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REVIEW ARTICLE

Predicting Drug Extraction in the Human Gut Wall: Assessing Contributions from Drug Metabolizing Enzymes and Transporter Proteins using Preclinical Models

Sheila Annie Peters[1] · Christopher R. Jones[2] · Anna-Lena Ungell[3] ·
Oliver J. D. Hatley[4]

Abstract Intestinal metabolism can limit oral bioavailability of drugs and increase the risk of drug interactions. It is therefore important to be able to predict and quantify it in drug discovery and early development. In recent years, a plethora of models—in vivo, in situ and in vitro—have been discussed in the literature. The primary objective of this review is to summarize the current knowledge in the quantitative prediction of gut-wall metabolism. As well as discussing the successes of current models for intestinal metabolism, the challenges in the establishment of good preclinical models are highlighted, including species differences in the isoforms; regional abundances and activities of drug metabolizing enzymes; the interplay of enzyme-transporter proteins; and lack of knowledge on enzyme abundances and availability of empirical scaling factors. Due to its broad specificity and high abundance in the intestine, CYP3A is the enzyme that is frequently implicated in human gut metabolism and is therefore the major focus of this review. A strategy to assess the impact of gut wall metabolism on oral bioavailability during drug discovery and early development phases is presented. Current gaps in the mechanistic understanding and the prediction of gut metabolism are highlighted, with suggestions on how they can be overcome in the future.

Key Points
A summary of current knowledge for the prediction of intestinal metabolism using in vivo, in situ, in vitro and mathematical models is provided.
A strategy for the prediction of intestinal extraction that can be applied in drug discovery and early development is outlined.
Gaps in current knowledge and technology that hamper the prediction of intestinal metabolism have been identified and a future direction proposed.

1 Introduction

Oral dosing is the preferred route of administration as it is cheap, convenient and safe for patients [1, 2]. However, oral drug bioavailability is often limited by first-pass extraction in the gut and liver requiring higher doses compared with intravenous administration. Poor oral bioavailability has led to the failure of many drugs. As such, pharmaceutical companies aim to minimize hepatic and intestinal metabolism through drug design during lead optimization (LO). Oral bioavailability (F) is defined as:

$$F = F_a F_G F_H$$

where F_a is the fraction of orally administered drug that is absorbed into the enterocytes, F_G is the fraction of drug

✉ Sheila Annie Peters
sheila-annie.peters@merckgroup.com

[1] Present Address: Translational Quantitative Pharmacology, BioPharma, R&D Global Early Development, Merck KGaA, Frankfurter Str. 250, F130/005, 64293 Darmstadt, Germany

[2] Oncology Innovative Medicines DMPK, AstraZeneca, Alderley Park, UK

[3] Investigative ADME, Non-Clinical Development, UCB New Medicines, BioPharma SPRL, Braine l'Alleud, Belgium

[4] Simcyp Limited (A Certara Company), Blades Enterprise Centre, Sheffield, UK

escaping first-pass metabolism in the enterocytes, and F_H is the fraction of drug escaping first-pass hepatic metabolism and biliary secretion. Absolute oral bioavailability ($F_a \times F_G \times F_H$) is determined by comparing the drug exposure (area under the plasma concentration–time profile) following oral administration with that after intravenous administration, assuming that first-pass metabolism from organs other than liver and gut can be neglected.

Intestinal metabolism can occur in the gut lumen as well as in enterocytes. In the gut lumen, microflora-mediated reduction [3] and hydrolysis [4–6] can be important but are often overlooked. Among the drug metabolizing enzymes (DMEs) expressed in human enterocytes [7], cytochrome P450 (CYP450), uridine diphospho-glucuronosyltransferases (UGTs) and sulphotransferases (SULTs) are the most important (Table 1). CYP3A is localized predominantly in functionally mature enterocytes at the villous tips, which result from migration of immature enterocytes in the crypts, a process that takes approximately 2–6 days [7]. Recently, meta-analysis has indicated that turnover of

mature enterocytes in preclinical species and humans takes between 2.4 and 3.5 days [8]. The total mass of CYP3A in the entire small intestine based on homogenates from isolated enterocytes has been estimated to be less than 1 % of the liver [9, 10]. However, the intestine contributes significantly, or even equal, to hepatic CYP3A in the overall first-pass metabolism of several drugs (e.g. cyclosporine, midazolam and verapamil) [11–13]. Extensive presystemic intestinal metabolism is probably due to anterior positioning of the intestine with respect to the liver; the high luminal drug concentration and the long intestinal transit times that provide an increased opportunity for substrate–enzyme interaction, especially given the potential synergistic interplay of CYP3A and P-glycoprotein (P-gp) on dual substrates [14–16]. Additionally, unlike hepatic metabolism, presystemic metabolism is not limited by plasma protein binding or blood perfusion rates, but rather by permeability across enterocytes [17].

Poor oral bioavailability and extensive first-pass metabolism predisposes a patient population to potential

Table 1 Major CYP450 enzymes identified in the human gut according to published protein abundance data

Enzyme class	Enzyme isoform (% of total)	References
CYP P450	CYP3A4 (33–87)	[9, 266]
	CYP2C9 (4–38) and CYP2C19 (0.5–7)	
	Other isoforms:	[9, 44, 195]
	CYP1A1 (only detected in $n = 3$)	
	CYP2D6 (0.2–4)	
	CYP3A5 (7–26)	
	CYP2E1 (not detected)	
	CYP2J2 (0.2–4)	
	Contributions (ranges) were calculated as a percentage of the total immunoquantified CYP450 ($n = 31$ donors)	
UGTs	UGT1A7 (21)	[149]
	UGT1A1 (18)	
	UGT1A9 (16)	
	UGT1A8 (15)	
	UGT1A4 (13)	
	UGT1A10 (11)	
	UGT1A6 (6)	
	UGT1A3 (not detected)	
	Eight UGT1A isoforms were evaluated and their mean contributions were calculated as a percentage of the total amount of immunoquantified UGT ($n = 3$ donors)	
SULTs	SULT1B1 (36)	[140, 148]
	SULT1A3 (31)	
	SULT1A1 (19)	
	SULT1E1 (8)	
	SULT2A1 (6)	
	Five SULT isoforms were evaluated and their mean contributions were calculated as a percentage of the total amount of immunoquantified SULT ($n = 6$ donors)	

CYP450 cytochrome P450, *UGTs* uridine diphospho-glucuronosyltransferases, *SULTs* sulphotransferases

toxicity arising from high doses as well as large interindividual variability [18] in exposures. In addition, extensive first-pass metabolism increases the risk of drug–drug interactions (DDIs) [19–23], the magnitude of which depends on the fraction escaping metabolism of both the substrate and the inhibitor [24] (if both the substrate and inhibitor are metabolized by the inhibited enzyme). The DDI risk arising from first-pass extraction can also be transporter-mediated [25–28]. These risks highlight the need for robust, quantitative models for predicting drug extraction through the gut wall and liver, backed by a sound understanding of the underlying mechanisms.

After decades of research, hepatic drug metabolism is well-understood. Only in recent years has there been an increased effort to understand intestinal first-pass extraction. Among the factors limiting intestinal availability ($F_a \times F_G$), intestinal metabolism can be a major determinant [29] but is poorly understood. This is due to a number of confounding factors affecting it; namely, drug transit through the gastrointestinal tract, drug permeability, solubility and intestinal blood flow. Species differences in the isoforms, regional abundances and activities of DMEs [9, 30, 31] and transporters [32, 33], as well as the interplay of enzyme-transporter proteins and overlap of substrate and inhibitor specificity [34], make it difficult to establish good preclinical models for this process. Knowledge gaps on enzyme abundances and lack of empirical enzyme activity scaling factors are the main limitations of in vitro methods for the evaluation of intestinal metabolism. The large interindividual variability and inaccurate estimation of human intestinal extraction, even when intravenous pharmacokinetic (PK) data are available, pose additional challenges to the validation of both in vitro and in vivo models [35, 36]. Due to its broad specificity and high abundance in the intestine, CYP3A is the enzyme most frequently implicated in human gut metabolism, and therefore the most studied. Focusing on CYP-mediated processes, this review will summarize current knowledge on the advantages and limitations of preclinical models (in vivo, in situ, in vitro and mathematical) available for quantitative prediction of intestinal availability. This review will also outline a strategy applicable in drug discovery and early development, identify gaps in current understanding and propose future directions for the prediction of human gut-wall metabolism.

2 Human In Vivo F_G

Seminal work from clinical studies with CYP3A drug substrates such as cyclosporine A [11, 37, 38] and midazolam [12, 39, 40] established the role of gut-wall metabolism in limiting human oral bioavailability. Substantial intestinal extraction was demonstrated in patients after sampling portal vein blood following intraduodenal drug administration during the anhepatic phase of liver transplantations. However, interpretation of this PK data is challenging given confounding factors attributable to the use of anaesthetics, surgery and the often poor condition of patients. These studies, as with in situ regional perfusions [41, 42], are rarely undertaken in humans for ethical, technical and cost reasons [21, 43].

