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Cryopreserved human intestinal mucosal epithelium: a novel in vitro experimental system for the evaluation of enteric drug metabolism, P450 induction, and enterotoxicity

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Nonstandard Abbreviations:

CERM™: Cryopreserved enterocytes recovery medium

HQM™: Hepatocyte/enterocyte incubation medium

CHIM: Cryopreserved human intestinal mucosa

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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 were isolated from the small intestines of four human donors. The small intestines were first dissected into duodenum, jejunum and ileum, followed by collagenase digestion of the intestinal lumens. The isolated mucosa were gently homogenized to yield multiple cellular fragments followed by cryopreservation in a programmable liquid cell freezer and stored in liquid nitrogen. After thawing and recovery, CHIM were found to retain robust P450 and non-P450 drug metabolizing enzyme activities, to demonstrate dose-dependent induction of transcription of CYP24A1 (approx. 300-fold) and CYP3A4 (approx. 3-fold) by vitamin D3, and induction of CYP3A4 (approx. 3-fold) by rifampin after treatment for 24 hours. Dose-dependent decreases in cell viability quantified by cellular ATP content was observed for naproxen and acetaminophen, with a higher enterotoxicity observed for naproxen, consistent with what is observed in humans in vivo. The 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.

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Introduction

Oral administration is the preferred route of drug delivery due to its convenience and non-invasiveness. Bioavailability of an orally-administered drug is a combination of both enteric and hepatic events. Drug entrance into the 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.

As 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 the well-established in vitro hepatic experimental systems including human liver microsomes, cDNA-expressed CYP isoforms, and human hepatocytes. Of these in vitro hepatic systems, hepatocytes are considered the “gold standard” due to the 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, Li et al. 2003; Badolo, Rasmussen et al. 2010), intrinsic hepatic clearance (Di, Trapa et al. 2012; Menochet, Kenworthy et al. 2012; Baudoin, Prot et al. 2013; Peng, Doshi et al. 2016), metabolite profiling (Bursztyka, Perdu et al. 2008), metabolic enzyme pathway identification (Yang, Atkinson et al. 2016), inhibitory and inductive drug-drug interactions (Doshi and Li 2011; Mao, Mohutsky

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et al. 2012), transporter-mediated drug efflux (Kanda, Takahashi et al. 2018), and hepatotoxicity (Li 2014; Li 2015; Zhang, He 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. PBPK approaches in combination with various databases and software have been applied successfully for the translation of in vitro observations to clinical events (Shaffer, Scialis et al. 2012; Marsousi, Desmeules 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. Recently we have demonstrated successful cryopreservation of partially purified human enterocytes isolated via collagenous digestion of the small intestine (Ho, Ring et al. 2017). The cryopreserved human enterocytes exhibit various drug metabolizing enzyme activities including various P450 and non-P450 pathways and have been used in the evaluation of intestinal clearance drug-drug interaction potential of orally administered drugs with promising results (Yan, Wong et al. 2017).

During the isolation of enterocytes, we noticed that collagenase digestion would 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 intestinal mucosal epithelium (CHIM) as an additional in vitro model of the small intestine. Our expectation is that CHIM, with all the key intestinal cell types represented and distributed like that 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, entertoxicity, and enteric pharmacology.

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We report here our initial results showing that the isolated intestinal mucosa from human small intestines can be isolated and cryopreserved as multicellular fragments to retain viability and functions. The thawed cryopreserved human intestinal mucosa (CHIM) were found to exhibit P450 and non-P450 drug metabolizing enzyme activities, were responsive to the enterotoxicity of acetaminophen and naproxen, and showed robust (approx. 300-fold) induction of CYP24A1 transcription by vitamin D3 and moderate (approx. 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.

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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, Pennsylvania). Benzydamine N-oxide, 7-hydroxycoumarin sulfate potassium salt, kynuramine hydrobromide, and N-acetyl sulfamethazine were obtained from Santa Cruz Biotechnology (Dallas, Texas). 4-acetamidobenzoic acid, p-acetamidophenyl β-D-glucuronide sodium salt, 4-aminobenzoic acid, benzydamine hydrochloride, chlorzoxazone, coumarin, dextromethorphan hydrobromide, 6β-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, 6α-hydroxypaclitaxel, acetaminophen glutathione disodium salt, midazolam, 1'-hydroxymidazolam, and 4-hydroxy-S-mephenytoin were obtained from Toronto Research Chemicals (Toronto, Canada). All other drug metabolizing enzyme substrates were obtained from Sigma Aldrich (St. Louis, MO).

