/5-1	11.27	18.60	8.40	2.51	4.02	BQL	0.67	0.37	1.89	1.54
CYP3A4/5-2	146.77	195.83	99.43	116.30	38.14	2.28	12.41	10.78	25.43	25.54
CYP2J2	3.49	2.71	5.73	2.41	2.38	2.14	4.18	1.71	3.00	2.55

BQL, below limits of quantification.

Non-P450 Drug-Metabolizing Enzyme Activities. The non-P450 drug-metabolizing enzyme pathways evaluated included ECOD, UGT, SULT, FMO, MAO, AO, NAT1, NAT2, and carboxylesterase (CES) 2. Results are shown in Table 8. Quantifiable activities were observed for all pathways evaluated except for AO. MAO had the highest activity, which ranged from similar to higher than that observed for CYP3A4 (testosterone 6 β -hydroxylation).

P450 Induction

P450 induction was evaluated based on transcription. After a 24-hour treatment duration, dose-dependent induction of CYP3A4 mRNA by rifampin and 25-hydroxyvitamin D₃ (25-OH-D₃) and of CYP2A4 mRNA by 25-OH-D₃ was observed. For CYP3A4, the maximal fold of induction was approximately 3-fold for both inducers. An approximately 300-fold induction of CYP2A4 mRNA was observed for 25-OH-D₃ (Fig. 3).

In Vitro Enterotoxicity Assay with CHIM

Dose-dependent cytotoxicity was observed for both acetaminophen and naproxen in CHIM isolated from the three donors (Fig. 4). The

calculated IC₅₀ values are shown in Table 9. Results show that naproxen consistently demonstrated higher enterotoxicity than acetaminophen, as shown by the lower IC₅₀ values for all CHIM lots evaluated.

Discussion

We report here our success in isolating and cryopreserving intestinal mucosa from the duodenum, jejunum, and ileum of human intestines to retain drug metabolism activity, responsiveness to P450 inducers, and gastrointestinal toxicants.

Upon collagenase digestion, the intestinal mucosa detached from the intestine as large sheets consisting mainly of intestinal villi (Fig. 1). The sheets of intestinal mucosal epithelia were gently homogenized into small, multicellular fragments before cryopreservation. Light homogenization was necessary to allow the CHIM suspension to be delivered with a multichannel pipette into the wells of 96-well plates for experimentation.

CHIM was found to retain P450 and non-P450 drug metabolic enzyme activities characteristic of the small intestine. As reported previously by others using intestinal microsomes (Paine et al., 1997; Perloff et al., 2003; Yang et al., 2004), and by us in cryopreserved

TABLE 6

Distribution of P450 isoform activities in CHIM using midazolam 1'-hydroxylation as CYP3A4 activity

Results are expressed as the percentage of the arithmetic sum of the specific activities (in picomoles per minute per milligram protein) of the P450 isoforms evaluated.

Isoform	Total Activities									
	Donor 1			Donor 2			Donor 3	Donor 4		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Combined	Duodenum	Jejunum	Ileum
CYP1A1	4.67	2.65	7.26	4.34	5.75	21.22	17.62	10.85	1.38	1.55
CYP1A2	16.33	10.77	19.10	35.02	22.85	16.40	23.59	34.75	44.12	31.32
CYP2A6	0.03	0.01	0.02	0.05	0.08	0.59	0.00	0.00	0.00	0.00
CYP2B6	3.11	2.41	4.09	3.45	2.72	6.41	10.92	10.07	7.47	6.15
CYP2C8	0.27	0.28	0.35	0.22	0.22	0.48	0.76	1.36	0.93	1.94
CYP2C9	11.24	29.37	33.16	7.33	12.01	4.35	2.40	4.32	2.83	4.66
CYP2C19	15.34	7.32	3.17	35.25	11.02	0.00	1.52	3.43	7.04	9.02
CYP2D6	0.58	0.35	0.00	0.77	0.35	0.00	0.00	0.29	0.00	0.43
CYP2E1	1.16	0.81	1.46	0.08	3.67	11.87	2.58	0.00	0.00	0.53
CYP3A4/5-1	36.09	40.17	18.66	6.87	25.95	0.00	5.60	6.21	13.98	16.72
CYP2J2	11.18	5.85	12.72	6.61	15.39	38.70	35.01	28.72	22.23	27.68
Sum of all isoforms	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

