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Invited Review

Absolute abundance and function of intestinal drug transporters: a prerequisite for fully mechanistic *in vitro–in vivo* extrapolation of oral drug absorption

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ABSTRACT: The use of whole body physiological-based pharmacokinetic (PBPK) models linked with *in vitro-in vivo* extrapolation (IVIVE) of kinetic parameters from laboratory experiments, has become embedded within many of the pharmaceutical industry and is used even as part of regulatory submissions. These include the influence of transporter proteins on drug disposition, a subject for which we have witnessed an increasing awareness. A combination of the development of high-powered analytical techniques and antibody-based technology, together with a realization that an understanding of absolute transporter protein abundances together with activity can potentially enhance the modelling of transporter kinetics by PBPK–IVIVE link models. This review summarizes the mechanistic approaches to integrate suitable non-biased *in vitro* transporter kinetic data relevant to the intestine (i.e. 'intrinsic' K_i, 'intrinsic' K_m), by *in vitro* system modelling for these kinetic inputs with the advantages of, and challenges for, generating these data for input into PBPK models. This step is considered as a prerequisite for mechanistic modelling of the oral absorption for drugs that are substrates for transporters. Various approaches are provided to integrate intestinal transporter expression into PBPK models with a perspective on the incorporation of the absolute abundance/activity of transporters to enhance the predictive power of the models. We define the key intestinal tissue and functional expression-based scaling factors required. The objective is to use these for facilitating the extrapolation from *in vitro* intestinal transporter assays to the *in vivo* system, using absolute quantification methodologies. The models could be used to elucidate the complex relationship and relative importance of metabolizing enzymes and transporters in drug disposition and toxicity. Copyright © 2012 John Wiley & Sons, Ltd.

Key words: transporter scaling; absolute abundance; drug transporters; human intestine; IVIVE; modelling and simulation; PBPK; ISEF-T

Introduction

As it has been demonstrated in the recent issue of this journal, the use of whole body physiological-based pharmacokinetic (PBPK) models, linked with *in vitro–in vivo* extrapolation (IVIVE) of kinetic parameters from laboratory experiments, is becoming more embedded into practices within many of the pharmaceutical industry, and it is used even as part of regulatory submissions [1–3]. The IVIVE practices include the prediction of oral drug absorption, accounting for gut metabolism and the simultaneous assessment of the potential influence of drug

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transporter proteins, a subject with an increased awareness amongst the science community [4,5]. We present an overview of the challenges in predicting oral drug absorption involving transporters, interpreting *in* vitro transporter kinetics for the purpose of IVIVE in order to link PBPK and IVIVE models for intestinal transporters, and highlighting the future possibilities of harnessing transporter protein abundances within an IVIVE–PBPK framework due to the recent advances made in measuring the absolute abundance level of relevant transporters in the gut wall.

Challenges in Predicting Oral Absorption Involving Transporters

The majority of therapeutic agents today are administered orally, since oral administration is usually preferred by the patient and can encourage compliance. In addition, oral formulations normally cost less to produce, even when poor drug solubility or degradation by enzymes or acidic conditions in the gastrointestinal (GI) tract need to be addressed. Thus, predicting the rate and extent of oral drug absorption accurately is a prerequisite of modern drug development. However, this becomes a significant challenge when it requires a detailed understanding of the complex role of drug transporters in the gut wall and the interplay between the physicochemical properties of a drug and the physiological parameters that define the absorptive barrier (the gut mucosa) and its capacity to metabolize the drug. A range of physiological factors essential to gut function will markedly impact on drug absorption including, gastric emptying, intestinal transit time, gastrointestinal pH, fluid dynamics, intestinal segmental blood flow, the physiological response to feeding, gut mucosal and luminal metabolizing enzymes, membrane transporters and receptors. Furthermore, many of these parameters are subject to regional differences along the gut and the villus–crypt axis and exhibit also significant inter-individual variability [6].

The bioavailability (F) of a drug can be directly (or indirectly) influenced by transporters expressed on either the apical or basolateral membranes of the enterocytes/colonocytes, affecting both the fraction of drug absorbed across the apical membrane (f_a), the fraction of drug escaping gut wall metabolism (F_G) [7] and the fraction of drug escaping hepatic metabolism (F_H) occurring down-stream in the liver (Equation 1), the first-pass effect.

$$F = f_a \times F_G \times F_H = f_a \times (1 - E_G) \times (1 - E_H) \quad (1)$$

The processes that govern bioavailability including gut transporters can be of benefit or hindrance to drug action, depending on the compound in question. If the drug concentration at the target site (i.e. liver for statins) is too low due to an extensive first-pass effect, then pharmacological action of drug may not be conferred. Alternatively, a restricted first-pass effect allowing a higher drug concentration in systemic blood and, therefore, increased organ drug concentrations can lead to a greater potential for tissue toxicity (i.e. rhabdomyolysis). In addition, the role of transporters local to the site of statin-induced toxicity (i.e. for rhabdomyolysis, the sarcolemmal membrane of the skeletal muscle), has also been postulated to play a critical role [8]. Therefore, understanding the role of transporters during early drug discovery and development by means of *in vitro* studies is becoming more common, although the ability to interpret the results from these studies and to extrapolate them quantitatively to *in vivo* effects is fraught with many unresolved problems.

Physiological-based pharmacokinetic (PBPK) models

Traditionally, PBPK models contain the mass and volume of a number of compartments corresponding to different organs interconnected by blood flows, where differential equations govern the mass transfer of drug into and out of these organs to generate drug tissue concentration–time profiles. These compartments were historically treated as perfusion-limited. However, recently PBPK models have been extended to incorporate models for drug absorption that take into account various physiological elements of the GI tract (e.g. see Pang [9]). Moreover, in order to account for permeation and membrane transporter effects, many recent PBPK models treat the organs as permeability-limited compartments. Commonly used models to predict

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the partition of the drugs into tissues (such as those proposed by Poulin and Theil [10]) cannot be used for this purpose whilst models proposed by Rodgers and Rowland [11], which separate intra- and extra-cellular compartments, might be adopted easily for assessing diffusion-limited distribution and incorporation of efflux and/or influx transporters.

A major requirement in developing state of the art mechanistic PBPK models is to define and implement the elements that can influence the processes governing the fate of the drug within the tissues. It is critical when using modelling and simulation that the 'system parameters' describing the anatomy, physiology and genetics, their associated variability and covariates within a given population are separated from those of the 'drug parameters' [12,13]. In addition, and to limit model uncertainty, robust links between drug parameters measured *in vitro* and the observed *in vivo* outcome is necessary. Some of these in relation to transporters will be explored in later sections.

PBPK-IVIVE linked modeling and simulation approaches

The complexity of the human gut means that it cannot be reproduced easily within a laboratory setting. However, a host of *in vitro* assays together with the use of pre-clinical species are utilized in drug development to mimic the relationship between the intrinsic properties of the drug and the biological systems it encounters in man. Obtaining reliable *in vitro* permeability and transport data early in drug development permits the use of mechanistic *in silico* modelling and simulation techniques to scale from the *in vitro* setting to gauge the impact of transporter(s) on drug disposition within an *in vivo* 'virtual human' [14–16]. Hence, IVIVE links *in vitro* systems to the appropriate human *in vivo* system using algorithms and physiologically relevant scaling factors to bridge the gap between the *in vitro* and *in vivo* systems [17] in a PBPK model [18]. In early drug development an IVIVE approach allows assessment of the drug candidates *in silico*, without resorting to costly human administration, to improve safety and efficacy, and the selection of the most appropriate candidates and optimal dosing. This has implications for reduction in attrition at later stages.