Alternative clinical approaches rely on indirect estimation of human F_G from intravenous/oral area under the curve (AUC) data or drug–food interaction data. Comparison of AUCs following intravenous and oral drug administration is relatively straightforward, although assumptions often hinder interpretation [44]. For example, the extent of metabolic extraction in the intestine can be over-emphasized if (1) notable extrahepatic systemic clearance is left unaccounted [45]; (2) the blood:plasma ratio deviates significantly from unity [46]; or (3) first-order elimination is not conserved at each site of drug administration [47, 48]. Additionally, calculated F_G can be sensitive and biased according to the average value used for liver blood flow [49, 50]. When factors limiting oral absorption (efflux, low F_a) are minimal and transporter-mediated uptake is negligible [46, 51], the drug–food interaction method offers an attractive, pragmatic model for estimating the extent of F_G for CYP3A drug substrates. This is because certain fruit juices offer complete and exclusive presystemic inhibition of CYP3A in the small intestine, providing means for F_G to be more readily identifiable and separated from hepatic first-pass elimination [52–58]. Advantageously, the interaction approach requires only measurement of oral AUC in the presence and absence of inhibitor, avoids many of the confounding factors associated with the intravenous/oral method, and allows researchers to benefit from the abundance of clinical data available [46, 51]. Clinical studies helped to delineate the metabolic component in liver and intestine for drugs such as alfentanil, cyclosporine, felodipine, midazolam, nifedipine and tacrolimus [46, 52, 54–56, 59, 60]. Grapefruit juice (GFJ) has been the most popular CYP3A inhibitor in food–drug interactions trialled thus far. The extent of clinical drug interactions with GFJ can be variable, depending on the GFJ strength and duration of administration [51, 61, 62]. Understanding underlying mechanisms and kinetics should aid study design and control exposure to inhibitory agents (furanocoumarins) mediating the interactions [57, 58, 61, 63–65]. In turn, this should address interstudy reproducibility and erroneous estimation of F_G [21]. Detailed discussion of the advantages, limitations and underlying assumptions of these in vivo models are elaborated elsewhere [21, 46, 51]. Uncoupling F_G and F_a remains a significant challenge to any clinical approach, as

does factoring out potential contributions from nonhepatic tissues [43].

3 Regional Differences in Intestinal Metabolism and Transport in the Human Gut

Compounds that have their maximum absorption in different regions of the gut are likely to have very different F_G, even if they have comparable metabolic liabilities. This is probably due to regional differences in drug uptake into enterocytes, efflux and intestinal metabolism arising from regional variation in the luminal environment (e.g. pH, composition of intestinal fluids) [66–68] and epithelial membrane (e.g. surface area, expression of enzymes and transporters) [67]. Information on regional expression of enzymes and transporters is important for understanding the in vivo plasma PK profile and possible DDIs with co-medications.

It is well known that passive diffusion varies in humans and preclinical models according to the physicochemical properties of the drug in question and the region of intestine [69, 70]. Lipophilic compounds have the same or higher permeability coefficients in the lower bowel compared with the upper small intestine. With hydrophilic molecules the trend is reversed [69, 70], partly due to differences in the membrane lipid composition and the tightness of the tight junctional area [70]. Regional differences in drug uptake into the enterocytes can also occur due to differences in intracellular metabolism along the gut. Higher rates of intracellular metabolism can cause sink conditions inside the cells. A concentration gradient maintained across the membrane favors increased uptake, as has been suggested for indinavir [71].

Efflux proteins impact gut metabolism by reducing the intracellular drug concentrations exposed to DMEs in the enterocytes. At high doses, efflux proteins may become saturated, allowing a larger proportion of drug to pass the membrane efficiently. Expression of the main ABC transporters is heterogeneous along the gastrointestinal tract (Table 2) [72–74]. It is well known that multidrug resistance protein 1 (MDR1) [ABCB1, P-gp] is preferentially expressed toward the lower parts of the human small intestine (ileum) [74–76], while multidrug resistance-associated protein 3 (MRP3) [ABCC3] is expressed at higher levels than MDR1 along the small and large intestines [74]. In contrast, the efflux protein MRP2 (ABCC2) is expressed at relatively high levels in the small intestine but at extremely low levels in the colonic regions [74]. The other important efflux protein, breast cancer resistance protein (BCRP) (ABCG2), has higher levels than both MRP2 and MRP3 in the small intestine, but very low levels in the colonic regions [77, 78].

The solute carrier (SLC) proteins, including PepT1 (SLC15A), MCT1 (SLC16A), OATPs (OATP2B1; SLC0), OATs (SLC22A), OCT/OCTN (SLC22A) and the recently described PMAT (SLC29), may add significantly to uptake of the drug into enterocytes for many compounds with lower lipophilicities ([72], and refs. therein). These are also affected by food constituents as well as genetic polymorphisms and disease states [79, 80]. OATP2B1 has been found to have an unexpected influence on the absorption of the drug aliskiren [81]. The compound has affinity for MDR1 as well as CYP3A4, and inhibition of these mechanisms was expected to increase its bioavailability. However, in a study involving 11 healthy volunteers, a reduction of the absorption of aliskiren was found, when coadministered with GFJ, due to additional inhibition of the uptake transporter OATP2B1 by GFJ. Thus, prediction of drug absorption and DDI in the clinic gets further complicated for substrates of uptake transporters.

The regional distribution of these SLC proteins in the intestine, as well as species differences, are largely unknown. However, PepT1 is reported to be highly distributed in the proximal intestine of humans and many animal models [72, 82], and MCT1 is well known to be highly abundant along the whole gastrointestinal tract. The abundances of other SLC proteins tend not to be significant [78, 83, 155]. Further details can be found in the excellent review by Estudante et al., and references therein [72].

The abundance and catalytic activity of the main human CYP450 enzymes is generally highest in the proximal regions (i.e. duodenum and proximal jejunum), declining towards the lower ileum after a slight increase from the duodenum to the jejunum (Table 2) ([84–87], and refs. therein]). Data on regional gene expression and enzymatic activity are readily available for CYP3A4 and members of the CYP2C family, but are less well-characterized for other CYP450s. Paine et al., measured CYP450 protein levels along the gastrointestinal tract in 31 human donors and found that after CYP3A4, the most abundant enzyme was CYP2C9, then CYP2C19 with low levels of CYP2J2 and CYP2D6 [9]. Western blot data indicated that concentrations of CYP3A4 and CYP2C isoforms decreased dramatically towards the distal small intestine, with CYP2C levels falling faster compared with CYP3A4 [9, 86]. Information available on the expression of CYP450s in the human colonic enterocyte is limited, and is contradictory depending on the technique used [88–90]. Using messenger RNA (mRNA) and protein analysis, Bergheim et al., [91] found expression levels of CYP2C, CYP2E1 and CYP3A5 significantly differed between different regions of the large intestine, with CYP2C significantly higher in the ascending colon, and CYP2E1 and CYP3A5 significantly lower. In contrast, others failed to detect any significant levels of CYP4502C8–10 and CYP2E1 protein in colonic tissue [63, 64].

Table 2 Regional distribution of important phase I and II enzymes and ABC transporters along the small and large intestine in humans and common preclinical species

Species	DME	Regional distribution in the intestine	References	Transporter name (gene name)	Regional distribution in the intestine	References
Human	CYP3A	3A4 highest expressed of all CYPs, duodenum < jejunum > ileum, colon	[9, 267]	P-gp, MDR1 (ABCB1)	Apical; proximal < distal small intestine > colon	[74, 77, 78, 83]
		3A5 small intestine < colon (major CYP in colon)				
		3A5 in some cases high in proximal intestine				
Rat	Cyp3a1/2	Duodenum > jejunum > colon	[154]	P-gp, mdr1 (ABCB1)	Apical; proximal < distal small intestine > colon	[76, 78]
Dog	Cyp3a12/26	Very low levels, no regional difference	[159]	P-gp, mdr1 (ABCB1)	Apical; highest in jejunum, low in colon and duodenum	[159]
Human	CYP2B6	Not expressed	[10]	MRP2 (ABCC2)	Apical; proximal > distal jejunum > ileum >colon	[74]
Rat	Cyp2b1	Higher or similar expression as for 3A, duodenum < jejunum > ileum, colon	[154]	mrp2 (ABCC2)	Apical; proximal > distal	
Human	CYP2C9/19	Second most abundant, proximal intestine	[9]	MRP3 (ABCC3)	Basolateral; high level overall, proximal < distal	[74, 78]
Rat	Cyp2c6	Duodenum = jejunum < ileum < colon	[154]	mrp3 (ABCC3)[a]	Basolateral; proximal < distal; highly expressed in the colon	[160]
Human	CYP1A1	No or variable low expression	[9]	BCRP (ABCG2)	Apical; high level in whole intestine higher than MRP2 and MDR1, jejunum > ileum, colon, duodenum	[74]
Rat	Cyp1a1	Main CYP in the small intestine		bcrp (ABCG2)	Highest in mid jejunum	[162]
Dog		No reports found	[90]	BCRP (ABCG2)	Tenfold higher than MDR1 and proximal ≫ ileum, colon	[159]
Human	UGT	Similar activity in both small intestine and colon	[157]			
Rat	UGT	Proximal small intestine > distal small intestine	[159]			
Dog	UGT	High level throughout the intestine and colon	[90]			
Human	SULT	Similar activity in both small intestine and colon	[157]			
Rat	SULT	Proximal small intestine > distal small intestine	[9]			
Dog	SULT	No reports found				