TABLE 7

Distribution of P450 isoform activities in CHIM using testosterone 6 β -hydroxylation as CYP3A4 activity

Results are expressed as the percentage of the arithmetic sum of the specific activities (in picomoles per minute per milligram protein) of the P450 isoforms evaluated.

Isoform	Total Activities									
	Donor 1			Donor 2			Donor 3	Donor 4		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Combined	Duodenum	Jejunum	Ileum
CYP1A1	0.87	0.55	2.40	1.05	1.80	15.04	8.88	3.94	0.50	0.43
CYP1A2	3.06	2.23	6.32	8.50	7.13	11.62	11.90	12.62	16.07	8.69
CYP2A6	0.01	0.00	0.01	0.02	0.41	0.00	0.00	0.00	0.00	0.00
CYP2B6	0.58	0.50	1.35	0.84	0.85	4.55	5.50	3.66	2.72	1.71
CYP2C8	0.05	0.06	0.12	0.05	0.07	0.34	0.38	0.50	0.34	0.54
CYP2C9	2.10	6.08	10.97	1.78	3.75	3.08	1.21	1.57	1.03	1.29
CYP2C19	2.87	1.52	1.05	8.55	3.44	0.00	0.77	1.24	2.57	2.50
CYP2D6	0.11	0.07	0.00	0.19	0.11	0.00	0.00	0.11	0.00	0.12
CYP2E1	0.22	0.17	0.48	0.02	1.15	8.41	1.30	0.00	0.00	0.15
CYP3A4/5-2	88.03	87.61	73.08	77.40	76.89	29.12	52.40	65.92	68.66	76.90
CYP2J2	2.09	1.21	4.21	1.60	4.80	27.43	17.65	10.43	8.10	7.68
Sum of all isoforms	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

enterocytes (Ho et al., 2017), the major P450 isoform activities were contributed by CYP3A4, especially for testosterone 6 β -hydroxylation, which was approximately 10-fold higher than that observed for midazolam 1'-hydroxylation. CYP1A2, CYP2C9, CYP2C19, and CYP2J2 represent the non-CYP3A isoforms with substantial activities. In general, CYP1A1 activity was lower than that for CYP1A2 and similar to CYP2B6. Minimal, near undetectable activities were observed for CYP2A6, CYP2D6, and CYP2E1 (Table 4). This overall relative distribution of CYP450 isoform activities was similar for all three regions of the small intestine (Tables 6 and 7). The P450 "pie" constructed for the duodenum, jejunum, and ileum using testosterone 6'-hydroxylation for CYP3A4 activity showed CYP3A4 as the most active P450 isoform in the small intestines, as previously reported by Paine et al. (2006). It is interesting to note that Paine et al. (1997) previously reported decreasing CYP3A4 activity from the duodenum to ileum. In our study, we observed the following intestinal regional variations in CYP3A4/5: 1) donor 1: jejunum > duodenum > ileum for both midazolam 1'-hydroxylation and testosterone 6 β -hydroxylation;

donor 2: jejunum > duodenum > ileum for midazolam 1'-hydroxylation and duodenum > jejunum > ileum for testosterone 6 β -hydroxylation; and 3) donor 4: ileum $\geq$ jejunum > duodenum for both pathways. Our results with the limited donors therefore show that, in general, the jejunum had the highest activity compared with the duodenum and ileum. Individual differences in regional differences may occur, presumably due to various host factors, including death and disease. In our laboratory, individual differences and regional differences among individuals will be further defined with intestines isolated from additional donors.