Human intestine. Human small intestines were obtained from the International Institute for the Advancement of Medicine (IIAM, Exton, PA) as tissues intended but not used for transplantation. The small intestines were collected and stored in University of Wisconsin solution 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, Hansen et al. 2000; Hansen, Borlak et al. 2000). The following lengths (post-pyloric sphincter) were used to aid the dissection of the various regions of the small intestine

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for enterocyte isolation: duodenum: 26 centimeters, jejunum: 2.5 meters and ileum: 3.5 meters. The intestines were recovered with a warm ischemic time of less than 15 minutes, and 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 the study, three (Donors 1, 2 and 4) were dissected into duodenum, jejunum, and ileum portions. For Donor 3, the entire small intestine from 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 Hank's Balanced Salt Solution to remove intestinal contents followed by digestion with an isotonic buffer containing 0.25 mg/mL of type I collagenase (Sigma-Aldrich). The intestinal mucosal epithelia released from the intestinal lumen were partially purified by differential centrifugation (100xg, 20 minutes). The isolated intestinal mucosal epithelia were resuspended in approximately 20x volume of HQM and gently homogenized with a loose-fitting Dounce homogenizer 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 followed by cryopreservation using a programmable liquid nitrogen cell freezer and stored in the vapor phase of liquid nitrogen maintained at <-150°C.

Recovery of CHIM. CHIM vials were removed from liquid nitrogen storage and thawed in a 37°C water bath for approximately 2 min. The contents of each individual vial were decanted into a 50 ml conical tube containing Cryopreserved Enterocyte Recovery Medium, (CERM™, In Vitro ADMET Laboratories, Columbia, MD) that was pre-warmed in a 37°C water bath. The thawed CHIM were recovered by centrifugation at 100 x g for 10 min at room temperature. After centrifugation, the supernatant was removed by decanting. A volume of 5 mL of 4°C Hepatocyte/Enterocyte Incubation Medium, (HQM™, In

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Vitro ADMET Laboratories, Columbia, MD) was added to the intact pellet of enterocytes at the bottom of the conical tube followed by briskly repipetting 5 times with a P1000 micropipet 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 Table 1 for P450 isoforms and Table 2 for non-P450 drug metabolizing enzymes. Incubations of CHIM and metabolism substrates were performed in a cell culture incubator maintained at 37°C with a humidified atmosphere of 5% CO_2. A volume of 50 uL of drug metabolizing enzyme substrates at 2x of the final desired concentrations was added into 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°C, followed by addition of CHIM at a volume of 50 uL per well to initiate the reaction.

The reaction plates were then incubated at 37°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 the subsequent LC/MS-MS analysis.

Evaluation of gene transcription. Gene transcription was quantified based on reverse transcription-real time PCR (RT-PCR) using the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Total RNA was extracted using the mini RNeasy kit (QIAGEN, www.qiagen.com) according to instructions provided by the manufacturer for isolation of total RNA from the hepatocytes. At first, reverse transcription (RT) was performed with approximately 200 ng of isolated RNA using the High Capacity cDNA Reverse Transcription Kit (Thermol Fisher Scientific Inc., www.thermofisher.com). Subsequently, quantitative PCR analysis was performed on RT reactions using gene specific primer/probe sets and the

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Taqman Fast Universal PCR Master Mix (Thermol Fisher). The relative quantity of the target gene was compared with that of the reference transcription of glucose 6-phosphate dehydrogenase (GAPDH) as determined by the ΔΔCT method and as previously described (Fahmi, Kish 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 of Hepatocyte Incubation Medium (IVAL, Columbia, MD). The CHIM suspension was added to each well of the 96-well plates followed by addition of 50 uL of the toxicants (acetaminophen and naproxen) at 2X of the final desired concentration. The CHIM cultures were then incubated in a CO_2 cell culture incubator kept at 37° C in a highly humidified atmosphere of 5% CO_2. After an incubation duration of 24 hrs., cell viability was determined based on cellular ATP contents as previously described. Results are presented as relative viability which is a ratio of the cellular contents of treated cultures to that of solvent control cultures.

Quantification of protein concentration. As 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, Waltham, MA) per instructions specified by the manufacturer.