Oral drug absorption and the role of transporters

The absorption processes after oral administration can be summarized as the permeation of a compound across tissue membranes. Orally administered drugs have to overcome numerous barriers prior to reaching their intended target *via* the systemic circulation, and understanding the processes that influence the concentration of drug at the target is of primary importance in drug development. A range of drug transporter proteins expressed in the gut, liver, kidney and brain can impact directly or indirectly upon the pharmacokinetic processes of absorption, distribution, metabolism, excretion and toxicity (ADME-T), thus in turn playing a key role in the disposition and efficacy of drugs [19,20]. The bioavailability of an orally administered drug is dependent to a large extent on the gut mucosal barrier which is composed of the unstirred boundary layer (UBL), the mucus layer, the acid microclimate at the luminal surface of the epithelium, and a polarized layer of epithelial cells (enterocytes), linked by tight junctions that form the structural barrier limiting absorption of potentially toxic substances (xenobiotics), including drugs, into the portal circulation. The drug can, in this process, also be metabolized (either in gut lumen or gut wall). The epithelium expresses a variety of transporter proteins for endogenous compounds and xenobiotics capable of secreting compounds back into the gut lumen that provides an additional 'functional' barrier to the efficient oral absorption of therapeutics. Absorptive transporters (apical uptake or basolateral efflux), mainly involved in the absorption of nutrients, are also present in the gut mucosa [21], and these can be targeted to enhance drug absorption [22].

A key consideration for drugs interacting with transporters in drug development and in the clinical setting is their potential role in the presentation of dose-dependent non-linear pharmacokinetic outcomes. Identifying and understanding the mechanism responsible for these phenomena is of particular importance given that the drug's absorption and subsequent disposition may be critical to efficacy and toxicity. The interaction of a drug with a transporter protein is a saturable process whereby an increase in the concentration of the drug at its binding site leads to a less than proportional increase in the flux of the drug and hence lower

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absorptive permeability for apical uptake transporters, or conversely an increase in overall absorptive permeability if the interaction is *via* an apical efflux transporter. Therefore, human intestinal transporter proteins are likely to play a role in dose non-linearity with orally administered drugs. It was demonstrated some years ago that increasing the oral dose of talinolol is associated with more than proportionate systemic exposure in humans [23]. It is also shown that the addition of the P-glycoprotein (P-gp, or gene product of the multidrug resistance protein 1, MDR1) inhibitor verapamil reduces secretory flux in the intestinal enterocyte-like epithelial cell line Caco-2. Therefore, the likely mechanism responsible for talinolol dose non-linearity is due to the saturation of intestinal secretory (efflux) processes. In contrast to the phenomena observed for talinolol, UK-427,857 (maraviroc) demonstrated a less than proportional exposure upon administration of an escalating oral dose in humans [24]. This might have different causes including induction of efflux transporters such as MDR1 or the activation of MRP2 (multidrug resistance-associated protein 2) *via* an allosteric binding mechanism thus enhancing apical efflux [25]. Further examples where induction of efflux transporters led to non-linearity are also provided later in this review.

Intentional targeting of intestinal influx transporters can be used to enhance the absorption of a compound *via* pro-drug strategies, leading to an increased bioavailability. The pro-drug of acyclovir, valcyclovir, relies on oligonucleotide influx transport by peptide transporter 1 (PEPT1), to enhance its permeability across the apical membrane of the enterocyte. However, when the active influx process saturates upon administration of high oral valcyclovir doses, there is an unexpected non-proportional reduction in the C_{max} and AUC and a delayed T_{max} [26].

In recent years there has been a focus on microdose studies to negate the requirement for non-clinical toxicity studies [27,28]. The translation of pharmacokinetics from these microdosing studies to those displayed at therapeutic concentrations could be problematic if gut wall transporters are involved in the absorption. The orally administered microdose may not saturate the active transport or metabolic processes but the therapeutic doses might. This leads to non-linear pharmacokinetics for certain compounds. Therefore, the use of microdosing strategies requires a good quantitative understanding of the role of gut wall transporters and gut wall metabolism.

The potential to saturate a transporter is generally based on its affinity and functional capacity. Intestinal influx transporters, i.e. MCT1 and PEPT1 are low affinity (high K_m)/high capacity transporters whereas efflux transporters are generally rather high affinity (low K_m)/low capacity and will tend to saturate more readily than influx transporters in the same membrane [22]. Physiologically, this is a useful combination as it enables the cell to efficiently control the concentration of an unknown exogenous compound like a toxin within the cell. Theoretically, for a compound like quinidine that has a high passive permeability and interacts with both apical influx and efflux transporters, it is likely that any non-linearity is based on the efflux process saturated at lower concentrations rather than saturation of the influx process. Some transporter-mediated drug–drug interactions (tDDIs), whether involving absorption or disposition, can lead to adverse drug reactions or loss of efficacy. These are becoming of increasing concern to the pharmaceutical industry and regulatory bodies [4,5,20,29,30]. However, it should be noted that tDDIs are often confounded by metabolism-based DDIs (mDDIs). This follows since in most cases transporters and metabolic enzymes compete for the parent compound and its metabolite(s). Several transporters and metabolizing enzymes such as CYPs, UGTs and SULTs are expressed along the intestine and require consideration [22,31–34]. The scope of clinically relevant interactions within the intestine have been reviewed in detail recently [19] and encompass co-administered drugs, dietary constituents and herbal remedies such as grapefruit juice and St John's wort. This paper will focus on the transporter aspect of well-known key drug transporters responsible for changes in drug absorption.

The transporter related problems for oral absorption does not apply to all drugs that are substrates for transporters. These may be related to the balance between the passive permeability and active transport, or the saturation of the transporters at therapeutic doses. Nonetheless, since many new drug candidates no longer belong to biopharmaceutical classification I (highly permeable and highly soluble), *in vitro* screening to understand the role of drug transporters in gut wall permeability, hepatic

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efflux and uptake or renal secretion has become a valuable tool in estimating tDDIs. Although, this is not a routine practice in early drug development, its use could be a potentially cost effective means to reduce adverse events or loss of efficacy in clinical practice due to tDDIs [20,30,35,36]. The approach is encouraged in the latest FDA draft guidance for the conduct of drug–drug interaction studies [30] and the final EMA guideline on the investigation of drug interactions [5], despite the on-going debate on quantitative methods which should be applied to information obtained from such *in vitro* measurement.

In Vitro Transporter Kinetics for the Purpose of IVIVE

Parameters required from in vitro transporter assays

To devise an effective mechanistic IVIVE strategy, incorporation of separate elements for the passive permeability and transporter-mediated flux is necessary. Subsequently, 'drug parameters' are defined for each process within appropriate *in vitro* experiments and used in conjunction with the 'system parameters' to predict the behaviour of the drug *in vivo*. When integrating drug parameters into a model, the key processes including active kinetics, are often described by Michaelis-Menten kinetics (K_m, J_{max} or $CL_{int,T}$) as shown in Equation (2). Although it has been asserted that drugs pass through membranes only by active uptake processes [37], this is not a commonly held view. Hence, applying active transport models to measured *in vitro* data can take place only after deconvoluting the effects of passive diffusion (CL_{PD}). Moreover, the effects of the unbound fraction at the binding site require delineation before using models such as those shown in Equation (2):

$$CL_{int,T} = \frac{J_{max}}{K_m + C} \quad (2)$$

where $CL_{int,T}$ is the intrinsic clearance due to active transport, K_m is the Michaelis constant, J_{max} (or sometimes termed V_{max}) is the maximal flux capacity of the transport protein and C is unbound drug concentration (at the transporter binding site).

In vitro cell based systems are used routinely to elucidate passive and active transporter processes acting to influence drug permeability. Caco-2 cells grown to confluent monolayers in bicameral filter systems endogenously express the majority of the relevant transporters also expressed in the human intestine *in vivo* [38]. Alternatively, sub cell lines of dog and pig cell lines, Madine Darby Canine Kidney (MDCK-II) and Lilly Laboratories Cells - Porcine Kidney Nr. 1 (LLC-PK_1), selected due to their low endogenous transporter expression of mdr1, breast cancer resistance protein (bcrp) and multidrug-associated protein 2 (mrp2) are transfected with human transporter proteins (single, double and even triple transfected cells), and are used to determine the impact of transporter proteins on drug transport [39]. To delineate the CL_{PD} from K_m, J_{max} or $CL_{int,T}$ in Caco-2 or human-transporter transfected cells, compounds inhibiting a specific transporter isoform are applied to the system with the flux remaining after inhibition assumed to be passive. Inhibition of active processes can be problematic due to the lack of specificity of transporter inhibitors for certain isoforms [40], therefore inhibitor usage is not always an optimal method to determine CL_{PD}. Compounds are also used under the assumption that the compound can saturate the transporter in soluble non-toxic concentrations which is not always the case, for example, digoxin or vinblastine (limited solubility at high saturating concentrations), and can give rise to toxicity, for example, quinidine (toxicity issues at high concentrations) [41,42].