The highest non-P450 drug-metabolizing enzyme activity was observed for MAO, with activities for all four individuals higher than those observed for CYP3A4 quantified by testosterone 6 β -hydroxylation. UGT, FMO, NAT1, NAT2, and CES2 activities were also in abundance, similar to that observed for CYP2C9. AO activities were mostly undetectable (Table 8). That UGT (Cubitt et al., 2009), FMO (Yeung et al., 2000), NAT (George, 1981), and CES (Crow et al., 2007) activities are present in the small intestine has been previously reported.

Fig. 2. P450 "pie" of the distribution of P450 isoform activities of CHIM isolated from the duodenum, jejunum, and ileum of donor 1. Testosterone 6 β -hydroxylation activity was used to represent CYP3A4 activity to construct the pie chart. The largest section of the pie is CYP3A4 (gold). The other isoforms are as follows, starting with the top of the pie from left to right separated spatially and also by color: CYP2J2 (light gray), CYP1A1 (dark blue), CYP1A2 (orange), CYP2A6 (gray; mostly absent), CYP2B6 (dark gold), CYP2C8 (blue), CYP2C9 (green), CYP2C19 (greyish blue), CYP2D6 (light gold; narrow section), and CYP2E1 (gray; narrow section).

TABLE 8
Non-P450 drug-metabolizing enzyme activities of CHIM isolated from the duodenum, jejunum, and ileum of donors 1, 2, and 4 and from the entire small intestine (from duodenum to ileum) of donor 3

Metabolic Pathway	Activity									
	Donor 1			Donor 2			Donor 3	Donor 4		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Combined	Duodenum	Jejunum	Ileum
<i>pmol/min per milligram protein</i>										
ECOD	0.74	5.75	1.56	0.15	0.08	0.05	0.92	0.73	0.53	1.50
UGT	6.88	8.60	22.19	14.46	2.56	BQL	0.02	0.36	0.70	0.32
SULT	0.07	0.15	0.07	6.88	1.24	BQL	0.01	2.64	2.33	0.66
FMO	7.06	6.02	8.87	8.59	5.41	3.55	9.25	9.12	10.40	6.53
MAO	303.64	480.99	740.74	790.12	207.30	18.06	81.59	213.17	448.71	147.49
AO	0.03	0.02	0.05	0.01	BQL	BQL	0.04	BQL	BQL	0.02
NAT1	5.45	10.87	36.61	94.20	15.84	3.46	15.16	1.92	1.82	1.14
NAT2	3.38	3.99	5.60	3.30	1.32	0.76	1.98	1.31	1.81	1.62
CES2	1.63	1.22	2.56	0.83	0.60	0.50	2.41	0.96	1.42	1.45

BQL, below limits of quantification.

An exciting discovery is that P450 induction could be observed in CHIM, thereby representing the only reported P450 induction system with primary enterocytes. Upon 24-hour incubation with

1, 25-dehydroxyvitamin D3 (1,25(OH)D₃), dose-dependent induction of CYP24A1 transcription was observed (approximately 300-fold induction). CYP24A1 is the P450 component of the 25-OH-D₃-24-hydroxylase

Fig. 3. Induction of CYP3A4 transcription by rifampin and 1,25(OH)₂D₃ (top and middle graphs) and induction of CYP24A1 transcription by 1,25(OH)₂D₃ (bottom graph). Error bars represent the S.D. of results from triplicate treatments. 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

Fig. 4. Acetaminophen and naproxen enterotoxicity in CHIM isolated from the duodenum, jejunum, and ileum of donor 1. The results show that naproxen was consistently more toxic than acetaminophen, consistent with human *in vivo* findings. The IC_{50} ratio was calculated by dividing the IC_{50} value of acetaminophen by that of naproxen. APAP, acetaminophen.