The abundance of the different proteins in the different regions was mainly reported as levels of mRNA, immunoquantification and enzyme activities

CYP cytochrome P450, *UGT* uridine diphospho-glucuronosyltransferase, *SULT* sulphotransferase, *P-gp* P-glycoprotein, *MDR1* multidrug resistance protein 1, *MRP2* multidrug resistance-associated protein 2, *MRP3* multidrug resistance-associated protein 3, *BCRP* breast cancer resistance protein, *mRNA* messenger RNA

[a] Refers to rat and mouse

The most obvious difference between the small intestine and colonic tissue is the content of CYP3A4 and CYP3A5. In the small intestine, CYP3A5 is detected only at low levels [89] and may be absent in some individuals [9], while in colonic tissues, very low levels of CYP3A4 and higher relative expression of CYP3A5 were reported. Thus, CYP3A5 constituted the major CYP3A isoform in this tissue [88]. The overall lower rate of hydroxylation in the colonic region compared with the proximal jejunum was confirmed by van de Kerkhof et al., using a mixture of

CYP450 substrates [CYP3A4/5 substrate midazolam (CYP3A4/5), followed by CYP2C9 (diclofenac) and CYP2D6 (bufuralol)] [90].

Regional differences in the abundance of phase II enzymes in the gut are not well-understood. van de Kerkhof et al. have reported similar activity for UGT and SULT enzymes [based on 7-hydroxy-coumarin (7-HC) conjugation] in both the proximal jejunum and the colon. They also report that glucuronidation efficiency in the gut was approximately sixfold higher than sulphation in both regions [90]. As with all quantitative approaches to measuring transporter and DME protein abundances, comparison of expression levels of different phase I and II enzymes between regions of the small intestine and colonic tissue, and between different studies, may be difficult given the different techniques used for quantification, i.e. protein quantitation using Western blot or liquid chromatography–tandem mass spectrometry (LC–MS/MS), as well as immunohistochemistry, mRNA and enzymatic activity using selected probes.

4 Combined Action of Drug Metabolizing Enzymes (DMEs) and Transporters in the Gut

Co-localization of CYP3A and MDR1 in the enterocyte along the crypt villus axis, overlapping substrate specificities and poor oral bioavailability of their joint substrates [15], have lead scientists to suspect an interplay between ABC transporters and members of the CYP450 enzyme families in the intestinal membrane affecting intestinal absorption and metabolism. It may also explain some DDIs which cannot be rationalized through either protein acting alone [15, 72].

P-gp is situated in the apical membrane of the enterocyte and efflux substrates from inside the cell towards the intestinal lumen. The CYP3A enzymes are located within the endoplasmic reticulum. It is therefore suggested that P-gp may regulate the intracellular concentration of dual substrates of P-gp and CYP3A. One might speculate that increasing P-gp levels in the proximal to distal direction serves to recycle its substrate and aid the efficient elimination of harmful molecules by reducing the intracellular drug concentration to levels below DME saturation in the proximal region, and increase the residence time of its substrate for metabolism in the distal gastrointestinal tract. Although demonstrated in vitro [90], some authors have pointed out that the extent of such synergistic effects in vivo is minimal [92]. The extent of activity of DMEs and P-gp are determined by the drug concentration at the site of the absorption, which in turn is related to solubility (biopharmaceutical classification) [73] and regional stability of the substrate in the gut lumen. Murakami and Takano

suggested that the biopharmaceutics classification system (BCS) Class I compounds that are readily water-soluble and have high permeability will be rapidly absorbed in the upper part of the small intestine by passive diffusion [73]. Consequently, these compounds can be extensively metabolized due to higher CYP450 expression in the proximal small intestine and lower dependence on P-gp and/or BCRP [29]. However, BCS class II and III, as well as intravenous compounds with poor solubility and/or permeability, are likely to be absorbed in the lower parts of the intestine where metabolism can be substantial if they are also substrates of P-gp/BCRP [73]. In conclusion, compounds with high permeability that are typically absorbed in the upper region of the small intestine can escape the combined action and potentiation of metabolism and efflux, while moderate and low-soluble compounds have an increased potential to be involved in the recycling action of transporters.

Metabolites formed by CYP450s can be efficiently transported to either the mucosal or blood side of the enterocyte by the action of either MDR1, BCRP or the MRPs. The metabolite of ropivacain was secreted to a larger extent on the luminal side of the human jejunum compared with the ileum [93]. Although the transporters involved were not identified, examples such as this clearly highlight the need for a greater understanding of the interplay between enzymes and transporters in the intestinal tract.

5 Preclinical Models for the Prediction of F_G

Various preclinical models of the intestine have been described (see Table 3). If appropriately integrated into DMPK strategies for optimization of drug absorption, distribution, metabolism and excretion (ADME) properties, it may provide more reliable prediction of human first-pass oral clearance, bioavailability and PK profile. Models should be considered in terms of their complexity, the mechanistic understanding they provide, and their clinical translation, e.g. quantitative prediction of the fraction of drug escaping metabolism in the human gut wall.

Often there is a trade-off between the different in vitro, in situ or in vivo models. On the one hand, in vivo models retain the native architecture of the small intestine and physiologically relevant expression profiles of DMEs, cofactors and transporter proteins. They offer integration of dynamic processes such as the mesenteric blood circulation and mucous layer coupled with function to study complexities arising from simultaneous metabolism–transporter interplay [15, 94, 95]. Notable attractions with in situ techniques are they closely mimic the in vivo situation yet provide a unique opportunity to study intestinal events in

Table 3 In vivo, in situ and in vitro methodologies to assess F_G, or the composite parameter $F_a \times F_G$ (intestinal availability). Typical assay throughput and potential reagent amounts for in vitro assays have been provided. # Number of in vitro incubations calculated on the basis of incubating test compounds in a total volume of 250 μL and at a protein concentration of 1 mg/mL

Technique	Matrix	Calculated parameter for interpretation or gut wall metabolism	Advantages and limitations of techniques	Assay throughput and tissue requirements
Recombinant expression systems	Isolated enzymes	F_G	• Commercially available, easy to use and amenable to long term storage (-80°C). Assay format suitable for high throughput metabolism studies. • Can be used to study metabolism with a specific enzyme, although potential to miss other routes of metabolism. • In vivo like functionality of the protein may be compromised within the expression system.	Amenable to >96-well format. Typically purchase 0.5 ml aliquots at 10mg/ml total protein (0.5-1 nmol CYP450 enzyme/ml). #Equates to approximately 40 test incubations.
Sub-cellular fractions	Microsomes	F_G	• Commercially available, applicable to all species, easy to use and amenable to long term storage (-80°C). Assay format suitable for high throughput metabolism studies. • Information on physiological scaling factors published but lacking standardised methodology and consensus for optimal enzyme isolation procedure as well as metabolism incubation conditions. • Fractions contain multiple enriched enzymes in their native configuration. Absence of cytosolic enzymes (microsomes) and drug transporters, co-factors not at physiological concentrations. • Less physiologically relevance although data can be used directly for prediction of F_G using minimal as well as complex PBPK models. The data can also aid interpretation of more complex in vivo models.	Amenable to >96-well format. Typically 0.5 ml aliquots at 10 mg/ml microsomal protein available commercially. #Equates to approximately 40 test incubations. Experience in house: approx. 150 incubations from pooled rat preparations (n=9 rats)
	S9 fractions	F_G		
Intact cells	Isolated enterocytes	F_G	• Commercially available, whole cell models do not require addition of cofactors. • Harvests likely to be contaminated with other intestinal cell types. Enzymes are not enriched, lower activities typical making bioanalysis more challenging. Very low expression of phase 1 metabolic enzymes (Caco-2 cells). • Limited information published on physiological scaling factors and poor data reproducibility between laboratories.	Medium/low throughput. Commercial availability of enterocytes limited. With Caco-2 typically use 24-well plate format (seeded at 80,000 cells per well).
	Other cell lines e.g. Caco-2	F_G		
Tissue explants	Precision cut tissue slices	F_G	• Applicable to animal and human tissue (except isolated tissue perfusions and everted sac), although not commercially available. • Can investigate regional differences in metabolism. All cell types and mucus layer retained, although muscularis mucosa also present which adds an artificial barrier to compound absorption in precision cut slices and everted sacs. • Bidirectional movement of compounds can be assessed (excepting tissue slices) in addition to combined effects of transporters and metabolic enzymes. • Limited tissue viability (~2-3 hours) may prevent evaluation of slowly metabolised compounds or enzyme induction. • Difficult to separate the absorption and metabolic components. • Some approaches (Ussing Chamber, isolated tissue perfusion) require a sensitive analytical technique because of compound dilution in the diffusion chambers.	Medium/low throughput. PCTS: 6 or 12-well formats, typically 20-30 slices (~4mg protein/slice) can be prepared from a 3cm core or from a human tissue sample (~2cm²). Using chamber: typically uses 160mg protein per incubation chamber, with a maximum of 8 chambers that can run in parallel per experiment. Everted sac: 1 test incubation per everted sac.
	Ussing Chamber	E_G		
	Everted sac	$F_a \times F_G$		
	Isolated tissue perfusion	E_G		