LC/MS/MS Analysis. Upon thawing, the samples were centrifuged at 3,500 rpm for 5 minutes. An aliquot of 100 µL of supernatant from each was transferred to a 96 well plate and was diluted with 200 µL of deionized water with mixing before LC/MS/MS analysis. CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 (midazolam 1'-hydroxylation), CYP3A4 (testosterone 6β-

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hydroxylation), ECOD, UGT, SULT, FMO, MAO, AO, and NAT2 metabolites, as well as acetaminophen metabolism were quantified performed by using API 5000 mass spectrometer with an electrospray ionization source (AB SCIEX, Framingham, MA) connected to Waters Acquity UPLC (Waters Corporation, Milford, MA) using LC/MS/MS MRM mode, monitoring the mass transitions (parent to daughter ion) as previously described (Ho, Ring et al. 2017). An Agilent Zorbax Eclipse Plus C18 column (4.6 x 75 mm i.d., 3.5 µm; 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: 0 to 1 min, increased B from 5 to 90%; 1 to 1.5 min, keep B at 90%; 1.5 to 1.75 min, decreased B to 5%; run-time 3 min. The gradient program for the negative ion mode was: 0 to 2.5 min, increase B from 30 to 95%; 2.5 to 3.0 min, keep B at 95%; 3 to 3.2 min, decrease B to 30%; run-time 4 min. For conjugates, the gradients and run time may be adjusted for better separation. Data acquisition and data procession were performed with the software Analyst 1.6.2 (AB SCIEX, Framingham, MA).

Data Analysis. Data are presented as mean and standard deviation of triplicate incubations derived using the Microsoft Excel 6.0 software. Statistical analysis was performed using student's t-test with the Microsoft Excel 6.0 software, with the probability of $p<0.05$ to be considered statistically significant. Specific activity (pmol/min/mg protein) of each drug metabolizing enzyme pathway was determined by dividing the total metabolite formed by the incubation time and normalized to protein contents. Graphpad Prism 6.0 was used for the determination of EC50 for the cytotoxicity of acetaminophen and naproxen.

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Results

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

Morphology, size, viability and yield of cryopreserved CHIM. The isolated epithelial consist mainly of individual villi. These relatively large mucosal fragments were gently homogenized to form smaller, multicellular fragments followed by cryopreservation. The morphology of the freshly isolated villi and the multicellular fragments of the thawed CHIM are shown in Fig. 1. As CHIM were multicellular aggregates, cell concentration could not be determined and protein content was used for data quantification. The protein contents of each vial of CHIM (1 mL total volume) are as follows:

1. *Donor 1: Duodenum (2.5 mg); jejunum (1.8 mg); ileum (3.9 mg)*
2. *Donor 2: Duodenum (1.8 mg); jejunum (4.0 mg); ileum (3.3 mg)*
3. *Donor 3: Duodenum/jejunum/ileum combined (1.6 mg)*
4. *Donor 4: Duodenum (1.7 mg); jejunum (1.2 mg); 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-b hydroxylation. CYP2C9 and CYP2C19 activities were higher than that observed for CYP2A6, CYP2B6, CYP2C8, CYP2D6, and CYP2E1. The relative distribution of the P450 isoform activities is shown in Table 6. The activity-

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based P450 “pie” using testosterone 6b-hydroxylation for CYP3A4 activity for CHIM from the duodenum, jejunum and ileum of Donor 1 is shown in Fig. 2.

Non-P450 drug metabolizing enzyme activities. The non-P450 drug metabolizing enzyme pathways evaluated included ECOD, UGT, SULT, FMO, MAO, AO, NAT1, NAT2 and CES2. Results are shown in Table 7. Quantifiable activities were observed for all pathways evaluated except for AO. MAO found to have the highest activity which ranged from similar to higher than that observed for CYP3A4 (testosterone 6b-hydroxylation).

P450 induction. P450 induction was evaluated base on transcription. Upon a 24-hr treatment duration, dose dependent induction of CYP3A4 mRNA by rifampin and 25-hydroxyD3 and CYP24A1 mRNA by 25-hydroxyD3 were observed. For CYP3A4, the maximal fold of induction was approximately 3-fold for both inducers. Approximately 300-fold induction of CYP24A1 mRNA was observed for 25-hydroxyD3. (Figure 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 8. Results show that naproxen consistently demonstrated higher enterotoxicity than acetaminophen as demonstrated by the lower IC50 values for all CHIM lots evaluated.

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Discussion

We report here our success in the isolation and cryopreservation of 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. The light homogenization was necessary to allow the CHIM suspension to be delivered with a multichannel pipet into the wells of 96-well plates for experimentation.