Additionally, verification of human transporter-dependent flux in transfected cells is achieved by correction for flux determined in background wild type or vector control (null-transfected cells). Nevertheless, there is still residual endogenous transporter expression within these cells, which is illustrated for absolute transporter abundances in wild type cells [43–45]. However, the endogenous contribution of canine apical transporters may be limited [39], but efflux ratio, the ratio of the apparent permeability (P_{app}), in the apical-to-basolateral (A-to-B) direction and P_{app} in the basolateral-to-apical (B-to-A) direction should still be interpreted with caution. Obtaining a passive flux/permeability coefficient that is due solely to the cell monolayer (transcellular permeability) and not system specific characteristics, the correction for

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paracellular permeability, the UBL and filter impedance is also employed [46].

Active transport processes are conventionally determined by bi-directional drug flux across multiple substrate concentrations, which can be transformed to transporter isoform-specific permeability or flux and incorporated into a one-site binding Michaelis-Menten equation, although a two-site binding model may be more appropriate for certain compounds and transporters [47]. Using non-linear regression, the apparent K_m and J_{max} can be estimated [48] under the assumption that the K_m, a biochemical constant, should be similar in the A-to-B, and B-to-A transport directions theoretically, if the buffer conditions on both sides are comparable (e.g. buffer pH). However, practical differences are observed where estimates of K_m are direction-dependent [48]. This is discussed in detail in the following section with further commentary also available in the recent review by Grandvuinet and colleagues [34].

Common pitfalls in determining transporter kinetics

The conventional two-step approach to determining transporter kinetics experimentally and statistically encompasses the following:

Step 1. The determination of the transport of a compound into a given compartment, i.e. the intracellular compartment, at multiple drug concentrations at a single time point to obtain 'raw uptake data'.

Step 2. The accumulation data from step 1 is transformed into rate data at different concentrations and regression procedures are used to transform the 'raw data' to kinetic data in the Michaelis-Menten model (Equation 2).

Some limitations of using a conventional approach for kinetic determination are as follows

(1) Nominal (initial) concentrations are used to describe drug transfer or P_{app}. Thus, an apparent permeability is reported and not an intrinsic permeability (P_{int}).
(2) Disregard for the unbound drug concentration at the transporter binding site(s), since the unbound fraction in the cell or membrane is not easy to measure.
(3) Disregard for time- and concentration-dependent integration of passive and active processes, to estimate kinetic parameters.
(4) Limited consideration for the experimental system, i.e. compartment volumes critical in governing the drug concentration directing active and passive processes.

The conventional approach has been shown to give rise to considerable bias dependent on the experimental system and the mathematical extraction of the kinetic data from the raw data (i.e. modelling of the *in vitro* data), leading to a direction-dependent determination in apparent K_m ($K_{m(app)}$), a 'K_m-shift'. The first instance of conventional kinetic determination reporting this phenomenon was demonstrated for eight compounds in Caco-2 cells grown in a filter system using the MDR1 substrates [48], where the authors describe a 3- to 8-fold higher affinity (lower $K_{m(app)}$) in the B-to-A transport direction compared with the A-to-B transport direction, a substantial discrepancy for this biochemical constant. It was postulated that this marked direction-dependent K_m-shift was the result of differing drug concentrations acting at the binding site when a drug was applied apically versus basolaterally, as a result of differences in passive permeability conferred by the dissimilar composition of the exofacial outer membrane leaflets at each membrane. Further studies reporting a 'K_m-shift' also suggested that membrane composition accounted for differences in $K_{m(app)}$ between Caco-2 and MDCK-II-MDR1 cells for vinblastine [49], while another study proposed that the lack of consideration for the diffusional barriers; the UBL and filter impedance accounted for $K_{m(app)}$ variability [50] and that transporter expression level resulted in a variable apparent $K_{m(app)}$ for quinidine, verapamil and vinblastine [51]. However, it is expected theoretically that only J_{max} is influenced by transporter expression.

Drug concentrations within the donor and acceptor compartments are dynamic, thus assuming a nominal drug concentration to describe drug transfer processes is likely to lead to kinetic inaccuracies. Theoretically, for efflux transporters where binding sites are thought to be located facing the cytoplasm or inner membrane leaflet [52], the critical concentration driving active efflux is the unbound intracellular or membrane concentration [39], therefore nominal concentrations in the donor compartment

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do not necessarily reflect the membrane or intracellular concentrations driving efflux. If the intracellular drug concentrations are substantially higher than the K_m, active efflux saturates, leading to increasing intracellular drug concentrations and a high outwardly directed concentration gradient, driving a greater passive transfer of drug from the cell to the apical compartment, until equilibration is reached [53]. If the appropriate unbound concentrations in all compartments are not considered at regular time increments, the synergistic active and passive processes are not accurately considered for subsequent statistical evaluation to estimate kinetics. Determining unbound intracellular drug concentration is a considerable challenge, particularly at short time point increments (1–5 s increments) at the beginning of *in vitro* studies (i.e. < 30 s from initiating flux). However, early time points, seconds (!), are not practical in any laboratory setting and thus can often only be estimated mathematically. These early time points may be critical in uptake transport investigations, where ascertaining initial drug transfers between compartments will govern subsequent concentration dependent processes [54]. On the contrary, evidence is recently presented that lag times, potentially due to the UBL, for transfer of drug across membranes have also been reported for poor and highly permeable compounds in filter grown MDCK-II cells [38], therefore the *in vitro* system under study requires careful consideration when defining experimental protocols. For apically or basolaterally localized influx transporters, defining the unbound concentration at the binding site is a less onerous task since the binding sites are proposed to be located in extracellular hairpin domains which form a gating mechanism for substrate flux *via* SLC proteins [55] or His residues that are postulated to play a role in pH-sensitive substrate transport [56], thus unbound drug concentration in the extracellular media can be evaluated by sampling the donor/receiver compartments.

Utilizing a conventional approach where time-dependency is not considered, it is no surprise that kinetic bias has been reported. If biased data are incorporated into an IVIVE model erroneous outcomes are likely. An example of optimizing Caco-2 MDR1 transport kinetics for incorporation into a PBPK model is given recently [57], where the analytical solution was not described, but the justification for optimizing was due to eradicating *in vitro* system bias when using extracellular media concentrations to estimate the intrinsic $K_{m(int)}$.

Defining in vitro transporter kinetics via mathematical models

Having established that drug transporter kinetic determinations are influenced by the experimental system, research has focused on using mathematical models to describe drug concentration–time courses at transporter binding sites in order to accurately determine the intrinsic kinetics of active processes (Figure 1). Using a global kinetic approach to simulate the time course of drug concentrations in multiple compartments of the experimental system was undertaken by Kalvass and Pollack some years ago [58]. More recently, Tachibana *et al*. [59] applied equations to a 3-compartment mathematical model describing the Caco-2 Transwell systems apical, cellular and basolateral compartments. Permeability data were utilized where $K_{m(app)}$ was previously correlated to increasing MDR1 expression for quinidine, verapamil and vinblastine [51] and the intracellular drug concentration–time course was simulated. Defining the unbound concentrations within the cell allowed the $K_{m(int)}$ to be estimated and resulted in $K_{m(int)}$ values that were independent of MDR1 expression, consistent with the notion of K_m as a biochemical constant. Solutions have also been acquired using 3-compartment models to describe a saturable component operating metformin permeation in Caco-2 cells [60]. While the interactions of the endogenous compound estrone-3-sulphate with an apical efflux transporter, probably BCRP and a simultaneously acting unknown rate-limiting basolateral influx transporter have also been described using simulation [61].