→ Closer physiological similarity to in vivo situation

versus

← Greater mechanistic understanding of assay

Table 3 continued

Technique	Matrix	Calculated parameter for interpretation or gut wall metabolism	Advantages and limitations of techniques	Assay throughput and tissue requirements
In vivo models (animal and human)	IV/Oral PK studies	$F_a \times F_G$	• Technically straightforward and moderate throughput in animals. More challenging in humans (development costs and time). • Option to cassette and analyze multiple compounds in parallel, providing metabolic routes are not compromised by DDIs. • Oral dose is not administered at site of absorption. Only provides an indirect measurement of F_G and it is difficult to separate absorption and metabolic components. • Native architecture of small intestine intact and physiologically relevant expression profiles of DMEs, co-factors and transporter proteins retained. Required for validation and IVIVE of in vitro approaches. • Interpretation can be confounded by assumptions including applying a single value for liver blood flow, that the liver is the only eliminating organ contributing to IV clearance.	Medium throughput. Possible to cassette dose multiple compounds per animal (typically up to n=5).
	PK studies using chemical inhibitors	F_G	• Technically straightforward in animal models, but lower throughput. More challenging in humans from clinical design perspective (e.g. to ensure complete and selective inhibition of CYP3A). • Potential to selectively inhibit liver or intestinal enzymes (particularly in rat), although approach is less well established for non CYP3A substrates. When intestinal enzymes are inhibited assumed that other enzyme pathways do not compensate. • Applicability limited to CYP3A substrates in humans. Difficult to separate F_a and F_G components.	Low throughput. 1 compound per animal.
	In situ gut loop	$F_a \times F_G$	• Native architecture and function of the small intestine retained (correct physiology in terms of enzyme expression and localisation and blood flows). Can investigate regional differences in metabolism.	Time consuming, low throughput. 1 compound per animal.
	In situ single-pass perfusions	$F_a \times F_G$	• Time consuming, technically difficult procedure requiring surgery. Scarcely used in humans given ethical issues and limited availability of healthy volunteers or patients. • Anaesthetics can influence kinetics. Difficult to separate absorption and metabolic components.	
	In situ re-circulating perfusions	$F_a \times F_G$	• Approach not valid for compounds with permeability limitations. Extraction is assumed given it is a perfusion rate limited model.	
	Gene knockout mice	F_G	• Mouse models are commercially available. • Possible to create selective knockout models of enzymes such as CYP3A in specific organs such as liver and/or intestine. • Knockout of metabolic pathway can be vulnerable to compensation mechanisms e.g. up-regulation of other CYP450s.	Very expensive, low throughput. 1 compound/assay.
	Genetically modified (humanized) mice	F_G	• Mouse models are commercially available. • Possible to selectively introduce human enzyme isoforms such as CYP3A4 in specific organs such as liver and/or intestine. • Can evaluate relative impact of intestinal versus hepatic metabolism attributable to a single enzyme. Can assess interplay between a CYP450 enzyme and s transporter protein, like P-gp. Can assess DDI associated with combining with co-medications. • Models limited in terms of quantitative prediction of clinical oral drug exposures.	Very expensive and low throughput. 1 compound/assay.
	Portal vein sampling	$F_a \times F_G$	• Enables direct sampling proximal to liver but requires surgical procedure, unethical in humans.	Expensive, low throughput. 1 compound/assay
	Anhepatic models	$F_a \times F_G$	• Requires surgery. Scarcely used in humans due to ethical issues. Patients frequently used in human studies and may not reflect kinetics in healthy population. Diversion of liver ensures only intestinal contributions are studied.	Very expensive, low throughput 1 compound/assay

← Closer physiological similarity to in vivo situation

versus

← Greater mechanistic understanding of assay

CYP450 cytochrome P450, PBPK physiological-based pharmacokinetic model, PCTS precision cut tissue slices, IV intravenous, PK pharmacokinetic, DDI drug–drug interaction, DMEs drug metabolizing enzymes, IVIVE in vitro to in vivo extrapolation, P-gp P-glycoprotein

isolation, e.g. absence of biliary excretion and enterohepatic recirculation; however, this isolation could compromise the interpretability of the results. Furthermore, species differences in enzymes and transporters (Table 2) can make them unreliable for human F_G prediction. In vitro models employing human-specific systems lack native architecture, but, in combination with mathematical models, hold the promise of robust prediction, provided there is a correlation between in vitro and human in vivo.

5.1 In Vivo and In Situ Animal Models

Gut-wall metabolism has been studied in several animal models [96–102]. By varying sites of drug administration (oral, intraportal and intravenous routes are common but intraperitoneal is also used) and PK sampling, the available fractions in liver and intestine can be calculated from comparison of AUCs under first-order conditions [103–107]; however, comparison of intravenous and oral AUCs after sampling at one site is more straightforward and routinely applied [108–110]. As such, similar issues described with the indirect approaches are to be expected. Despite differences in the CYP450 isoforms expressed in the rat compared with human (see the following paragraphs), a good correlation [root mean square error (RMSE) = 0.19] between rat and human F_G has been reported using a set of 11 CYP3A-metabolized compounds that had both intravenous and oral PK data [111]. Ten of these 11 compounds studied had human F_a of 0.8 or higher. As rat is a good model for human oral drug absorption, it follows that any differences in the intestinal availability should arise from intestinal efflux or metabolism. For BCS class I compounds, good solubility ensures sufficient concentrations for the saturation of efflux transporters. A high permeability rate ensures a low residence time within enterocytes, resulting in a rate of metabolic extraction that is limited by the rate of drug permeation through the enterocytes, rather than by the intrinsic ability of the intestinal DMEs. With permeability-limited intestinal extraction, any differences in enzyme isoform, abundance or activity have limited impact on the metabolic extraction, especially since CYP450s with broad substrate specificity abound in both human and rat, and their regional distributions are similar in both species. However, for hepatic extraction, lower hepatic concentrations compared with the gut lumen implies a greater role for transporters, while species differences in plasma protein binding and blood flow rate contribute to varying exposures to uptake and efflux transporters, as well as to DMEs, heightening the impact of differences in transporter and enzyme isoform, abundance and activities on hepatic extraction. This may explain why the rat is a good model for the prediction of human F_G but is a poor predictor of human oral bioavailability [112]. A strong correlation was also reported between cynomolgus monkey and human F_G for human CYP3A [113]. However, the cost and ethical concerns limit the availability of monkey PK studies. Animal models may be useful in the prediction of human F_G for CYP3A-metabolized compounds, but not much is known about their utility for other intestinal DMEs.

In situ approaches include the perfused gut loop, single-pass and recirculating intestinal perfusions, portal vein cannulations, portacaval shunts and transpositions [114–121]. These techniques open the possibility of studying route-dependent intestinal metabolism following systemic and luminal drug presentation. This has been shown with acetaminophen, morphine and enalapril [96, 98, 106, 118, 122, 123]. Naturally-occurring tissue structure and physiology are retained, notwithstanding surgical manipulation and effect of anaesthetics [114, 124, 125]. Minimal interference of intestinal function and architecture means optimal tissue viability is maintained [106]. A full complement of endogenous DMEs and transporter proteins are present. Discrete segments of the small intestine can be evaluated to assess the impact of regional differences in DMEs or transporter expression and gut physiology. Intestinal metabolism can be more rigorously evaluated if drug concentrations are measured after sampling from mesenteric or portal veins [126]. Detailed methodologies are provided elsewhere [114, 127–130]. In situ models have several advantages over in vivo models. For instance, bypassing the stomach means acidic compounds are unlikely to precipitate, and therefore dissolution rates do not confound intestinal drug concentrations and resultant plasma levels [94]. These models can be exploited to investigate metabolism–transporter interplay, as exemplified with midazolam, indinavir and UK-343,664 [48, 131, 132]. Various aspects need to be controlled [133], including luminal flow rate and thickness of the unstirred water layer (UWL), which can be rate-limiting for rapidly absorbed compounds [106]. In spite of their utility, isolating perfused organs from, or within, the laboratory animals typically requires specialized surgical procedures. As such, these approaches are arguably more labour-intensive, time-consuming and costly compared with other in vivo and in vitro approaches.