enzyme that catalyzes the conversion of 25-OH-D₃ and 1,25-dihydroxyvitamin D₃ into 24-hydroxylated products, which constitute the degradation of the vitamin D molecule. Dose-dependent induction of CYP3A4 by both 1,25(OH)D₃ and rifampin was observed, with a maximum approximately 3-fold induction (Fig. 2). Rifampin and 1,25(OH)D₃ induction of CYP3A4 via the vitamin D receptor and pregnane X receptor pathways was previously reported in small intestinal cell lines (Kolars et al., 1992; Thummel et al., 2001; Thompson et al., 2002; Zheng et al., 2012) and in small bowel biopsies of patients treated with rifampin *in vivo* (Kolars et al., 1992). CYP3A induction in the small intestines *in vivo* may have physiologic consequences, including enhanced metabolism of vitamin D as well as orally administered drugs that are substrates of CYP3A4 and CYP24A1. In the application of hepatocytes for the evaluation of hepatic P450 induction, mRNA is a relevant *in vitro* endpoint that allows the estimation of *in vivo* effects (e.g., decrease in plasma half-life and plasma area under the curve) (Fahmi et al., 2008, 2010; Youdim et al., 2008; Einolf et al., 2014). Our results with mRNA in CHIM therefore may be used similarly in the estimation of *in vivo* enteric metabolic clearance. It is our intention to further optimize the experimental conditions to allow the measurement of enteric P450 induction using both mRNA and activity as endpoints.

Enterotoxicity is a known adverse effect of orally administered drugs. Nonsteroidal anti-inflammatory drugs, for instance, are known to cause upper gastrointestinal tract damage (Biour et al., 1987; Semble and Wu, 1987). Because drug metabolism is a key determinant of toxicity due to metabolic activation and detoxification, an *in vitro* enteric system with drug metabolism capacity similar to that in the gastrointestinal tract *in vivo* would be ideal for the early evaluation of gastrointestinal toxicity in drug development. We therefore embarked on the development of an *in vitro* enterotoxicity assay with CHIM using two nonsteroidal anti-inflammatory drugs known to be associated with gastrointestinal toxicity, acetaminophen (Rainsford and Whitehouse, 2006) and naproxen (Curtarelli and Romussi, 1973). Both acetaminophen and naproxen have been associated with upper gastrointestinal bleeding and perforations. Although intestinal gastrointestinal ulcerations are commonly associated with acid reflux and *Helicobacter pylori* infection,

TABLE 9

IC_{50} values for acetaminophen and naproxen in CHIM isolated from human donors 1 (duodenum, jejunum, and ileum), 2 (duodenum and jejunum), and 3 (combination of all three small intestine regions)

Drug	IC_{50}		
	Duodenum	Jejunum	Ileum
	mM		
Acetaminophen	2.25	1.22	1.20
Naproxen	0.35*	0.39*	0.36*
IC_{50} ratio	6.36	3.09	3.35

The IC_{50} ratio was calculated by dividing the IC_{50} for acetaminophen by that for naproxen. The results show that naproxen was consistently more cytotoxic than acetaminophen for all three regions of the small intestine.

* $P < 0.05$ (asterisks represent IC_{50} values obtained from naproxen that are significantly different from those obtained for acetaminophen).

enteropathy has also been associated with enterocyte cytotoxicity. CHIM may represent a physiologic relevant experimental system for the evaluation of cytotoxicity-related enteropathy. Treatment of CHIM (from the duodenum, jejunum, and ileum of donor 1) with acetaminophen and naproxen led to dose-dependent decreases in viability quantified by cellular ATP contents (Fig. 4). The IC_{50} value of naproxen (0.35–0.39 mM) was significantly lower than that for acetaminophen (0.92–1.18 mM), with acetaminophen having an IC_{50} value three to six times that for naproxen. Our results with CHIM are consistent with clinical findings showing that naproxen has higher enterotoxicity than acetaminophen (Lewis et al., 2002). Our results therefore suggest that CHIM can be useful in evaluation of the enterotoxic potential of orally administered drugs, especially for drugs that may be activated or detoxified by enteric metabolism, such as acetaminophen (Laine et al., 2009; Jaeschke and McGill, 2015; Jiang et al., 2015; Miyakawa et al., 2015) and naproxen (Miners et al., 1996; Rodrigues et al., 1996; Tracy et al., 1997). In our laboratory, we have initiated a study on the role of drug-metabolizing enzyme activities on the cytotoxicity of acetaminophen and naproxen on CHIM as an experimental approach to evaluate the usefulness of this novel experimental system in the definition of key pathways for toxic metabolite formation and/or detoxification. *In vitro* enteric systems such as CHIM should be useful in the assessment of enterotoxic potential, which can be used in the assessment of *in vivo* enterotoxicity upon appropriate physiologically based pharmacokinetic modeling considering key *in vivo* factors including rate of transit, drug dissolution, and available drug concentration at various regions of the intestinal tract.

