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TITLE PAGE

SHORT COMMUNICATION

In Vitro-In Vivo Extrapolation Scaling Factors for Intestinal P-glycoprotein and Breast Cancer Resistance Protein: Part II. The Impact of Cross-Laboratory Variations of Intestinal Transporter Relative Expression Factors on Predicted Drug Disposition

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RUNNING TITLE PAGE

Running Title: Impact of Cross-Laboratory Differences of Intestinal REF on IVIVE-PBPK

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ABBREVIATIONS – BCRP (Breast Cancer Resistance Protein); BPh (Bertin Pharma); fa (fraction of dose absorbed); HV (Healthy Caucasian Volunteer population); IVIVE (*In Vitro-In Vivo* Extrapolation); J_{max} (maximal flux capacity of the transporter protein); PBPK (Physiologically-Based Pharmacokinetic); P-gp (P-glycoprotein); REF (Relative Expression Factor); REF_{iBCRP} (intestinal Relative Expression Factor for Breast Cancer Resistance Protein); $REF_{iP\text{-}gp}$ (intestinal Relative Expression Factor for P-glycoprotein); SITT (Small Intestinal Transit Time); TC (Theoretical BCRP Compound); UoM (University of Manchester).

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ABSTRACT

Relative Expression Factors (REFs) are used to scale *in vitro* transporter kinetic data *via In Vitro–In Vivo* Extrapolation linked to Physiologically-Based Pharmacokinetic (IVIVE-PBPK) models to clinical observations. Primarily two techniques to quantify transporter protein expression are available, immunoblotting and LC-MS/MS. Literature collated Relative Expression Factors (REF) ranged from 0.4 to 5.1 and 1.1 to 90 for intestinal P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP), respectively. The impact of using human jejunum-Caco-2 REFs, for P-gp ($REF_{iP\text{-}gp}$) and BCRP (REF_{iBCRP}), generated from the same samples, employing different proteomic methodologies from independent laboratories, on PBPK outcomes was assessed. A 5-fold decrease in $REF_{iP\text{-}gp}$ for a single oral dose of digoxin resulted in a 1.19- and 1.31-fold higher plasma AUC and C_{max}, respectively. All generated $REF_{iP\text{-}gp}$ values led to simulated digoxin C_{max} values within observed ranges; however, combining kinetic data generated from a different laboratory, with the 5-fold lower $REF_{iP\text{-}gp}$, could not recover a digoxin-rifampicin DDI; emphasising the necessity to obtain transporter-specific kinetic estimates and REFs from the same *in vitro* system. For a theoretical BCRP compound, with absorption taking place primarily in the jejunum, a decrease in the REF_{iBCRP} from 2.22 (University of Manchester) to 1.11 (Bertin Pharma) promoted proximal intestinal absorption, while delaying t_{max} 1.44-fold. Laboratory-specific differences in REF may lead to different IVIVE-PBPK outcomes. To understand the mechanisms underlying projected PK liabilities, it is important to assess the potential impact of bias on the generation of REFs on an inter-individual basis within a target population.

INTRODUCTION

In Vitro-In Vivo Extrapolation linked to Physiologically-Based Pharmacokinetic (IVIVE-PBPK) models aim to predict profiles of drug disposition dynamically. This is accomplished by incorporating 'drug' data, generated *in vitro* and physico-chemical knowledge, together with 'systems' data in a population (Rostami-Hodjegan, 2012). Kinetic data (i.e., J_{max} and K_m) describing the active transport processes generated from cell systems can also be included in IVIVE-PBPK models. In order to scale these data to *in vivo*, human and *in vitro* system transporter protein expression or activity data are also required in combination with physiological, demographic and genetic information (Rostami-Hodjegan, 2012). Intestinal transporter IVIVE scaling factors have to date (Neuhoff et al., 2013a) been generated based on Western blotting, a relative quantitative technique to quantify transport expression (Troutman and Thakker, 2003a). Yet, absolute transporter protein abundances quantified by LC-MS/MS have recently been explored for hepatic application in IVIVE-PBPK (Vildhede et al., 2014).

In this study, we provide a systematic analysis of the mRNA and protein expression data available in the literature for generating the Relative Expression Factor (REF), an IVIVE scalar that describes the ratio of *in vivo* to *in vitro* systems transporter expression for human jejunum and Caco-2 monolayer P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP). We also evaluate the impact of intestinal P-gp and BCRP REFs generated by different laboratories and methodologies on drug absorption in a PBPK model.