Over the past few years there have been numerous reports with increasing model complexity (up to five compartments) to describe the characteristics assumed to be important to the mechanics of drug transport within a two chamber-filter system such as the Snap- or Transwell systems. Analytical solutions have been sought to determine the effect of differences in basal and apical membrane surface areas on kinetic estimations [62]. The UBL and pH-gradient at the apical membrane have been incorporated into models for drugs possessing a high CL_{PD} and where ionization requires consideration to

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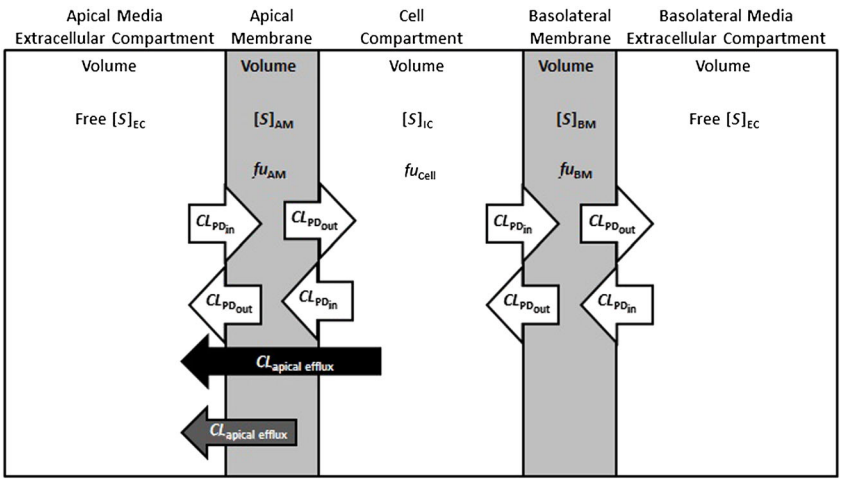

Figure 1. An example of a five-compartment mathematical model for the consideration of apical active efflux clearance ($CL_{int,T}$) from intracellular and membrane sites, passive diffusion into (CL_{PDin}) and out (CL_{PDout}) of the apical and basolateral membrane at both the inner and outer membrane leaflets, with the unbound/free fraction of drug in the cell (fu_{cell}) to determine the intracellular substrate concentration $[S]_{IC}$, the apical membrane $[S]_{AM}$ and basal membrane $[S]_{BM}$ concentration and extracellular compartmental free drug concentrations (Free $[S]_{Ec}$). Experimentally observed permeability data at multiple concentrations and time points can be integrated into a model describing the experimental system. An analytical solution is derived that provides the optimal fit to the observed data where the intrinsic kinetic parameters are obtained for input into an IVIVE model. This model can be extended to a seven-compartment model by accounting for the UBL on both sides of the cell, i.e. between the media and the membrane

evaluate the contribution of these parameters in determining transporter kinetics [50,63]. Korzekwa and co-workers found that there was improved prediction of experimentally observed compartmental concentrations, including unbound intracellular concentrations, when explicit modelling of the partition of drug into the apical and basolateral membrane was included in a compartmental model [39].

Key components to model drug transport-permeability time courses using real examples *in vitro* involve: sampling at multiple time points and concentrations in the donor and receiver compartments; estimating or measuring the unbound fraction of drug in the extra and intracellular milieu and defining the structure of the experimental system. The unbound fraction in the cell (fu_{cell}) can be difficult to measure experimentally. Nonetheless, the monolayer could be lysed to release intracellular contents with the addition of the drug to the lysed cellular components [64]. Drug partitioning between lysed cell components and the supporting media is then assessed to estimate fu_{cell}. Furthermore, plasma protein binding measurement could be used as a surrogate for fu_{cell} or quantitative structure activity relationships (QSAR) models driven by logP and protein content for estimation of binding [65], or more mechanistic estimations using tissue composition with drug physicochemical descriptors are available [66]. Monolayer volumes could be measured by determining individual cell volumes by flow cytometry [67] or isotope dilution [68] and scaling this to the number of cells on the monolayer.

Integrating the available concentration–time course data into a model provides a distinct advantage in that assays using inhibitors are not necessary. This eradicates any issues surrounding the lack of specificity for certain transporter isoforms to provide estimates of passive permeability.

It is therefore advocated that transporter kinetics determined using a global kinetic approach are undertaken to provide robust kinetic parameters for input into IVIVE models to reduce the potential for erroneous outcomes when extrapolating apparent *in vitro* kinetic data to the *in vivo* system. A modelling and simulations approach can also aid in recognizing processes that have not been identified to facilitate further work on these as yet unidentified mechanisms acting on the drug, in other words each outlier might supply further answers to the problem and should therefore be treated with extra attention.

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PBPK-IVIVE Linked Models of Intestinal Transporters

In line with the rapid advancement in the development of PBPK models is the ever increasing need for high quality biological data [69], including drug transporter protein expression and activity data to enhance the proximity of the model's system parameters to the *in vivo* milieu, and is critical in developing predictive models of transporter-mediated drug disposition. In addition to the definition of *in vivo* expression data, the IVIVE approach requires not only scaling factors to bridge any mechanistic gap between the *in vitro* systems used to mimic the *in vivo* system, but also organ-based scaling factors. The categorization of these scaling factors is shown in Table 1. For any biological data incorporated into a PBPK model to generate scaling factors and drive functionality, there must be assurance as to the suitability of the data, and that any confounding factors associated with sample/tissue quality and methodological bias are factored into analysis prior to implementation, therefore care should be taken when interpreting data from the literature for implementation into a mathematical model.

Structure of a mechanistic model for intestine

To model the intestine mechanistically it is necessary to account for the region-specific anatomical and physiological differences that the compound encounters as it transits distally upon oral administration. This is achieved by representing the regions of the gut as compartments. The small intestine in a mathematical model generally contains seven segments, including one duodenal, two jejunal and four ileal segments, with each compartment potentially distinctive from one another based on defined system parameters. Many of the advanced models are based on adaptations of the original compartmental absorption and transit (CAT) model that accounts for small intestinal transit time (SI), permeability, radii are the same for all compartments, gut metabolism is neglected, passive diffusion is mainly responsible for drug absorption across the gut wall, the compound is in the solution (instantaneous dissolution, luminal degradation is neglected, and the only site of absorption is the small intestine, i.e. neither colon nor stomach is catered for [70,71]). Two of the most sophisticated models currently available are the advanced dissolution, absorption and metabolism (ADAM) model as recently reviewed [14,16] and the advanced compartmental absorption and transit model (ACAT) [57]. Both models demonstrate the utility of the IVIVE approach to effectively address simultaneous gastrointestinal transporter–metabolism interplay by simulation.

Tissue scaling factors for IVIVE of intestinal oral absorption

The IVIVE approach for predicting drug pharmacokinetics relies on scaling factors from cells and subcellular fractions to bridge the mechanistic gaps

Table 1. A description of scaling factors derived to permit IVIVE for transporters

In vitro–in vivo scalars	Description
Relative mRNA/protein expression factors (REF)	A system-based scalar describing the difference in expression for a transporter between the *in vitro* and *in vivo* system based on relative mRNA and/or protein quantitation
Relative activity factors (RAF)	A drug-based scalar describing the difference in activity between the *in vitro* and *in vivo* system based on $CL_{int,T}$ or V_{max} [97]
Between species scalars	A drug-based scalar describing the difference in transporter clearance between species for example, rat and human [144]. Often also named RAF
Absolute protein expression and/or activity scalars	System-based scalars describing the difference in expression and/or a combination of expression and activity for a transporter between the *in vitro* and *in vivo* system based on absolute protein quantitation and/or the associated activity [97]. These would lead to possible inter-system extrapolation factors for transporters (ISEF-T)
Organ scalars	
Membrane protein scalars	Employed to convert the *in vitro* derived transporter $CL_{int,T}$ to estimate whole organ $CL_{int,T.}$ These could be scalars for the whole organ (e.g. membrane protein per intestine, MePPI) or for segments/parts of the organ (i.e. jejunum, duodenum, colon)
Cellular scalars	Employed to convert the *in vitro* derived transporter $CL_{int,T}$ from cellular systems to estimate whole organ $CL_{int,T}$ (e.g. HPGL, PTCPGK)

PTCPGK, proximal tubule cell per gram of kidney.