CHIM were found to retain P450 and non-P450 drug metabolic enzyme activities characteristic of the small intestine. As reported before by others using intestinal microsomes (Paine, Khalighi et al. 1997; Perloff, Von Moltke et al. 2003; Yang, Tucker et al. 2004), and by us in cryopreserved enterocytes (Ho, Ring et al. 2017), the major P450 isoform activities were contributed by CYP3A4, especially for testosterone 6b-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. CYP1A1 activity was in general 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 (Table 6). The P450 "pie" constructed for duodenum, jejunum and ileum using testosterone 6'-hydydroxylation for CYP3A4 activity showed CYP3A4 as the most active P450 isoform in the small intestines as previous reported by Paine et al. (Paine, Hart et al. 2006). It is interesting to note that the

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previous reported decreasing CYP3A4 activity from duodenum to ileum (Paine, Khalighi et al. 1997). In our study, the following were observed for intestinal regional variations in CYP3A4/5:

- Donor 1: jenunum>duodenum>ileum for both midazolam 1'-hydroxylation and testosterone 6b-hydroxylation
- Donor 2: Jejunum>duodenum>ileum for midazolam 1'-hydroxylation and duodenum>jejunum>ileum for testosterone 6b-hydroxylation;
- Donor 4: ileum >jejunum>duodenum for both pathways

Our results with the limited donors therefore show that in general, the jejunum had the highest activity when compared to duodenum and ileum, and that individual differences in regional differences may occur, presumably due to various host factors including die and diseases. In our laboratory, individual differences and regional differences among individual will be further defined with intestines isolated from additional donors.

For the non-P450 drug metabolizing enzyme activities, the highest was observed for MAO, with activities for all four individuals higher than that observed for CYP3A4 quantified by testosterone 6b-hydroxylation. UGT, FMO, NAT1, NAT2 and CES2 activities were also in abundance, similar than that observed for CYP2C9. AO activities were mostly undetectable (Table 7). That UGT (Cubitt, Houston et al. 2009), FMO (Yeung, Lang et al. 2000), NAT(George 1981), and CES(Crow, Borazjani et al. 2007) activities are present in the small intestine have been previously reported.

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

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enzyme that catalyzes the conversion of 25-hydroxyvitamin D3 (25-OH-D3) and 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) 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 VDR and PXR pathway has been previously reported in small intestinal cell lines (Kolars, Schmiedlin-Ren et al. 1992; Thummel, Brimer et al. 2001; Thompson, Jurutka et al. 2002; Zheng, Wang et al. 2012) and in small bowel biopsies of patients treated with rifampin in vivo (Kolars, Schmiedlin-Ren et al. 1992). CYP3A induction in the small intestines in vivo may have physiological consequences including enhanced metabolism of vitamin D as well as orally-administered drugs that are substrates of CYP3A4 and CYP24A1. In the application of hepatocyte for the evaluation of hepatic P450 induction, mRNA is found to be a relevant in vitro endpoint that allows the estimation of in vivo effects (e.g., decrease in plasma T1/2 and plasma AUC) (Fahmi, Boldt et al. 2008; Youdim, Zayed et al. 2008; Fahmi, Kish et al. 2010; Einolf, Chen 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. NSAIDS, for instance, are known to cause upper gastrointestinal tract damages (Biour, Blanquart et al. 1987; Semble and Wu 1987). As 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 upon the development of an in vitro enterotoxicity assay with CHIM using two NSAIDs known

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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. While intestinal gastrointestinal ulcerations are commonly associated with acid reflux and h. pylori infection, enteropathy has also been associated with enterocyte cytotoxicity. CHIM may represent a physiological 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 IC50 of naproxen (0.35 – 0.39 mM) was statistically significant to be lower than that for acetaminophen (0.92 – 1.18 mM), with APAP having an IC50 value 3 – 6 times that for naproxen. Our results with CHIM is consistent with clinical findings that naproxen has a higher enterotoxicity than acetaminophen (Lewis, Langman et al. 2002). Our results therefore suggest that CHIM can be useful in the evaluation of enterotoxic potential of orally administered drugs, especially for drugs that may be activated or detoxified by enteric metabolism like APAP (Laine, Auriola et al. 2009; Jaeschke and McGill 2015; Jiang, Briede et al. 2015; Miyakawa, Albee et al. 2015) and naproxen (Miners, Coulter et al. 1996; Rodrigues, Kukulka et al. 1996; Tracy, Marra et al. 1997). In our laboratory, we have initiated a study on the role of drug metabolizing enzyme activities on the cytotoxicity of APAP 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 system 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 PBPK modeling considering key in vivo factors including rate of transit, drug dissolution, and available drug concentration at various regions of the intestinal tract.