Whereas certain processes, such as passive diffusion, can be relatively well-predicted from animals [94, 106, 134–139], pronounced species differences in expression of DME isoforms, substrate selectivity and abundance along the gastrointestinal tract [30, 31, 113, 140–142] implies that human F_G prediction from preclinical species is not always feasible. The differences in relative protein expression of individual CYP450 isoforms in rat, dog, monkey and human small intestines are presented in Fig. 1. These species differences have led to substantial
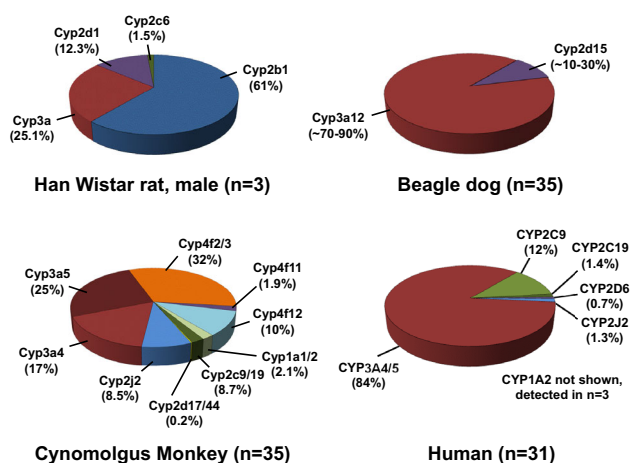
Fig. 1 Small intestine CYP450 pie charts for rat, dog, monkey and humans. The mean percentage contributions of individual CYP450 enzymes were calculated from total immunoquantified CYP450 protein (dog, monkey, human) or total spectrally determined CYP450 (rat). Analysis was based on published data: rat [154], dog [254, 255], monkey [268] and humans [9]. Additionally, CYP4F2 protein was detected (∼7 pmol/mg protein) in human small intestine microsomal fractions [269]. This has been excluded from the human CYP450 pie chart because the total intestinal CYP450 content has not been reported, precluding comparison of relative abundances of individual enzyme isoforms between studies. *CYP450* cytochrome P450

differences in apparent $F_a \times F_G$ as well as F_G [30, 143–145]. Metabolism studies, using drug substrates for human CYP450s [141, 143, 146] and UGT enzymes [142, 147], have generally reported poor correlation between human and animal F_G. Certain DMEs appear to be selectively expressed in human intestines, including UGT1A8, UGT1A10 and SULT1A3 [140, 148, 149]. To the best of our knowledge, there is no known animal orthologue of SULT1A3 or UGT1A10 [147]. Additionally, there are known species differences for other enzyme classes expressed in the gut, such as the carboxylesterases [3, 4, 150, 151]. If a drug is shown to be a substrate for one of these enzymes then predicting human F_G using animal data would be questionable.

Species differences in regional enzymatic profiles exist within the intestine (Table 2). The regional decrease in CYP3A in humans, as a function of distance along the small intestine from the duodenum to the ileum [9, 85], is similar to the rat small intestine [152–154]. Interestingly, in the rat intestine Cyp2b1 is highly expressed, whereas the equivalent isoform in humans (CYP2B6) is not expressed [9]. Rat Cyp2b1 is present at much higher protein levels in the upper parts of the intestine, whereas in humans the equivalent isoform (CYP2D6) is not [154]. The trend in the regional expression of rat Cyp2c isoforms (such as Cyp2c6) is opposite to that of Cyp3a, with higher expression towards the lower bowel [154] (Table 2). In contrast

to being absent in the human small intestine, the extra-hepatic enzyme Cyp1a1 has been reported by some authors to be the predominant CYP450 isoform expressed in rat small intestine, together with Cyp3a [9, 154–156]. The glucuronidation/sulphation ratio can differ between regions according to individual species. In rat colon and proximal jejunum, the ratio was 16 and 23, respectively [157], showing a clear species difference in conjugation activity and regional difference compared with humans (see Sect. 3 and van de Kerkhof et al. [90]).

Regional expression of Mdr1b in rat intestine is similar to regional expression of MDR1 in the human small intestine and colon [74–76, 78, 158] (Table 2). Contrary to this, canine intestinal Mdr1 expression is highest in the jejunum and very low in the lower parts of the ileum and colon [159]. For the MRP2 transporter, humans are similar to rat, with high expression in the proximal small intestine and very low levels in the lower bowel [74, 78, 83]. Abcc3 (Mrp3) is highly expressed in the colon of rats [160], and mice, at both the mRNA and protein levels [161] (Table 2). Interestingly, MRP3 (the corresponding transporter gene in humans) has the highest level of all the efflux proteins expressed along the intestinal tract [74]. In mouse, the expression of bcrp is higher in the mid jejunum compared with the rest of the intestinal tract [162]. A similar cellular location (basolateral in enterocytes) found in mice, rats and humans further supports a high degree of conservation for ABC transporters amongst eukaryotes.

5.2 Knockout and Transgenic Mouse Models

In vivo rodent models with intestinal or hepatic enzyme gene knockdown or replacement have demonstrated the impact of intestinal versus hepatic elimination [163–168]. These knockout (KO) and/or genetically modified (GM), transgenic (TG) mouse models have been established to create more reliable in vivo systems to study and predict human response to novel chemical entities (NCEs) [163, 169–173]. For example, the importance of CYP3A metabolism in the intestine and liver has been illustrated with docetaxel in KO mice lacking all Cyp3a genes [Cyp3a (−/−)] [174]. When the CYP3A anticancer drug was administered intravenously to Cyp3a (−/−) mice, a sevenfold increase in systemic exposure was observed compared with wild-type. After oral dosing, an 18-fold higher systemic exposure was reported. Similar findings were reported for lopinavir and triazolam (CYP3A) [175, 176], debrisoquine (CYP2D6) [177] and tolbutamide (CYP2C9) [178]. This highlights the critical role intestinal CYP3A plays in human first-pass oral clearance and bioavailability.

Unfortunately, compensatory mechanisms arising from expression of host (murine) DMEs may confound data interpretation from KO models. For instance, clearance of

midazolam was expected to be severely reduced in Cyp3a KO mice. In spite of this, metabolism was only marginally altered versus wild-type [179]. The revelation that several murine Cyp2c isoforms were significantly upregulated in Cyp3a KO mice, and could catalyze formation of midazolam 1′- and 4′-hydroxymidazolam, helped rationalize these results [179]. In contrast, no such effect was seen with triazolam, apparently a more selective Cyp3a substrate in mouse [176]. These compensatory mechanisms are likely to be drug- and species-dependent. In vitro studies characterizing background metabolism may prevent assessment of drugs susceptible to elevated host DMEs. Alternatively, exciting development of a viable mouse model in which all murine CYP450 genes have been deleted could avoid this issue altogether [180]. Interestingly, TG mice have been generated that are capable of expressing human CYP3A4 in the intestine and/or the liver on top of a mouse Cyp3a KO background [174]. As demonstrated with docetaxel and lopinavir, these humanized mice can provide mechanistic insight into the separate and combined roles of intestinal and hepatic human CYP3A, and the interplay with transporters such as P-gp in vivo [163, 175].