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between the *in vitro* and *in vivo* system using expression-based scaling factors (Table 1). An area within IVIVE that has been heavily studied is that of the metabolic scaling factors that are necessary for scaling a CL_{int} per mg protein within the *in vitro* assay, for example, hepatic, intestinal or renal microsomal preparations, to a whole organ CL_{int}. Commonly used scaling factors for liver, intestine and kidney are hepatocytes per gram of liver (HPGL), microsomal protein per gram of liver (MPPGL), cytosolic protein per gram of liver (CPPGL), microsomal protein per whole intestine (MPPI) and microsomal protein per gram of kidney (MPPGK) which are required to scale metabolic clearance predominantly from cytochrome P450 (CYP450) and uridine glucuronosyltransferase (UGT) enzymes in hepatocyte, liver, intestinal and kidney microsomal preparations, respectively [72]. However, microsomal preparations are unsuitable for transporter determinations and hence there are relatively few data for membrane protein scalars, which are required for scaling transporter kinetics to whole organs. Recent studies by Tucker *et al*. and Deo *et al*. [73,74] broach this field and provide a crude membrane protein scalar for the liver (35.8 ($n = 13$) and 41.6 mg ($n = 51$) crude membrane protein per gram of liver, respectively). Additionally, the Tucker group provides a crude membrane protein per cm^2 of duodenal mucosal surface area of $1344 \pm 737\,\mu g/cm^2$ [(mean ± SD), CV = 55%, $n = 14$] [73], to attempt mechanistic transporter scaling. However, a cell surface plasma/brush border membrane-specific protein scalar rather than a crude membrane scalar could be more appropriate due to functional transporter protein residence in the cell surface plasma membrane. Any differences between a crude membrane and plasma/brush border membrane scalar to the best of the authors' knowledge have yet to be reported. Ideally, scalars should be corrected for loss of protein throughout the procedure using key enterocyte protein markers, sucrase or villin; however, this is not necessarily common practice [72].

It is obvious that considerably more work is required in this area to determine membrane protein scalars in various intestinal regions from a considerably larger donor group to represent a heterogeneous population(s), including potential gender differences (i.e. BCRP) [75], the effects of ethnicity (e.g. in the case of the organic anion transporting polypeptide 2 (OATP1B1), MDR1 and BCRP) [76], phenotype [77] and induction of transporters due to drug exposure [78]. The issue of tissue quality also requires addressing, namely, cryopreserved versus fresh tissue, healthy and tissues potentially affected by a donors disease state, the time interval from tissue removal to the key stages of tissue processing and any effects of buffers.

A relative expression scaling approach

Incorporation of transporter expression into mechanistic intestinal models. There are known regional differences in transporter and metabolic enzyme expression along the human intestine [79,80]. For example, Figure 2 shows the expression profiles of MDR1, MRP2, BCRP and CYP3A4 as used within the current ADAM model. Therefore, for any transporter isoform the regional heterogeneity of drug transporter expression requires consideration when incorporating intestinal transporter expression data into a PBPK model. Representation of the regional heterogeneity of intestinal drug transporter expression in mechanistic intestinal models, and studies where these models are integrated within a full-PBPK model framework is limited [15,57,80,81]. These models incorporate expression derived from relative quantitative methods to describe ATP-dependent (ABC) efflux transporter expression or solute carrier (SLC) data from immunoblot densitometry or mRNA gene expression. Where mRNA expression is used, it is assumed directly to correlate to protein activity [79,80,82–92], but this may not always be the case [93]. A common practice when using relative expression data is to normalize the expression of a transporter throughout the intestine to a given segment, such as the first ileal segment as undertaken by Bolger and colleagues [15] or the first jejunal segment as implemented in the ADAM model [14]. In addition to transporter expression data, a variety of drug parameters are also utilized; CL_{PD}, the efflux ratio or Michaelis-Menten kinetics (Equation 2) were incorporated for simulations from *in vitro/in situ* systems such as the parallel artificial membrane permeability assay (PAMPA), Caco-2, MDCK-II, LLC-PK_1, rat intestinal perfusion and oocytes. The combination of transporter

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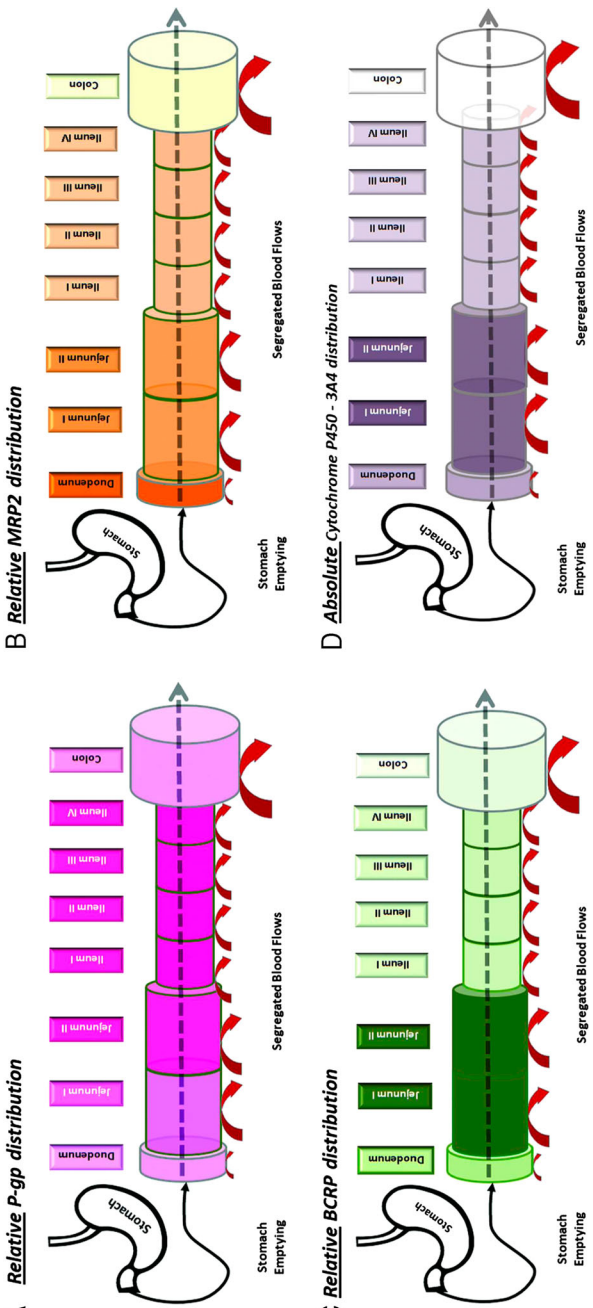

Figure 2. Schematic representation of the ADAM model, displaying the mechanistic segmentation of the GI tract into nine sections with segregated blood flows to each section. The abundance of various enzymes and transporters in each segment varies non-monotonically along the intestine as depicted by the varying intensity of the colour for each section, representing P-glycoprotein (MDR1) (A), MRP2 (B), BCRP (C) and CYP3A4 (D). The small intestine consists of seven segments where drug can dissolve, re-precipitate or be exposed to chemical degradation. Fluid dynamics (secretion and re-absorption), varying pH and bile salt concentrations in each section are considered. The first jejunal segment 'Jejunum I' is calculated according to body surface area [14] and contains a normalized MDR1 expression of 1 with the relative expression of all other segments set against the Jejunum I (Table 2). The increasing colour density indicates a higher relative MDR1 expression along the small intestine. The expression of MDR1 in the stomach is not considered within the current ADAM model

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protein and mRNA expression together with drug parameter data lead to successful capture of drug disposition.