CHIM represent an in vitro experimental system that can aid evaluation of enteric drug properties. Current in vitro experimental models include Caco-2 cells, IPS-derived intestinal cells which in general are deficient of drug metabolizing enzyme activities, especially the sub-optimal expression of CYP3A (Schmiedlin-Ren, Thummel et al. 1997; Cummins, Jacobsen et al. 2004; Negoro, Takayama et al. 2016), the most important drug metabolizing enzyme for enteric drug metabolism. 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, Ring 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, Reith et al. 1997; Li 2007; Li 2015).

It is also possible that CHIM can be used for the evaluation of additional enteric pharmacology and physiology, especially using transcription as endpoints. For instance, as CHIM contain 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, Dubuquoy et al. 2007).

One limitation of using CHIM for induction study is that the culture conditions only allows a culturing duration of 24 hrs, a property that likely reflects the short life span of enterocytes in vivo. In vivo, enterocytes are continuous formed from the intestinal crypts and migrate from the crypt surface and slough off at villus tip in a 3 days 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 the apoptosis if 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, Woo et al. 1975; Pantuck, Hsiao et al. 1976; Zhang, Jia 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 are also ongoing comparing drug metabolizing enzyme activities before and after cryopreservation. As of the time of this study, no substantial loss of enzyme activities due to cryopreservation have been observed (Li, A. P., 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.

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Authorship Contributions

Participated in research design: Li, A.P., Ho, D., Yang, Q.

Conducted experiments: Ho, D., Amaral, K., Alam, N., Mitchell, W., Yang, Q., Loretz, C.

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Figure legends.

Figure 1. Morphology of freshly isolated human intestinal mucosal epithelium (top microphotograph) and thawed CHIM (middle and bottom microphotographs) from the duodenum of the small intestines from Donor 1. The freshly isolated mucosal epithelia consisted mainly of intestinal villi (top; 50X magnification; brightfield microscopy). The epithelia were gently homogenized to smaller fragments for cryopreservation. CHIM recovered from cryopreservation consisted of smaller pieces to form multicellular fragments (middle; 100X magnification; phase contrast microscopy). In the bottom microphotograph, each of the square shown is that of a hemocytometer with a dimension of 200 uM (bottom; 100X magnification; brightfield microscopy).

Figure 2. P450 pie of the distribution of P450 isoform activities of CHIM isolated from the duodenum, jejunum and ileum of Donor 1. Testosterone 6-b hydroxylation activity is used to represent CYP3A4 activity to construct the pie chart.

Figure 3. Induction of CYP3A4 transcription by rifampin and 1,25(OH)2D3 (upper 2 charts) and induction of CYP24A1 transcription by 1,25(OH)2D3 (lowest chart). Error bars represent the standard deviations of results from triplicate treatments.

Figure 4. Acetaminophen (APAP) 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 APAP, consistent with human in vivo findings. IC50 ratio was calculated by dividing the IC50 of APAP by that of naproxen.

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Table 1: A summary of the P450 isoform-selective substrates and their respective metabolites quantified for CHIM

Isoform	Substrate	Substrate Conc. (μM)	Metabolites Quantified
CYP1A1	7-Ethoxyresorufin	20	Resozufin
CYP1A2	Phenacetin	100	Acetaminophen
CYP2A6	Coumarin	50	7-HC, 7-HC-Sulfate, 7-HC-Glucuronide
CYP2B6	Bupropion	500	Hydroxybupropion
CYP2C8	Paclitaxel	20	6α-hydroxypaclitaxel
CYP2C9	Diclofenac	25	4-hydroxydiclofenac
CYP2C19	*S*-Mephenytoin	250	4-hydroxy *S*-Mephenytoin
CYP2D6	Dextromethorphan	15	Dextrophan
CYP2E1	Chlorzoxazone	250	6-hydroxy chlorzoxazone
CYP3A4/5-1	Midazolam	20	1-hydroxymidazolam
CYP3A4/5-2	Testosterone	200	6β-hydroxytestosterone
CYP2J2	Astemizole	50	O-Demethyl Astemizole

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Table 2. A summary of the nonP450 pathway selective substrates and their respective metabolites quantified in CHIM