MATERIALS AND METHODS

***Literature Review of the Intestinal Expression Data of P-gp and BCRP*:** Starting with a previously reported meta-analysis that established human intestinal P-gp and BCRP regional-specific protein expression (Harwood et al., 2013), a new search for the relevant published

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data quantifying P-gp and BCRP mRNA or protein expression in human jejunum and filter-grown Caco-2 monolayers, using PubMed (http://www.ncbi.nlm.nih.gov/pubmed) was undertaken. The following keyword combinations were used in PubMed: human; jejunum; Caco-2; P-gp; MDR1; BCRP; ABCB1; ABCG2; mRNA; protein; expression; absolute; abundance; proteomics. Graphical data were extracted, where required, by GetData Graph Digitizer (http://getdata-graph-digitizer.com).

Generation of the Relative Expression Factors: A REF for P-gp or BCRP was generated, where P-gp or BCRP mRNA/protein expression for human jejunum and Caco-2 monolayers was available using the same technique within a laboratory, including reference genes. These were compared to REFs generated from two different LC-MS/MS workflows (two-independent laboratories; matching samples) for P-gp and BCRP in human jejunum and 21 day cultivated Caco-2 monolayers. Methodological details and individual values are provided in the companion study, Part I (Harwood et al., DMD).

Incorporating Intestinal REFs into IVIVE-PBPK Models: The impact of the laboratory-specific $REF_{iP\text{-}gp}$ and REF_{iBCRP} in virtual Healthy Caucasian Volunteers (HV) was assessed in a PBPK model (version 14.1, Simcyp, a Certara company, Sheffield, UK), containing the regional distribution of intestinal P-gp and BCRP, and their population variability (Harwood et al., 2013). P-gp and BCRP transport in the model is driven by the unbound intracellular enterocyte concentration and is multiplied by REF and the regional-specific transporter expression to yield effective permeability (Yang et al., 2007; Neuhoff et al., 2013a).

The Impact of P-gp and BCRP REF in IVIVE-PBPK: The impact of $REF_{iP\text{-}gp}$ values generated by the LC-MS/MS (University of Manchester (UoM); Harwood et al., DMD) compared to the immunoblotting approach (Troutman and Thakker, 2003a) on digoxin C_{max} was investigated using identical digoxin parameter inputs as the previously reported digoxin

IVIVE-PBPK model (Neuhoff et al., 2013a). Caco-2 derived J_{max} and K_m data (Troutman and Thakker, 2003b) was applied to intestinal and hepatic P-gp, assuming P-gp activity *in vitro* corresponds in healthy individuals to that *in vivo*, and that J_{max} is related to P-gp protein expression. Simulations were run with a single oral digoxin dose of 0.5 mg in 100 HV individuals to evaluate if kinetic data for digoxin generated in a Caco-2 system from another laboratory (Troutman and Thakker, 2003b) to the $REF_{iP\text{-}gp}$ from the UoM, could capture the observed digoxin-rifampicin DDI, *via* induction of intestinal P-gp (Greiner et al., 1999), thus verifying the correct contribution of the active transport built into the digoxin PBPK model.

The impact of laboratory-specific differences for REF_{iBCRP} on PK parameter predictions was evaluated using the Simcyp simulator. A permeable compound (TC; see Supplemental Information, Technical Note) with limited gut metabolism and a specific BCRP activity was administered orally (10 mg in-solution) to 100 HV individuals, with the default regional-specific BCRP expression within the PBPK model, as published by (Harwood et al., 2013).

RESULTS & DISCUSSION

$REF_{iP\text{-}gp}$ and REF_{iBCRP} Generation from Different Laboratories: According to our literature analysis, human jejunum mRNA and protein expression was identified in 19 studies for P-gp and 9 studies for BCRP (Table S1, Supplemental Information). Expression data for Caco-2 P-gp and BCRP from the same laboratory using the same protocol to generate a REF_{iPgp} or REF_{iBCRP} was found for 5 and 4 studies, respectively (Table 1). For P-gp, relative mRNA expression (RT-PCR) analysis enabled the generation of $REF_{iP\text{-}gp}$ from two laboratories in 3 studies (Taipalensuu et al., 2001; Seithel et al., 2006; Hilgendorf et al., 2007), as the data from Seithel et al., (2006) and Hilgendorf et al., (2007) used Caco-2 monolayers cultivated in the same laboratory for 23 and 16 days, respectively. A $REF_{iP\text{-}gp}$ from two independent laboratories that used Western blotting (Troutman and Thakker, 2003a; von Richter et al.,