A study by Bruyere and co-workers has highlighted the importance of obtaining regional intestinal transporter expression data for incorporation in PBPK models [80]. Levels of the apical efflux transporters MDR1 and BCRP were measured by immunoblot in 13 biopsies from various intestinal sites and the data implemented into the model was expressed relative to the ileum. The results demonstrated that accounting for the MDR1 distributions enhanced PBPK predictions of bioavailability for the development compound under study.

A study using the ADAM model utilizing regional MDR1 and CYP3A expression has been described by Darwich and co-workers (Figure 2) [16]. Simulations using a drug parameter space for eight compounds encompassing MDR1 intrinsic clearances from Caco-2 monolayers, ($CL_{int,T}$) and $K_{m(app)}$ were undertaken [48]. A relative expression factor (REF) was used as a scaling factor that accounted for differences in the *in vitro* Caco-2 and *in vivo* human jejunal MDR1 expression from immunoblot [94], with the intestinal regional distribution of MDR1 being based on a meta-analysis performed in an identical statistical approach as a recent study by Cubitt and colleagues [95] by incorporating a composite of mRNA and protein data (Table 2) [38,48,79,82–85,93]. The results of this analysis and others [57,79,80,93] demonstrate that there is certainly no consensus on the regional expression of MDR1 in the small intestine and colon using relative expression techniques. The results of further meta-analyses defining the distributions of MRP2 mRNA [82–84,86–88] and BCRP mRNA [38,83–85,89,90] are also provided in Table 2, with expression normalized to that in the first jejunal segment 'Jejunum I'. It is interesting to note that results of the meta-analysis of BCRP mRNA data (Table 2) from six studies do show a non-uniform regional distribution, implying that there is a disparity between mRNA and protein expression described for BCRP [80]. However, to fully elucidate this relationship, mRNA and protein samples should be matched, as done by Urquhart *et al*. 2008 [90].

Accounting for inter-individual transporter variability. Predicting the impact of inter-individual variability in transporter expression and function within a population rather than in the individual human is advantageous in order to appreciate particular individuals who are at the greatest or lowest risk of adverse events. Numerous studies were therefore analysed to gauge the variability of MDR1, MRP2 and BCRP protein expression in the duodenum/proximal jejunum to obtain the weighted mean coefficient of variation (CV) [79,87,90–92,97] for implementation into the ADAM model (Table 3). It is assumed that there is a constant variability for a given transporter in all intestinal segments. However, due to regional anatomical differences the overall variability for each transporter along the intestine is higher than in any of the segments alone which is in agreement with observed data. At present, regional intestinal distribution from reported intestinal PBPK models has relied upon either, a

Table 3. The weighted mean variability of MDR1, MRP2 and BCRP incorporated into the ADAM model

Protein	Mean CV%	Sample *n*	References
MDR1	65	38	[79,91,92,96]
MRP2	79	30	[87,90,91]
BCRP	63	29	[90,91]

Table 2. Regional intestinal efflux transporter mRNA and protein expression data implemented into the ADAM model (in Version 8/2008 and Version 9/2009)

	MDR1 [38,48,79,82–85,93] $n = 79$	MRP2 [82–84,86–88] $n = 78$	BCRP [38,83–85,89,90] $n = 79$
Duodenum	0.51	1.41	0.47
Jejunum I	1	1	1
Jejunum II	1.46	1	1
Ileum I	1.5	0.60	0.59
Ileum II	1.51	0.60	0.59
Ileum III	1.52	0.60	0.59
Ileum IV	1.51	0.60	0.59
Colon	0.57	0.02	0.13

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mixture of mRNA and protein expression data or only on protein data from relatively few donors, which is possibly not representative of a wider population.

Relative expression factors (REFs) for scaling.Expression-based scaling factors have been utilized in IVIVE and incorporated into PBPK models for many years [97]. The inter-system extrapolation factor (ISEF) was devised to correct for differences in enzyme abundance and activity (per unit of CYP450 isoform) in recombinant expression systems and human liver. However, transporter expression scaling factors at present are not nearly as sophisticated due to the relative lack of transporter abundance data and paucity of good quality kinetic data owing to a lack of transporter specific substrates, definition of experimental set ups and their interpretation, as well as the information on transporter isoform effects on activity. To account for any transporter expression differences in the *in vitro* system (Caco-2, MDCK-II) and the human jejunum, IVIVE scaling factors have been generated using immunoblot densitometry to provide REFs for each transporter in Caco-2, MDCK-II system for MDR1, MRP2 and BCRP (Equation 3, Tables 4, 5) and are incorporated into published work and Simcyp Simulator libraries [16].

$$REF = \frac{In\ \ Vivo\ \ Expression}{In\ \ Vitro\ \ Expression} \quad (3)$$

A substantial advancement in defining expression scaling factors, however, will arise from absolute transporter abundance data generated in both the human and the *in vitro* systems, where models can progress from this relative-based semi-mechanistic approach. Further information on the designed transporter features of many 'off the shelf, ready to use' PBPK models currently available are provided by Bouzom *et al*. [69].

Table 5. Weighted mean relative expression factors (REF) based on immunoblot densitometry studies described in Table 4

Transporter	MDR1	MRP2	BCRP
User	1	1	1
Caco-2	0.99	1	1.19
MDCK II	1.49	2.12	1

Using absolute transporter abundances as a scaling factor

Technical considerations. In the past 4 years, significant advances within proteomics using mass spectrometric techniques, [98–100], and to a limited extent quantitative immunoblotting [73], have enabled the determination of drug transporter protein abundances within human [101–109] and pre-clinical tissues [101,102,104,110–112]. This was not possible previously due to the difficulty in purifying these integral membrane proteins, and the lack of antibody standards to quantify protein abundance in biological samples. To coordinate with mammalian tissue abundance measurements there has also been a focus on characterizing *in vitro* systems used routinely in drug development for determining the impact of drug transporters on drug disposition, for example: (i) isolated and sandwich cultured hepatocytes [101,102,107,113], (ii) immortalized cells lines such as Caco-2 [113], (iii) transfected human immortalized cell lines such as HEK293 [109,114,115] and (iv) transfected dog/pig/insect cell lines expressing human or (iv) pre-clinical species transporter proteins [43–45,116].

The first and most studied tissue for determining transporter absolute abundance is the human liver.

Table 4. Studies used to derive relative expression-based scaling factors (REFs)

In vitro system	Transporter	REF Jejunum I	Reference	Comments: Scalar measured relative to...
Caco-2	MDR1	0.78	[145]	Human enterocytes, western blot ($n = 5$)
Caco-2	MDR1	2.04	[94]	Jejunal samples, western blot ($n = 1$)
Caco-2	BCRP	1.07	[89]	Duodenal samples, mRNA (normalized to villin) ($n = 14$)
Caco-2	BCRP	1.19	[145]	Human enterocytes, western blot ($n = 5$)
MDCK-II-MDR1	MDR1	1.49	[94]	Jejunal samples, western blot ($n = 1$)
MDCK-II-MRP2	MRP2	2.12	[87]	Duodenal samples, western blot ($n = 10$)