DME Pathway	Substrate	Substrate Conc. (μM)	Metabolites Quantified
ECOD	7-Ethoxycoumarin	100	7-HC, 7-HC-Sulfate, 7-HC-Glucuronide
UGT	7-Hydroxycoumarin	100	7-Hydroxycoumarin Glucuronide
SULT	7-Hydroxycoumarin	100	7-Hydroxycoumarin Sulfate
FMO	Benzydamine HCl	250	Benzydamine-N-Oxide
MAO	Kynuramine HBr	160	4-hydroxyquinoline
AO	Cabazeran	20	4-Hydroxycabazeran
NAT1	4-Aminobenzoic Acid	200	N-Acetyl-p-aminobenzoic acid
NAT2	Sulfamethazine	100	N-Acetyl-sulfamethazine
CES2	Irinotecan	50	SN38

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Table 3: Primers used for RT-PCR quantification of transcription. GAPDH represents the house keeping gene used for normalization of the transcription of CYP3A4 and CYP24A1.

Target Gene	Direction	Primer Sequence
GAPDH	F	AAGGTGAAGGTCGGAGTCAA
	R	AATGAAGGGGTCATTGATGG
CYP3A4	F	TTTTGTCCTACCATAAGGGCTTT
	R	CACAGGCTGTTGACCATCAT
CYP24A1	F	GGTGACATCTACGGCGTACAC
	R	CTTGAGACCCCCTTTCCAGAG

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Table 4. Demographic information of the donors of the small intestines used in the preparation of CHIM for the study.

	Donor 1	Donor 2	Donor 3	Donor 4
Gender	Male	Male	Male	Male
Age (years)	20	48	59	20
Race	Caucasian	Caucasian	Hispanic	Caucasian
Cause of death	CVA, Anoxia, Non-MVA	Anoxia	CVA 2nd to ICH	Asphyxiation
BMI	30.62	21.04	30.27	26.34
Smoking	No	No	Yes	No
Alcohol	No	No	No	No
Substance abuse	Yes	No	No	No

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Table 5: P450 isofom-specific activities of CHIM isolated from the duodenum, jejunum and ileum of Donor 1, Donor 2 and Donor 4, and from the small intestine (from duodenum to ileum) of Donor 3. (BQL: below limits of quantification)

	Activity (pmol/min/mg protein)									
	Donor 1			Donor 2			Donor 3	Donor 4		
Isoform	*Duodenum*	*Jejunum*	*Ileum*	*Duodenum*	*Jejunum*	*Ileum*	*Combined*	*Duodenum*	*Jejunum*	*Ileum*
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

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Table 6. Distribution of P450 isoform activities in CHIM using midazolam-1'hydroxylation (top chart) and testosterone 6b-hydroxylation (bottom chart) as CYP3A4 activity. Results are expressed as percent of the arithmetic sum of the specific activities (pmol/min/mg protein) of the P450 isoforms evaluated.

	Percent of Total Activities									
	Donor 1			Donor 2			Donor 3	Donor 4		
Isoform	*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

	Percent of Total Activities									
	Donor 1			Donor 2			Donor 3	Donor 4		
Isoform	*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	0.01	0.02	0.41	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

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Table 7. Non-P450 drug metabolizing enzyme activities of CHIM isolated from the duodenum, jejunum and ileum of Donor 1, Donor 2 and Donor 4, and from the entire small intestine (from duodenum to ileum) of Donor 3. BQL: Below limits of quantification.

	Activity (pmol/min/mg protein)									
	Donor 1			Donor 2			Donor 3	Donor 4		
Metabolic Pathway	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Combined	Duodenum	Jejunum	Ileum
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

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Table 8. IC50 values for acetaminophen (APAP) and naproxen in CHIM isolated from human duodenum, jejunum, and ileum of Donor 1; duodenum and jejunum of Donor 2, and the combination of all three small intestine regions of Donor 3. Asterisks represent IC50 values obtained from naproxen that are statistically significant to be different ($p<0.05$) from that obtained for APAP. IC50 ration is calculated by dividing the IC50 for APAP by that for naproxen. The results show that naproxen was consistently more cytotoxic than APAP for all three regions of the small intestine.