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2009) is available, but not for LC-MS/MS quantification for either P-gp or BCRP, as the Caco-2 cell abundances reported by Oswald et al., (2013) were from plastic, not filter-grown cells (personal communication). The $REF_{iP\text{-}gp}$ based on mRNA expression ranged 7.1-fold and for LC-MS/MS quantification 2.6-fold. The REF_{iBCRP} of 90 (Taipalensuu et al., 2001) may result from low BCRP levels or variability in the housekeeping gene used (Seithel et al., 2006). The $REF_{iP\text{-}gp}$ and REF_{iBCRP} generated from P-gp and BCRP quantification by LC-MS/MS from two different laboratories for the same samples, UoM and Bertin Pharma (BPh) is provided in Table 1 (Harwood et al., DMD). The $REF_{iP\text{-}gp}$ (Troutman and Thakker, 2003a) from independent samples quantified by Western blotting was 5-fold higher than the $REF_{iP\text{-}gp}$ generated by the UoM (UoM-$REF_{iP\text{-}gp}$), while, the UoM-REF_{iBCRP} was approximately 2-fold higher than BPh (LC-MS/MS) and Altana AG (Western Blot) (von Richter et al., 2009).

Assessing the Sensitivity of $REF_{iP\text{-}gp}$ in IVIVE-PBPK: Both the UoM-$REF_{iP\text{-}gp}$ of 0.4 (Harwood et al., DMD) and the $REF_{iP\text{-}gp}$ of 2 (Troutman & Thakker, 2003a) led to digoxin C_{max} values within observed ranges after a single oral dose of 0.5 mg digoxin (Figure 1A), implying both $REF_{iP\text{-}gp}$ reflect realistic contributions of P-gp to estimate observed C_{max} values when using the J_{max} and K_m for P-gp reported by Troutman and Thakker (2003b). Using the UoM-$REF_{iP\text{-}gp}$ of 0.4 compared to the $REF_{iP\text{-}gp}$ of 2 led to a modest 1.31- and 1.19-fold lower mean C_{max} and AUC, respectively in 100 HV individuals (Figure 1B). A previous study showed that the observed digoxin-rifampicin DDI, which was attributed to a 3.5-fold increase in intestinal P-gp expression (Greiner et al., 1999), could be recovered using an IVIVE-PBPK strategy, in which the $REF_{iP\text{-}gp}$ of 2.0 was increased 3.5-fold to 7 after induction (Neuhoff et al., 2013b). A 3.5-fold increase in the UoM-$REF_{iP\text{-}gp}$ of 0.4 gave a $REF_{iP\text{-}gp}$ of 1.4, leading to a simulated under-prediction in the observed DDI (Figure 1C (no induction) & D (induction)). This indicates that the lower UoM-$REF_{iP\text{-}gp}$ (that is not derived from the same Caco-2 system in which the apparent kinetic data was generated) is not sufficient to recover

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the contribution of P-gp induction by rifampicin on digoxin plasma concentration in HVs. The inability to recover the observed DDI when using the UoM-$REF_{iP\text{-}gp}$ may result from lower P-gp expression and hence lower activity in the UoM Caco-2 systems. This can be due to laboratory differences in methods for expression quantification, Caco-2 cell cultivation and the variability in jejunum expression. To recover the activity shortfall when using the UoM-$REF_{iP\text{-}gp}$, a 4.3-fold increase in J_{max} (1874 pmol/min/cm^2), was required to recover the observed DDI (Figure 1E) after using the Nelder-Mead minimization method and weighted least squares algorithm in the simulators parameter estimation module (Jamei et al., 2014).