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Five studies, for example, have determined MRP2 and BCRP liver abundances in an overall 95 and 42 samples, respectively [73,74,101–103]. The abundances of MRP2 measured by Ohtsuki *et al*. [103] and Deo *et al*. [74] are approximately 2.5-fold greater than in the two studies carried out by Li *et al*. and Tucker *et al*. [73,101,102]. Caution should be exercised when implementing data into mathematical models, therefore ascertaining the potential confounding factors that could lead to the differences shown for MRP2 abundance in the liver from these studies requires appreciation. These differences may simply be based on inherent biological variability; however, other biological factors may also require attention such as the influence of age, gender, disease and drug history, with particular relevance to induction of transporters due to drug exposure (e.g. rifampicin [78], etravirine, maraviroc [20]). Procedural effects leading to methodological bias on endpoint outcomes require appreciation such as, harvesting procedures, buffer composition, fresh versus cryopreserved tissue and transporter stability in the membrane, as demonstrated by the intracellular sequestration of efflux transporters upon collagenase isolation of hepatocytes [117]. Furthermore, any time gap to undertaking the assay for either activity or abundance may affect the endpoint measures [118]. The membrane extraction technique may lead to bias, for instance, the employment of either a membrane extraction kit or differential centrifugation, or abundances determined in crude membranes (containing all associated membranes within a sample) compared with the plasma membrane (Figure 3). However, Deo and co-worker's study [74] may rule out a methodological bias as their MRP2 abundances were obtained using a kit extraction procedure similar to that used in the studies by Li and co-workers [101,102] to provide a native membrane for quantification that are similar to those for MRP2 abundances measured by Ohtsuki's group using plasma membrane [103] (Figure 3). As yet, no formal assessment has been published as to the impact of the membrane extraction method on transporter absolute abundance quantitation. A recent publication by Balogh *et al*. has also highlighted the importance of denaturing/digestion conditions and that optimization of these for each protein may be necessary to obtain more accurate transporter abundances for scaling factor development [109]. Furthermore, the purity of the synthetic peptide may introduce errors in endpoint abundance. There should be consideration for factors such as the labile nature of some peptide molecules, artifactual modifications affecting the peptide and whether the target peptide sequence is prone to post-translational modification or polymorphic sequence alterations [99,119]. In order to reduce the potential for endpoint abundance errors, the calibration of an unknown sample against a single synthetic peptide standard should be avoided, thus it is good practice to use at least two independent peptides for each transporter.

It is important to consider that when determining protein abundance that the assay measures protein that is resident in plasma/brush border membranes, rather than proteins that are associated with intracellular membranes [73]. Identification of plasma/brush border membrane-associated protein abundance and any potential transporter protein sequestrations that are redundant to function and not relevant for inclusion in PBPK models is critical, given that membrane extractions from kits have been shown to be contaminated with cytosolic fractions [120]. There should also be an awareness of additional loss of membrane-associated proteins with further purification of the preparation to obtain the plasma/brush border membrane, potentially leading to an altered quantification per mass of total membrane protein. The ultimate goal, therefore, is to establish the inherent abundance and biological variability with future work using matched samples across techniques that could inform scientists of the potential influence of methods on endpoint abundance measures [121].

From a tissue and cellular perspective, the heterogeneous nature of the intestine provides additional challenges to obtaining the intended membrane proteins for quantitation. The key transporters and metabolic enzymes reside within the enterocytes and are located in the mucosal layer; therefore homogenization of an intestinal tissue sample containing the entire tissue cross section is not appropriate for obtaining membrane protein preparations. Chelation techniques disrupting the enterocyte-basement membrane interactions are a common method to isolate enterocytes from the underlying mucosal tissue to provide a relatively uncontaminated sample for membrane extraction [122]. Alternatively, a mucosal scraping technique is applied to provide

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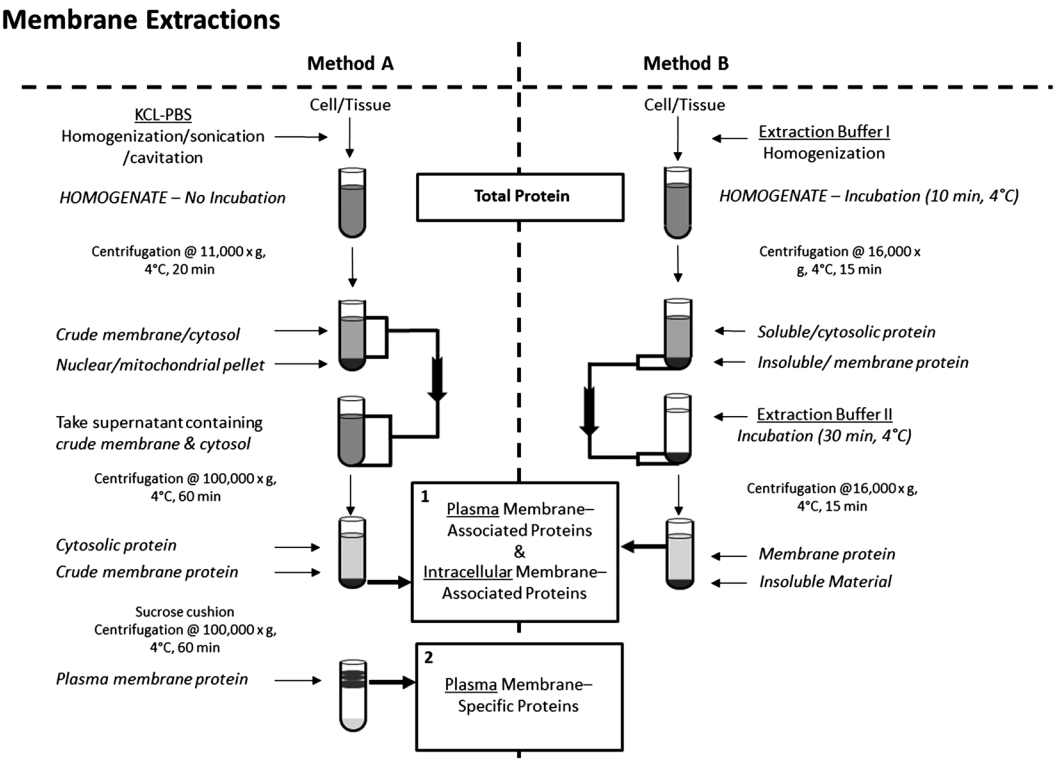

Figure 3. Schematic depiction of two methods to obtain membrane protein for subsequent transporter abundance quantitation. Method A describes a differential centrifugation technique and method B describes a kit-based technique to obtain membrane. For the mucosal tissue, ensure that the surface area of mucosa that is scraped/eluted is determined for tissue-based scaling factor derivation. Both methods A and B provide a crude membrane fraction Box 1, for protein abundance quantitation, however, if there are target proteins associated with intracellular membrane structures, analysis of this membrane fraction will measure these proteins in addition to plasma membrane-associated proteins. A sucrose density gradient procures a plasma membrane specific protein fraction. At each stage sampling should take place to account for protein loss to correct the final protein yield. To check for transporter protein sequestration, or transporters that are not associated with the plasma/brush border membranes, these non-plasma/brush border membrane fractions should also be quantified. Method A: Box 1, Transporter protein abundances in tissues and cells have been quantified using the crude membrane fraction from studies by Tucker *et al*. [73], Miliotis *et al*. [114]. Method B: Box 1, Transporter protein abundances in tissues and cells have been quantified using the crude membrane fraction from studies by Li *et al*. [101,102] Zhang *et al*. [44], Di *et al*. [45], Deo *et al*. [74] and Balogh *et al*. [109]. Method A: Box 2, Transporter protein abundances in tissues and cells have been quantified using the plasma membrane fraction from studies by Kamiie *et al*. [110], Niessen *et al*. [106], Ito *et al*. [111], Ohtsuki *et al*. [103], Uchida *et al*. [104], Sakamoto *et al*. [105] and Schaefer *et al*. [107]

a yield of enterocytes where histological examination could be used to confirm the acquisition of villi [123]. However, it is possible that other cell types potentially expressing the target proteins, i.e. red blood cells or leukocytes could contaminate the sample biasing abundance quantification. To provide assurance that abundance measures are founded on enterocytes and not contaminating cell types expressing the target proteins, marker proteins such as the leukocyte marker antibody or proteotypic peptides to CD45 [124] and glycophorin-A [125], could be incorporated into quantitative analyses to act as indicators of leukocyte and red blood cell contamination, respectively. Likewise, villin, an enterocyte-specific structural protein [126] that is routinely used within real time polymerase chain reaction (RT-PCR) mRNA assays used for enterocyte-specific normalization of target transcript [124], would be a valuable addition to quantitative proteomic assays.