CHIM represents an *in vitro* experimental system that can aid evaluation of enteric drug properties. Current *in vitro* experimental models include Caco-2 cells and induced pluripotent stem cell-derived intestinal cells, which in general are deficient in drug-metabolizing enzyme activities, especially the suboptimal expression of CYP3A (the most important drug-metabolizing enzyme for enteric drug metabolism) (Schmiedlin-Ren et al., 1997; Cummins et al., 2004; Negoro et al., 2016). Intestinal microsomes contain drug-metabolizing enzymes associated with the endoplasmic reticulum but lack cytosolic, mitochondrial, nuclear, and plasma membrane-associated drug-metabolizing enzymes. The CHIM model reported here and the cryopreserved enterocytes that we reported earlier (Ho et al., 2017) represent practical and physiologically relevant *in vitro* enteric models with “complete” drug-metabolizing enzyme pathways for the evaluation of enteric drug metabolism, akin to the use of cryopreserved hepatocytes for hepatic drug metabolism (Li et al., 1997; Li, 2007, 2015).

It is also possible that CHIM can be used for the evaluation of additional enteric pharmacology and physiology, especially using transcription as an endpoint. For instance, because CHIM contains

multiple enteric mucosal cell types, it may be useful for the evaluation of the onset and treatment of inflammatory-related events and diseases such as inflammatory bowel disease (Coste et al., 2007).

One limitation of using CHIM for induction studies is that the culture conditions only allow a culturing duration of 24 hours, a property that likely reflects the short life span of enterocytes in vivo. In vivo, enterocytes are continuously formed from the intestinal crypts and migrate from the crypt surface and slough off at the villus tip in a 3-day duration (Kaminsky and Zhang, 2003). It would be ideal to have an in vitro enterocyte culture recapitulating the entire process of the enterocyte life cycle, starting with the generation of enterocytes from the crypt cells, differentiation of the newly generated enterocytes, and apoptosis of the mature enterocytes.

Additional ongoing activities in our laboratory include the collection of CHIM from additional donors for the development of a CHIM bank for use in experimentation and for research into genetic and environmental factors affecting enteric drug-metabolizing enzyme activities (Hoensch et al., 1975; Pantuck et al., 1976; Zhang et al., 2013). We will continue to optimize conditions to prolong the life span of CHIM for enzyme induction and enterotoxicity studies, fully realizing that this may be difficult due to the limited life span of mature enterocytes in vivo. Studies comparing drug-metabolizing enzyme activities before and after cryopreservation are also ongoing. As of the time of this study, no substantial loss of enzyme activities due to cryopreservation has been observed (A. P. Li, personal communication).

Our results on the presence of P450 and non-P450 drug-metabolizing enzyme activities, responsiveness to P450 inducers, and sensitivity to gastrointestinal toxicants suggest that CHIM may represent a practical and physiologically relevant in vitro experimental model for the evaluation of enteric drug metabolism, drug-drug interactions, and drug toxicity.

Authorship Contributions

Participated in research design: Li, Ho, Yang.

Conducted experiments: Alam, Amaral, Ho, Loretz, Mitchell, Yang.

Performed data analysis: Li, Alam, Amaral, Ho, Loretz, Mitchell, Yang.

Wrote or contributed to the writing of the manuscript: Li, Alam, Amaral, Ho, Loretz, Yang. The authors also acknowledge the technical assistance of Nola Mahaney and Sharon Burkhardt.

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