5.3 In Vitro and Mathematical Models

'Bottom-up' quantitative predictions that use in vitro data in conjunction with mathematical models describing processes within the gastrointestinal tract have been implemented [181–185]. Several of the in vitro assays highlighted in Table 3 are simplistic in nature, take less time to complete, and are a fraction of the cost of animal experiments. This makes them eminently suited for screening NCEs and designing out presystemic metabolic liabilities. Models vary in complexity, from simple membrane preparations of individually recombinantly expressed enzymes [186–190] to subcellular fractions [12, 43, 45, 85, 153, 191–195], intact cells [196–201] to tissue explants. The latter retain their in vivo tissue architecture, albeit lacking physiological surroundings. Although relatively labour-intensive and lower throughput (see Table 3), working with whole tissue preparations such as precision cut tissue slices (PCTS) [157, 202–206], Ussing chamber [43, 69, 90, 93, 207], everted sac [126, 208–212] and isolated tissue perfusions [94, 197, 213–216] brings with it several advantages. The cell–cell contacts remain intact, all cell types are present and the DME systems, co-factors and transporters are available at physiologically relevant concentrations that more closely mimic the in vivo situation. With the isolated perfusion technique, variables such as temperature, pH, osmolality, blood pressure and flow can be controlled [217]. Metabolism on both sides of the intestine (luminal and vascular) can be studied. However,

maintaining tissue viability is the major issue with this approach [94]. As such, its application is generally limited to animal tissue, and short-term incubations (approximately 2 h post-excision) impeding assessment of slowly metabolized drugs or enzyme induction [43]. This can be circumvented in situ but is not without complication. Loss of tissue viability caused by insufficient oxygenation is also problematic for everted sacs (1–2 h) and Ussing chambers preparations (2–4 h) [218–220]. Nevertheless, everted sacs provide a fast and relatively inexpensive model for measuring regional differences in metabolism [106, 126]. The small volume inside the sacs offers analytical advantages over the isolated tissue perfusion and Ussing chamber, which due to sample dilution require sensitive bioanalysis to detect drug and/or metabolites [119, 126].

The Ussing chamber can be used with animal or human tissue providing a good model of drug absorption, transporter interactions, as well as metabolism, during passage across the gastrointestinal membrane [42, 69, 90, 219, 221–223]. Drug can be added to luminal or serosal sides, allowing bidirectional transport and metabolism kinetics to be studied in different sections of the intestine [106]. Detailed mechanistic interpretation from studies such as these may require additional insight from experimentation with enzyme inhibitors, radiolabelled drug, or separate consideration of metabolism and permeability, e.g. using intestinal microsomes and Caco-2 monolayers. Indeed the extent of drug extraction in human intestine (E_g) has been successfully predicted for testosterone, midazolam and ropivacaine using human in vitro Ussing experiments [69, 93]. PCTS have received growing attention now that reproducible production of very thin slices (between 250 and 450 μm) is possible. These maintain better viability [203] and retain a high drug biotransformation capacity [43]. PCTS are also suitable for studying regional differences in intestinal metabolism, as well as regulation of enzymes and transporters involved in drug disposition [206, 224]. Applicable to all species, investigations have been reported in mouse [225], rat [157] and human [90]. Additionally, other tissues can be examined, allowing the extent of metabolism in different organs to be compared [204]. Potential drawbacks include poor penetration of highly metabolized drugs into the slices inner cell layers, lag time in phase II metabolism, and nonspecific binding to the slices [202, 226].

Intestinal subcellular fractions (S9 homogenates or microsomes) are one of the more established in vitro approaches used in drug discovery. More information has been published on the physiological scalars (Table 4) and several investigators have explored quantitative prediction of F_G [49, 111, 227–231]. Commercial availability of animal and human samples, ease of storage and automation, make them an attractive option in terms of assay

speed, capacity and cost. The fractions contain multiple enriched enzymes in their native configuration for assessment of phase I and selective phase II metabolism [45, 113, 195, 231, 232]; however, they do not contain a full complement of DMEs and lack potentially important interactions with uptake and efflux transporters. Incubations require the addition of expensive co-factors for optimal DME activity, often at higher nonphysiological concentrations. Others have suggested metabolic rates in S9 and microsomal fractions can be much lower compared with matrix such as PCTS [43]. This may be attributed to poor recovery of enzymes through suboptimal preparation procedures [195]. Presented in Table 5 are 6β-hydroxy testosterone rat data (normalized to units of pmol/min/mg rat intestinal protein) including intrinsic clearance (CL_{int}) from in-house intestinal microsomes prepared under optimal conditions [233, 234]. Interestingly, in-house microsomes achieved broadly similar rates compared with PCTS and biopsies [43, 205, 225], and were much higher than previous microsomal preparations [212, 235]. This highlights progress made with DME extraction procedures, which, until recently, have limited the scalability of intestinal microsomes [192, 195]. Given the profound effect enzyme extraction procedure and incubation conditions can have on enzyme activity, consensus is needed on best practise before we can expect significant improvements to the accuracy and reproducibility of predictions.

In vitro models allow the function of metabolic enzymes, transporters and absorption processes to be studied in the gut wall. However, to apply this data to retrospectively explain, or prospectively predict, human oral PK ultimately requires

insight into the mechanisms influencing drug behaviour in vivo [132, 236–238]. As such, several mechanistic approaches of varying complexity have been described for in vitro to in vivo extrapolation (IVIVE), some of which are available commercially, e.g. GastroPlus^{TM} and Simcyp$^{®}$ [17, 92, 132, 238–241]. These mathematical translations (Fig. 2) can be relatively straightforward and 'minimal' models, such as Q_{Gut}, require only in vitro metabolic CL_{int} and cell permeability data to estimate F_G. Several groups have reported successful prediction of F_G in animals [242, 243] and humans [49, 227, 228, 244, 245] with this approach. Drugs with high in vivo extractions (F_G values <0.5) were less accurately predicted and may reflect inability of the Q_{Gut} model to account for changes in enterocyte drug concentration and therefore saturation of DME and efflux transporter processes [246]. However, to the authors' knowledge, no critical assessment of possible systematic underprediction of IVIVE similar to that reported for hepatic metabolism [247–249] is available in the literature. Improved F_G and oral clearance prediction was noted when the same set of drugs were evaluated using a physiological-based PK (PBPK) model. This was partly attributed to the model's ability to account for saturation of intestinal metabolism by using maximum velocity (V_{max}) and Michaelis-Menten constant K_m, the substrate concentration at which the reaction rate is half of V_{max} rather than CL_{int} [246].

Sophisticated PBPK models have been published, such as the segmental segregated flow model (SSFM), which encompass all salient variables, e.g. absorption, gastrointestinal transit, metabolism, transport and efflux [95, 98]. This allows route-dependent intestinal metabolism and

Table 4 Reported literature values of intestinal protein scalars in rat, dog and human

Scalar	Methodology	Rat	Dog	Human	References
Intestinal protein (mg/intestine)	PCTS	434	–	–	[204]
	Homogenization	324.3	–	–	[225]
	Elution	165.6	7970	970.8[a]	[197, 198, 254, 255, 270]
		124.89	4071		
	Scraping		–	–	
S9 protein (mg/intestine)	Homogenization	256.0[a]	–	28,476[a,b,c]	[271, 272]
	Elution	–	–	–	
	Scraping	–	–	17,540[c]	[85, 273]
Microsomal protein (mg/intestine)	Homogenization	17.1[b]	–	3155.1[a,b]	[225, 272]
	Elution	54.4[d], 16.5[a]	5459		[194, 197, 254, 255]
			1918		
	Scraping	156[a], 114[a]	–	2978	[85, 274]

[a] Assuming a total intestinal wet weight of 6.9 g [204], 307 g [254, 255] and 809 g [85] for rat, dog and human, respectively

[b] No correction for losses

[c] Based on summation of cytosolic and microsomal protein

[d] Extrapolated to total length (not reported)

zonal distribution of DMEs and transporters to be considered, as has been exemplified with morphine [98]. This in turn provides insight into the likely interplay between luminal transit, metabolism and active transport [250], and can be used in experimental design and to explore 'what if' scenarios [232, 236, 237, 251]. Comprehensive review of these dynamic, integrated modelling approaches have been provided elsewhere [238, 240, 252, 253]. Often, disadvantages perceived with these models relate to their inherent complexity and the level of detail required on parameters used in predictions. For example, transporter kinetic data (K_m and V_{max}) are often lacking, and abundance data for individual DMEs and transporters for the in vitro models and intact tissues are limited and hence restrictive to IVIVE [16, 246]. In response, proteomic- and mass spectrometry-based methods for protein quantification and establishment of scaling factors have started to emerge [32, 149, 254–256].