	IC50 (mM)		
Drug	Duodenum	Jejunum	Ileum
APAP	2.25	1.22	1.20
Naproxen	0.35*	0.39*	0.36*
IC50 Ratio	*6.36*	*3.09*	*3.35*

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Figure 1:

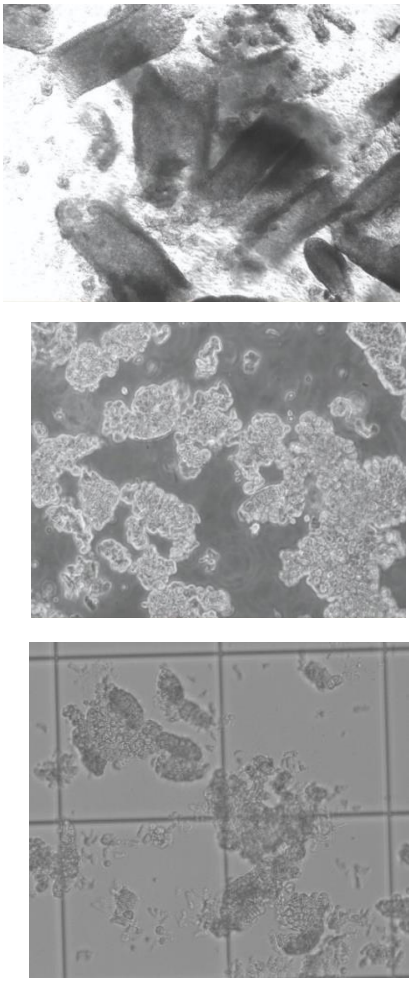

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Figure 2.

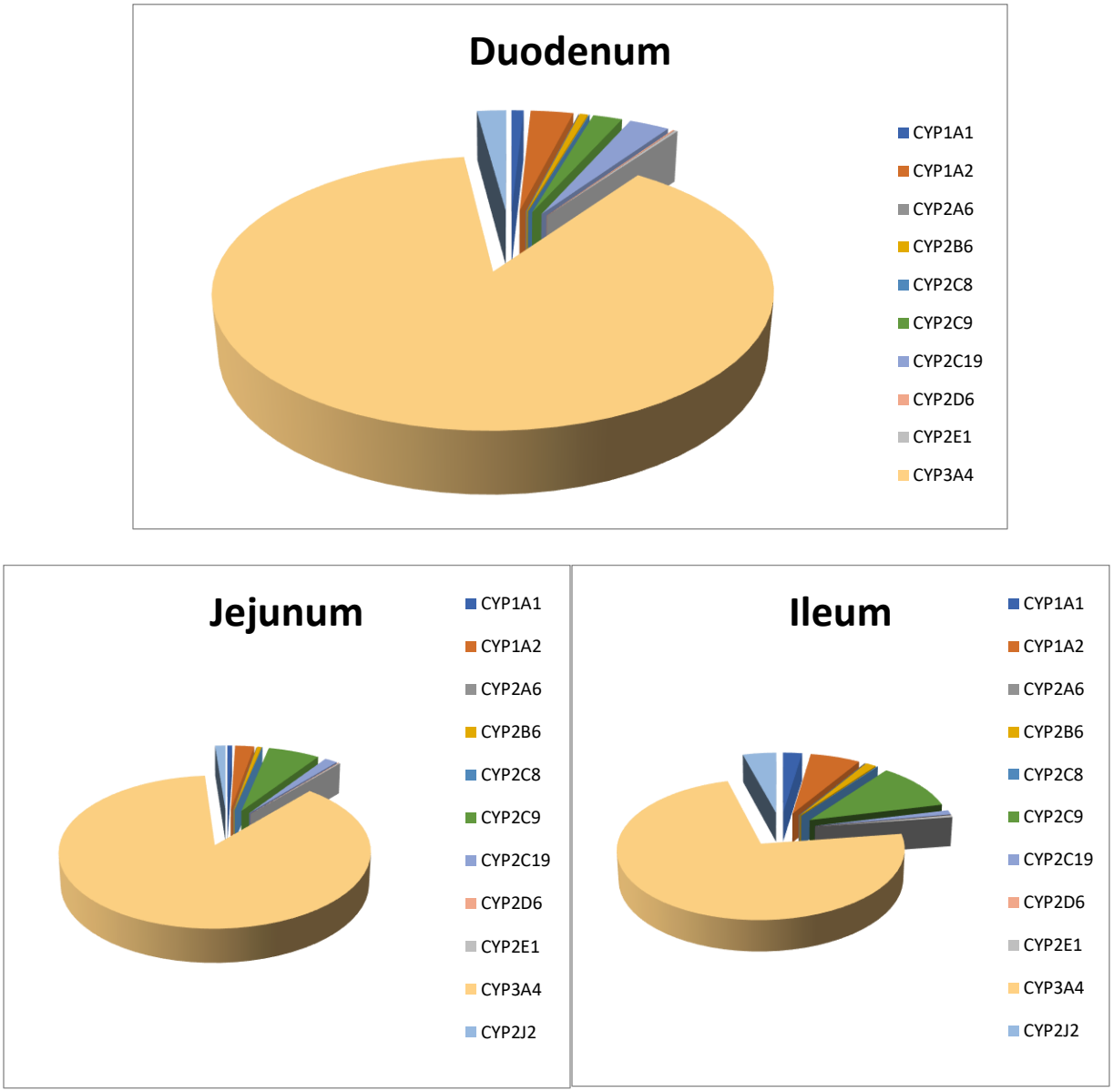

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Figure 3.