Assessing the Sensitivity of REF_{iBCRP} in IVIVE-PBPK: The sensitivity of regional-specific fraction of dose absorbed (fa) and enterocyte concentrations to REF_{iBCRP} generated by BPh (1.11) and the UoM (2.22) (Table 1; Harwood et al., DMD) was assessed for the BCRP test compound TC. Figure 2 shows the free segmental enterocyte concentration for TC, used as the driving force for apical efflux transporters. As expected, the lower BPh-REF_{iBCRP} leads to higher TC enterocyte concentrations in proximal regions than the higher UoM-REF_{iBCRP}. While, an increasing importance of intestinal BCRP UoM-(REF_{iBCRP}) results in higher TC absorption and higher enterocyte concentration in the distal intestine due to the efflux activity promoting TC retention in the gut lumen and transit to the colon, a region with 7.7-fold lower BCRP levels (Harwood et al., 2013). The higher REF_{iBCRP} has a limited impact on lowering C_{max} and AUC (1.22-fold & 1.03-fold, respectively), but increases t_{max} 1.44-fold to 2.8 hours and is in-line with clinical observations, where an inhibition of intestinal BCRP leads to a decrease in t_{max} (Schneck et al., 2004). Alongside differences in BCRP expression, inter-individual variability in system parameters like the small intestinal transit time (SITT; range 0.5 – 10 hours; Yu et al., 1996) also contribute to regional-specific fa (Figure S1, Supplemental Information). Acidic BCRP substrates, like rosuvastatin, are expected to possess higher enterocyte concentrations, as limited metabolism, low passive permeation and

apical uptake transporters operate (Li et al., 2012; Jamei et al., 2014). Therefore, increased BCRP expression alters t_{max} and regional absorption, while not limiting overall absorption and bioavailability. This is dissimilar to the cooperation of P-gp and CYP3A4 activities that facilitates a drug's repeated exposure to intestinal CYP3A4, increasing overall gut metabolism, reducing bioavailability (Wacher et al., 1998). To our knowledge, the current study is the first highlighting this difference of the co-localised transporters P-gp and BCRP.

Combining IVIVE scalars and activity data generated from different laboratories for ATP-dependent transporters, may not lead to successful IVIVE, while we postulate that laboratory-specific differences in REF may impact on the mechanistic understanding of projected PK liabilities (efficacy/toxicity). This is due to *in vitro* activity, reproducibility of *in vitro* assays, culture conditions and proteomic workflows. As discussed previously, direct translation of protein expression to activity may not always occur, therefore accounting for deviations in this linear relationship *via* activity-abundance scalars will be required (Harwood et al., 2013). Ideally, scaling factors should be defined on a laboratory-specific basis against a common reference and combined with activity data from the same system. However, it is improbable within an industrial setting that groups will possess a bank of human intestinal tissues by which to obtain the *in vivo* abundance for in-house intestinal REF generation. It is therefore advocated that commercially available pooled human intestinal microsomes (constituting ≥ 20 intestines) are utilized to generate a REF using the same proteomic methods as those used for quantifying *in vitro* system abundances used for determining activity. Alternatively, a link between human liver microsomes, intestinal microsomes and Caco-2 cells can be approached.

AUTHORSHIP CONTRIBUTIONS: ***Participated in research design:*** Harwood, Neuhoff, Warhurst, Rostami-Hodjegan

Conducted experiments*:* Harwood, Achour

Contributed new reagents & analytical tools*:* Russell, Carlson

Performed data analysis*:* Harwood, Achour, Neuhoff

Wrote or contributed to the writing of the manuscript: Harwood, Achour, Neuhoff, Warhurst, Rostami-Hodjegan

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FIGURE LEGENDS:

Figure 1. (A) Sensitivity of C_{max} to $REF_{iP\text{-}gp}$ for digoxin (single oral dose, 0.5 mg) in 100 HV individuals. The ranges of observed C_{max} values are given between the dashed lines and the $REF_{iP\text{-}gp}$ of 2.0 from Troutman and Thakker (2003a) (grey arrows) and the UoM-$REF_{iP\text{-}gp}$ (Harwood et al., DMD) (dashed arrows) are shown. (B) The mean digoxin plasma concentration when using the $REF_{iP\text{-}gp}$ of 2.0 (Troutman and Thakker, 2003a) and the UoM $REF_{iP\text{-}gp}$ of 0.4 in 100 HVs. (C) The observed and predicted plasma concentrations for digoxin (single oral dose, 1 mg) in 80 HV individuals after the dosing of rifampicin (600 mg, 11 doses, once daily) using a $REF_{iP\text{-}gp}$ of 2 (Troutman and Thakker, 2003a), (D) $REF_{iP\text{-}gp}$ of 0.4 (Harwood et al., DMD) and (E) of 0.4 after optimising the J_{max} of P-gp by parameter estimation. Key, Fig C-E: The thin grey lines represent mean values for 10 individual virtual trials of 8 individuals, males aged 21-37 years, the thick lines are the overall means of the virtual population (n = 80) and the dashed lines the 95^{th} and 5^{th} percentiles of the confidence interval. Open circles mark the observed digoxin concentrations when co-administered with multiple doses of rifampicin (Greiner et al., 1999).