6 Strategy

An integrated DMPK strategy has been proposed to identify the right compound in the right assay at the right time [257]. Assessing the oral absorption potential of a candidate drug (CD) is a key component to this strategy. Improvements made to preclinical models of gut-wall metabolism (Table 3), and quantitative, mechanistic understanding of processes governing the magnitude of F_G [132, 246, 250], present a compelling rationale for inclusion of a gut-wall metabolism strategy. The strategy outlined in Fig. 3 can be implemented along the drug discovery value chain. Briefly, this would entail profiling and designing out intestinal metabolic liabilities during lead identification (LI) and LO phases. If the potential for gut-wall metabolism still resided in the CD, then detailed PBPK modelling would be required for scenario setting and risk assessment. A decision tree to address gut-wall

Table 5 Metabolic rates of 6β-hydroxytestosterone formation obtained from selected rat intestinal tissue preparations

6β-Hydroxy testosterone (CYP3A)	Everted sac (fresh)	PCTS	Biopsies	S9 fraction-scraping	RIM-scraping	RIM-optimized elution using recovery factor
Testosterone incubation concentration (μM)	100	100	250	250	100	100
Reference for metabolic rate data	[212]	[205, 225]	[205]	[235]	[212]	[233]
6β-Hydroxytestosterone rate	83	25	5.9	1.2	42	113
Original units for rate data	pmol/min/mg protein	pmol/mg whole intestinal tissue protein	pmol/min/mg whole intestinal tissue protein	pmol/min/mg mucosal cytosol plus microsomal protein	pmol/min/mg mucosal microsomal protein	pmol/min/mg enterocyte microsomal protein
Intersystem factor	Not defined	1[a]	1[a]	0.5[a] [(0.5 + 0.13) × 0.8]	0.1[a] (0.15 × 0.80)	0.17[c] (0.53 × 0.33)
Recalculated rate (pmol/min/mg intestinal protein)	8.3[b]	25	33	0.6	4.2	19.5
Fold change compared with PCTS	0.04	1.00	1.32	0.02	0.17	0.78

Intersystem factors were used to recalculate the rate of formation data in units of pmol/min/mg intestinal protein (total intestinal protein from tissue, including muscle) to allow comparison between in vitro methods

CYP cytochrome P450, *PCTS* precision cut tissue slices, *RIM* rat intestinal microsomes

[a] The intersystem factors have been detailed previously [43]

[b] The intersystem factor was not reported for the everted sac rate data previously published [43]. However, the recalculated value has been included to facilitate a broader comparison (for the sake of completeness)

[c] A higher value (39.8 mg protein per gram intestinal wet weight) was determined using an optimized elution method [233]. Taking into account the same total protein content previously described (121 mg/g wet weight in intestinal tissue), of which 39.8 mg consisted of total epithelial protein, indicated that 33 % of the intestinal tissue consisted of epithelial cells (intersystem factor 0.33). Of these epithelial cells, 53 % of the total cellular protein was determined to be microsomal protein (10^6 enterocytes contained 0.45 mg of total cellular protein, of which 0.24 mg was microsomal protein: intersystem factor 0.53). With an optimized scraping method, the whole intestinal wall was used, with the exception of the muscle layers [233]. Accepting that the muscle layer in rat intestine approximated 20 % of the tissue weight [43] gave an intersystem factor of 0.8 × 0.33 = 0.26
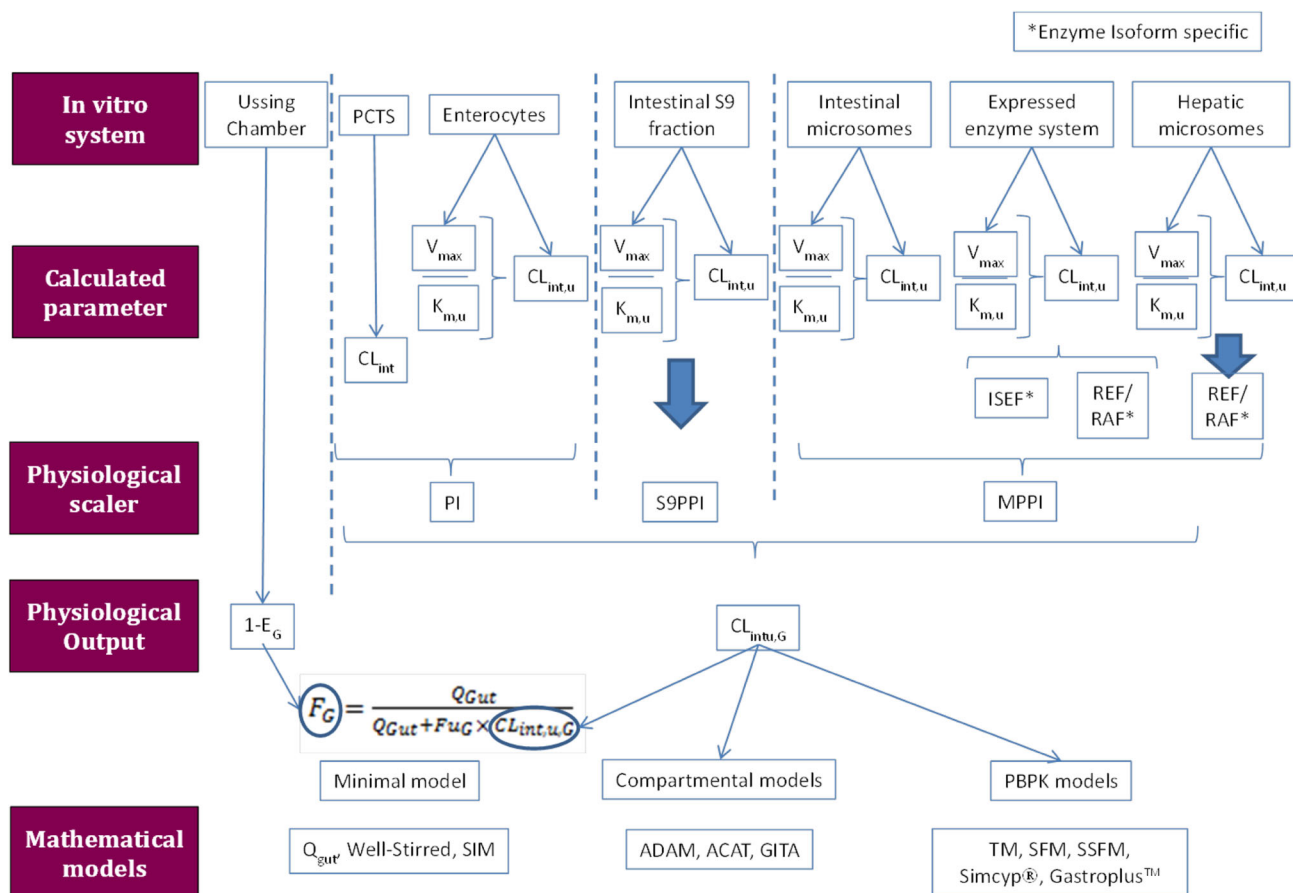
Fig. 2 Bottom-up approach for prediction of F_G using established in vitro systems and mathematical models of the gut. *ACAT* Advanced compartmental absorption and transit model [239, 275], *ADAM* advanced dissolution absorption metabolism model [92], *GITA* gastrointestinal transit absorption model [276, 277], Q_{gut} minimal static model of intestinal metabolism [227], *RAF* relative activity factor, *REF* relative expression factor, *ISEF* intersystem extrapolation factor [190, 201, 227, 278, 279], CL_{int} intrinsic clearance, *MPPI* microsomal protein per gram intestine, *PI* per intestine, *S9PPI* S9 protein per gram intestine, E_G extraction ratio in the gut [69], *PCTS* precision cut tissue slices, $CL_{int,G}$ intrinsic clearance per gram intestine, *SIM* small intestinal metabolism, *TM* traditional model, *SFM* segregated flow model, *SSFM* segmental segregated flow model

metabolism is provided in Fig. 4. Known structural motifs (e.g. metabolic handles for direct phase II metabolism or CYP3A substrates), metabolic instability in relevant in vitro systems, or intestinal loss indicated in animal intravenous/oral PK would trigger further evaluation. Signs of human intestinal metabolic instability would necessitate risk assessment. The potential magnitude of human F_G would be evaluated in relevant preclinical models (Table 3) factoring in the compounds physicochemical properties, biopharmaceutical classification and projected dose (panel 1, Fig. 4). A workflow (panel 2, Fig. 4) is proposed for situations whereby significant intestinal loss limits in vivo exposure in safety or efficacy models. Circumstances such as these may require additional studies to identify whether absorption (permeability/solubility), transporters and/or intestinal metabolism are responsible.