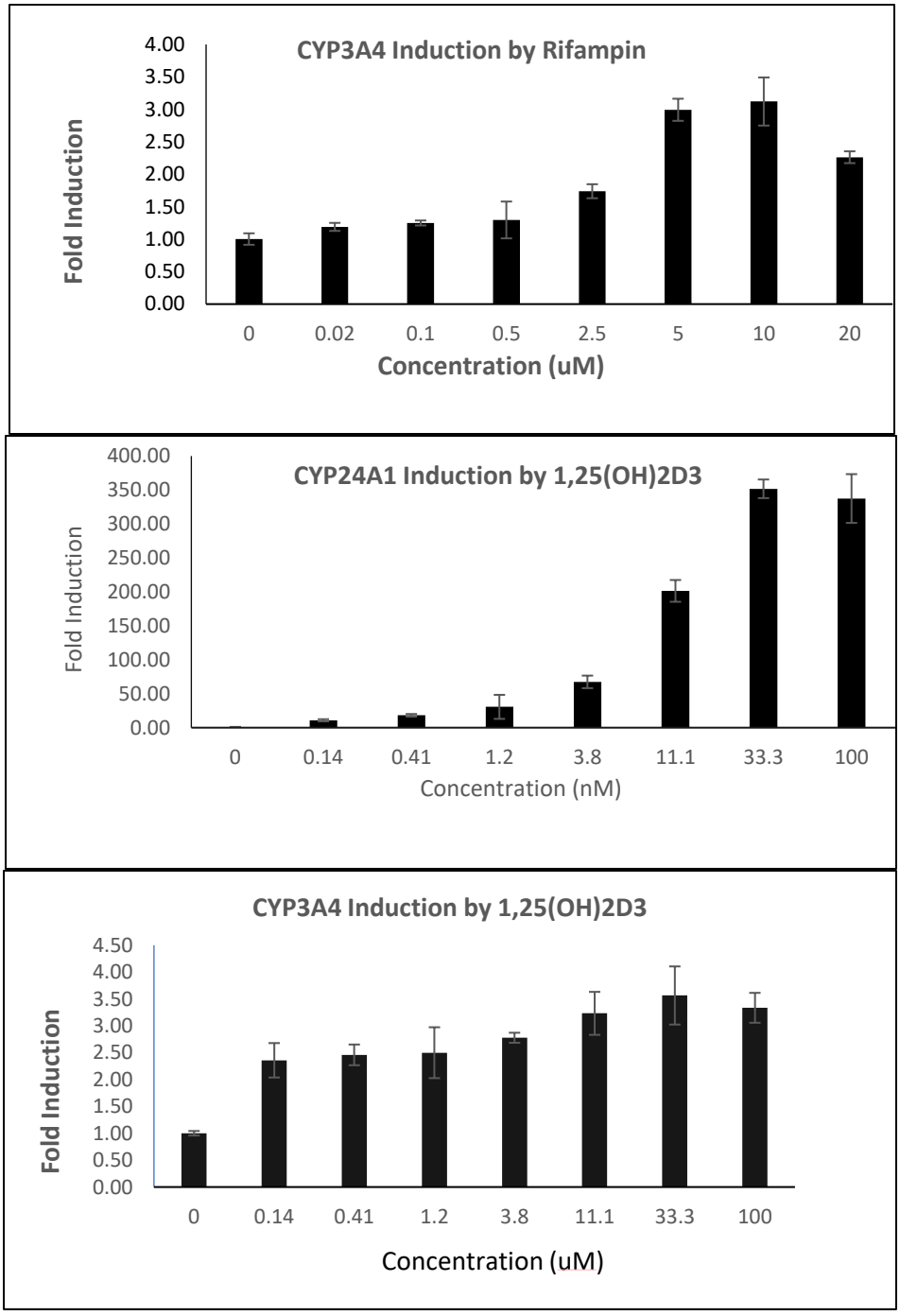

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Figure 4.