Figure 2. Simulated enterocyte concentration profiles for all intestinal segments of the model for the BCRP compound TC (10 mg oral, single dose, in-solution) in 100 HVs using the BPh REF_{iBCRP} (1.11) or UoM-REF_{iBCRP} (2.22) with a mean small intestinal transit time (SITT) of 3.34 hours, a passive apparent permeability of 115.2 x 10^{-6} cm/s and an intrinsic clearance for BCRP of 17 μL/min/cm^2.

Table 1. Generation of $REF_{iP\text{-}gp}$ and REF_{iBCRP} from human jejunum and Caco-2 from relative mRNA expression and protein abundance (relative & absolute) data available in the literature for P-gp (MDR1) and BCRP.

Transporter	Method	Reference standard gene/protein/peptide	Jejunum Abundance Mean (SD, sample *n*)	Caco-2 Abundance Mean (SD, sample *n*)	REF	Source
P-gp (MDR1)	RT-PCR	Villin	7.90 (± 1.4, *n* = 13)	11.0 (*n* = 1)	0.7	Taipalensuu et al., 2001[a]
	RT-PCR	Cyclophilin-A	0.63 (± 0.23, *n* = 4)	0.13 (*n* = 1)	5.0	Seithel et al., 2006[b]
	RT-PCR	Cyclophilin-A	1.1 (± 0.67 , *n*=5)	0.21 (± 0.04 , *n*=3 - 6)	5.1	Hilgendorf et al., 2007[b]
	Western blot	Not run in blot	2.12 (*n* = 1)	1.04 (± 0.16, *n* = 3)	2.0	Troutman and Thakker, 2003a[c]
	Western blot	Not run in blot	1.00 (*n* = 5, pooled)	1.29 (*n* = 2)	0.8	Von Richter et al., 2009[c]
	LC-MS/MS	AGAVAEEVLAAIR	1.89 (± 1.07, *n* = 3)	4.67 (± 0.47, *n* = 3)	0.4	Harwood et al., 2015b[d]
	LC-MS/MS	FYDPLAGK	0.77 (± 0.35, *n* = 3)	2.08 (± 0.19, *n* = 3)	0.4	Harwood et al., 2015b[e]
BCRP	RT-PCR	Villin	2.7 (± 1.4, *n* = 13)	0.03 (*n* = 1)	90	Taipalensuu et al., 2001[a]
	RT-PCR	Cyclophilin-A	0.38 (± 0.08, *n* = 4)	0.07 (*n* = 1)	5.5	Seithel et al., 2006[b]
	RT-PCR	Cyclophilin-A	2.36 (± 0.29, *n* = 5)	0.36 (*n* = 3 - 6)	6.6	Hilgendorf et al., 2007[b]
	Western blot	Not run in blot	1.00 (*n* = 5, pooled)	0.84 (*n* = 2)	1.2	Von Richter et al., 2009[c]
	LC-MS/MS	VIQELGLDK	(2.56 ± 0.82, *n* = 3)	(1.16± 0.04, *n* = 3)	2.2	Harwood et al., 2015b[d]
	LC-MS/MS	SSLLDVLAAR	(2.06 ± 1.11, *n* = 3)	(1.86 ± 0.14, *n* = 3)	1.1	Harwood et al., 2015b[e]

[a] units for expression of mRNA are number of transcripts per μg of total RNA, [b] units for expression given as relative units of target to reference gene ($2^{-\Delta Ct}$), [c] values for expression given as signal intensity as measured by densitometry image analysis, [d,e] units for abundance given as fmol protein/μg total membrane protein, [d] abundance determined in UoM, [e] abundance determined in BPh, [d,e,] the same samples were quantified.