Certain preclinical models may better complement the strategy and decision tree outlined above. During the LI and LO phases, scaled animal and human intestinal microsomal data could provide an efficient means to benchmark the risk of achieving low human F_G, and also help troubleshoot underlying causes of intestinal loss in PK species. These data can also be readily incorporated into PBPK models for quantitative prediction of F_G and simulation of oral PK profiles [258, 259]. Towards CD nomination, more physiological relevant models such as the Ussing chamber may be introduced, facilitating estimation of F_a in the context of metabolism/transporter interplay. Recently, this technique has shown value in predicting human absorption for drugs that are substrates for DMEs and/or transporters expressed in the gut wall [69].
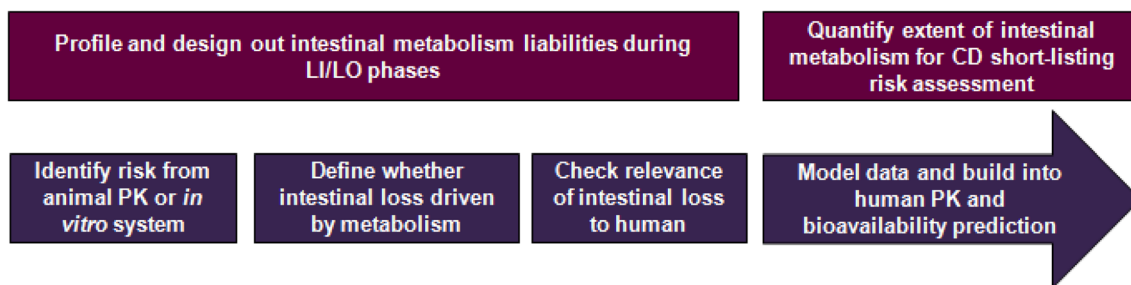
Fig. 3 Strategy to address the impact of gut-wall metabolism on oral bioavailability during drug discovery phases. *LI* lead identification, *LO* lead optimization, *CD* candidate drug, *PK* pharmacokinetics
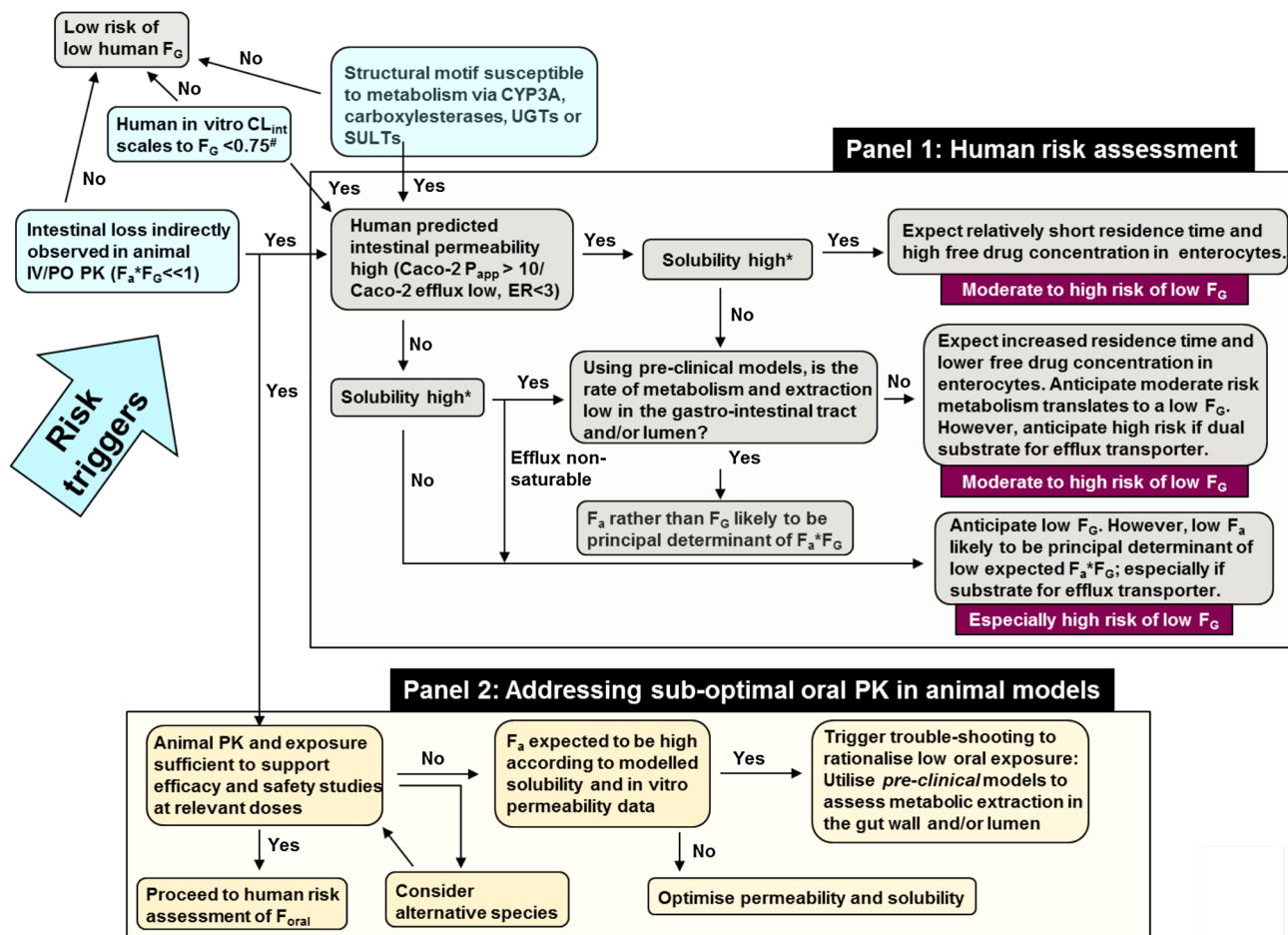

Fig. 4 Decision tree for risk assessment of gut-wall metabolism mediated via CYP450s, carboxylesterases, SULTs and UGTs. Note, as F_H approaches 1 calculation of $F_a \times F_G$ becomes sensitive to the value used for liver blood flow. Intestinal microsomal CL_{int} is scaled to a $CL_{int,u,G}$ then transformed using a model such as Q_{gut} to an estimated F_G. #For CYP3A substrates, physiological scaling factors accounting for relative CYP3A abundance in human liver microsomes and hepatocytes can also be applied to estimate $CL_{int,u,G}$. *The solubility range required for an acceptable F_a is dependent on dose. Solubility is frequently reassessed during drug discovery and early development phases and the F_G liability, in the context of F_a, can be revisited as understanding of an NCEs' solubility profile is developed. Initial measurement of thermodynamic solubility, potentially on amorphous material, would be replaced with solubility of crystalline

material, and then potentially solubility in biorelevant media (e.g., simulated intestinal fluids). Additionally, the pKa (ion class-dependent) can be assessed on representative compounds to allow simulation of F_a using established PBPK models [92, 239, 280–282]. *CYP450* cytochrome P450, *UGTs* uridine diphospho-glucuronosyltransferases, *SULTs* sulphotransferases, CL_{int} intrinsic clearance, Q_{gut} minimal static model of intestinal metabolism, *IV* intravenous, *PO* oral, *PK* pharmacokinetics, *NCEs* novel chemical entities, *PBPK* physiological-based pharmacokinetic, F_a Fraction of orally administered drug absorbed into enterocytes, F_G fraction of drug escaping first-pass metabolism in the enterocytes, *ER* efflux ratio, P_{app} apparent permeability, $F_a \times F_G$ intestinal availability, F_H fraction of drug escaping first-pass hepatic metabolism and biliary secretion, *NCEs* new chemical entities, $CL_{int,G}$ intrinsic clearance per gram intestine

7 Challenges and Future Perspectives

When two or more drugs are coadministered, the effects could be additive, synergistic or antagonistic due to DDIs affecting the absorption and/or therapeutic profile of the victim drug. Overlapping substrate specificities for multiple enzymes and transporters might also enhance the complexity of the absorption profile along the gastrointestinal tract. Thus, overall understanding is a result of complex interplay between physiological (e.g. enzymes and transporters, blood flow, region of the intestine, luminal fluid composition) and physicochemical factors (e.g. pKa, solubility, dissolution, lipophilicity, substrate to enzymes and/or transporters) characterizing the drug molecule. In addition, genetic polymorphisms in drug transporter and DMEs, as well as disease states, may be responsible for variability in the profile and adverse events arising from co-medication among patients, which may be different from healthy volunteers [260] and is difficult to predict from preclinical tools.

In this review, we have illustrated the PK complexity associated with oral administration of drugs linked to intestinal regional variation in DMEs/transporters, as well as species and model differences. Assessing whether clinical candidates have the right risk/benefit balance for patients can be challenging given the inherent complexities and difficulties in the early screening phase and translation into clinical use. Because of the complexity, PBPK modelling will be a crucial tool as it enables efficient integration of knowledge on compound behaviour with the dynamics of intestinal physiology in the preclinical models and humans [236]. However, for successful modelling, high-quality data from in vitro and in vivo preclinical tools needs to be generated (see above strategy). New bioanalytical tools for quantitatively analyzing DME [149, 255, 261, 262] and transporter [33, 256, 263, 264] isoform abundances are already available to improve the quantitative translation between preclinical animals and humans, and will benefit understanding [185, 256, 265]. Knowledge of the impact of pharmacogenomics and disease on regional intestinal availability and variability in underlying mechanisms is scarce. Alongside this, reports focused on back translation of clinical outcome that enable evaluation of the successes or failures of predictions, made from preclinical data, will be crucial to advancing understanding and selection of the best tools for future development activities.

Compliance with Ethical Standards

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