FIGURE 1

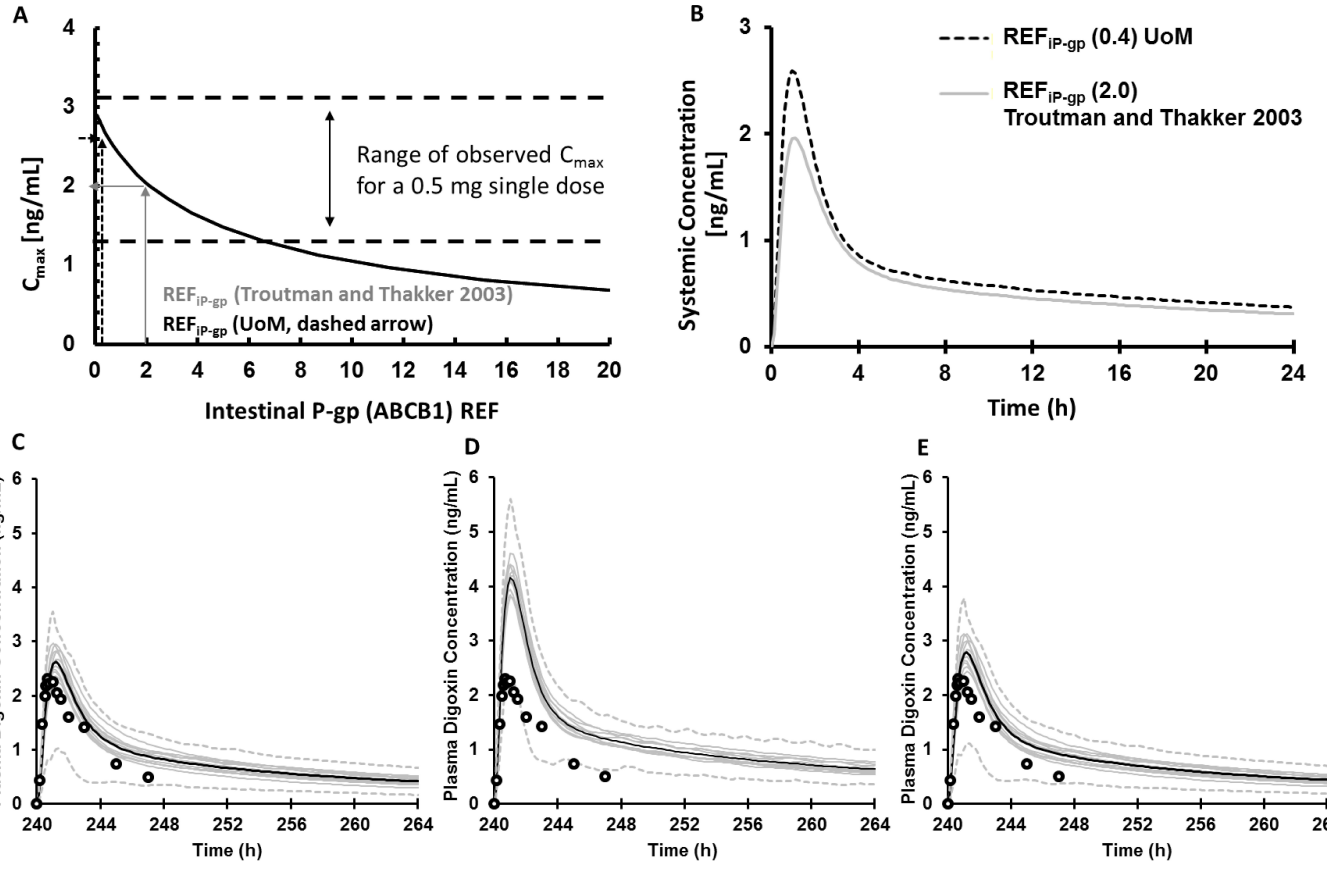
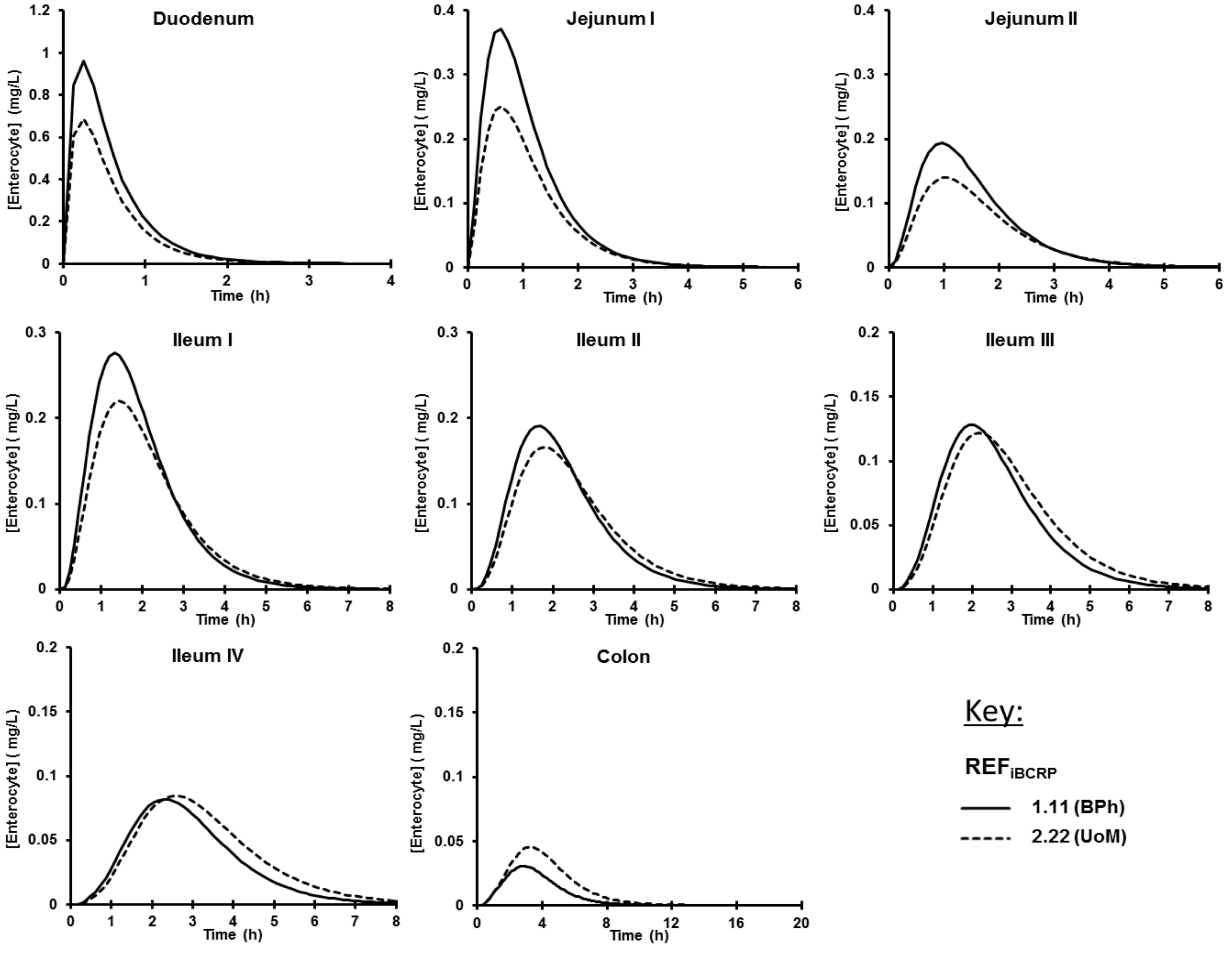

A
Range of observed C_{max} for a 0.5 mg single dose
$REF_{iP\text{-}gp}$ (Troutman and Thakker 2003)
$REF_{iP\text{-}gp}$ (UoM, dashed arrow)
C_{max} [ng/mL]
Intestinal P-gp (ABCB1) REF
B
$REF_{iP\text{-}gp}$ (0.4) UoM
$REF_{iP\text{-}gp}$ (2.0)
Troutman and Thakker 2003
Systemic Concentration [ng/mL]
Time (h)
C
D
E
Plasma Digoxin Concentration (ng/mL)
Time (h)

FIGURE 2


Duodenum
Jejunum I
Jejunum II
Ileum I
Ileum II
Ileum III
Ileum IV
Colon
[Enterocyte] (mg/L)
Time (h)
Key:
REF iBCRP
1.11 (BPh)
2.22 (UoM)