There are potential procedural differences that should be addressed, such as techniques for obtaining enterocytes; mucosal scraping and chelation/elution. It has been shown that there are activity

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differences between numerous CYP450 enzymes in intestinal microsomes procured from mucosal scraping and chelation/elution methods [127], suggesting that it is plausible that there are protein yield differences in the two techniques. Additionally, catalytic assays should be employed using brush border marker enzymes, alkaline phosphatase or sucrase to confirm enterocyte membrane enrichment from the total tissue homogenate. Therefore, the intestine is not a straightforward tissue on which to work and at present there is only a single study, Tucker *et al*., that has quantified the absolute abundance of drug transporter proteins in the intestine [73].

Determining the abundance of transporters. Within the immunoquantification study by Tucker and co-workers [73], the authors measured MDR1, MRP2 and BCRP abundance in the duodenum after using a mucosal scraping technique to obtain their crude membrane preparation (Figure 3). The abundances of BCRP and MDR1 are similar, whereas MRP2 shows a 5-fold lower expression when comparing the data for all three ABC-transporters to each other. This highlights the disparity in using relative expression and absolute abundance for the duodenum, where MRP2 relative expression is greater than MDR1 and BCRP (Table 2) which contradicts the relationship between these transporters' absolute abundance within Tucker's study. Theoretically, if efflux activity correlates to mRNA and protein expression for all three ATP-dependent transporters, for a drug with the same $CL_{int,T}$ for MDR1, MRP2 and BCRP a drugs efflux from the duodenal enterocytes will be somewhat greater for MRP2 than MDR1 or BCRP if using a model incorporating the relative expression data from Table 2; using the alternative approach in which the model employs absolute abundance from Tucker *et al*. [73] the drug's efflux based on MRP2 abundance will be approximately 5-fold lower than that for MDR1 and BCRP. The use of both models is likely to lead to very different pharmacokinetic outcomes.

Due to the limited human intestinal absolute transporter abundances currently reported, REFs based on absolute abundance data can only be applied to the duodenum [73]. It would be preferable to expand the number of studies measuring *in vitro* and *in vivo* abundances before applying absolute abundance REFs. Nevertheless, a rudimentary examination of absolute abundance data from quantitative immunoblot or LC-MS/MS analysis can provide ATP-dependent efflux transporter REF's for cells forming intact monolayers; Caco-2, MDCK-II and LLC-PK_1 cells expressing transfected MDR1 to the human duodenum (Figure 4). The REF data demonstrate that the absolute abundance within *in vitro* systems is considerably higher than the abundance *in vivo*. There is also a considerable difference when comparing a REF derived from relative quantitation methods to the absolute quantitation methods, where *in vitro* expression from the relative methods is closer to the MDR1 expression *in vivo*. Comparison of MDR1 absolute abundance between Caco-2 and MDCK-II-MDR1 indicates that the abundance of MDR1 is greater in Caco-2

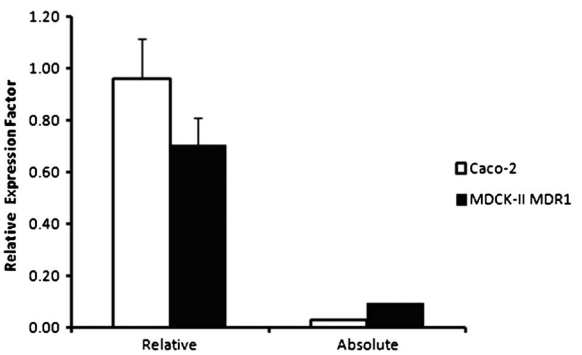

Figure 4. Relative expression factors (REF) (Equation 3) for MDR1 expression in the human duodenum relative to Caco-2 (white bars) and MDCK-II-MDR1 (black bars) expressing cells. The bars given as 'Relative' are derived from analysis of immunoblot optical densitometry from Troutman and Thakker [94] ($n = 1$, for human duodenum, $n = 3$ for both Caco-2 and MDCK-II-MDR1). Error bars represent ± standard deviation. The bars given as 'Absolute' are derived from analysis of human duodenal absolute abundance quantitation of MDR1 from quantitative immunoblot by Tucker *et al*. [73] ($n = 12$–14) relative to absolute abundances of MDR1 in Caco-2 by Miliotis *et al*. [114] (n = not stated), and the mean absolute abundances of MDR1 from two studies, Zhang *et al*. [44] ($n = 1$) and Di *ei al*. [45] (n = not stated). For all analysis relating to MDCK-II-MDR1 cells, correction for endogenous dog mdr1 expression/abundance in MDCK-II wild type cells was undertaken to provide expression/abundance of human MDR1. Additionally, an MDR1 REF from absolute quantitation of MDR1 in LLC-PK_1-MDR1 cells after quantitative immunoblot and LC-MS/MS analysis, Kamiie *et al*. ([110], $n = 4$–6) to the human duodenum, Tucker *et al*. ([73], $n = 14$) was determined with the mean REF of 0.0018 (not shown in the figure)

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compared with MDCK-II-MDR1. Two laboratories have independently shown, using relative immunoblot densitometry, the trend for greater levels of MDR1 in transfected MDCK-II cells compared with Caco-2 [94,128]. This also highlights the potential variability between different laboratories as well as in *in vitro* systems as described by Lee (on behalf of the 'P-gp IC_{50} working group') recently [129]. This further emphasizes the need for robust, absolute abundance quantification for each system used.

factor for transporters (ISEF-T) permits the correction for transporter driven flux or $CL_{int,T}$ per unit of transporter isoform in both the enterocyte and recombinant system. This enables the calculation of a transporter's catalytic rate (k_{CAT}) in both the enterocyte and recombinant system, advocating the combination of abundance and kinetic *in vitro* studies. At present the PBPK models utilizing relative expression data neglect the extrapolation of activity per unit of transporter.

$$CL_T ISEF\text{-}T_j = \frac{CL_{\text{int, }Tj}(Ent)/Transporter_j \;\; abundance \quad (Ent)}{CL_{\text{int, }T}(rhTransporter)_j/Transporter_j \;\; abundance \quad (rhTransporter)} \tag{4}$$

Further studies to establish absolute abundance in various intestinal regions as well as *in vitro* systems used to study transporter function are required to provide robust expression-based scaling factors. However, it is necessary to distinguish whether simple expression-based scaling factors are sufficient for success using an IVIVE approach or, if in addition to expression, accounting for the activity within the *in vitro* and *in vivo* system is also required as has been established for metabolizing enzymes.

Function versus expression in using scaling factors

Thus far, the review has addressed scaling factors based solely on transporter expression within membrane preparations. If parity to the ISEF

where $CL_T ISEF\text{-}T_J$ is the $CL_{int,T}$ inter-system extrapolation factor for transporters (unit-less) based on the derivation of the transporter isoforms (*j*) enterocytic intrinsic clearance $CL_{int,Tj}$ *(Ent)* (μl/min/cm^2) in, for example, an *ex vivo* intestinal mucosal preparation within an Ussing chamber system, the transporter isoform's enterocyte abundance (pmol transporter/cm^2 mucosal tissue), the recombinant transporter (*rh Transporter*) isoforms intrinsic clearance $CL_{int,Tj}$ in a bi-cameral filter system (μl/min/cm^2) and the *j*th transporter isoform's enterocyte abundance (pmol transporter/cm^2 filter grown cell monolayer).

The *ISEF-T* can then be incorporated into Equation (5) to calculate the unbound intrinsic clearance for a transporter isoform in any intestinal segment.

$$CLu_{GIS,\text{int}} = \left[\sum_{j=1}^{n}\left(ISEF - T_j \times \frac{J_{\max}(rhTransporter_j) \times Transporter\ Abundance_j(ent)}{K_m(rhTransporter_j)}\right)\right] \times MePPGIS \quad \times GISS.A \tag{5}$$

metabolic activity-expression scalar for transporters is to be achieved (Equation 4) further work is required to elucidate the amount of active transporter within the membrane, the 'functional transporter abundance'. An inter-system extrapolation

for each transporter isoform (*j*); rhTransporter indicates recombinantly expressed transporter; *MePPGIS* is the amount of membrane protein per gastrointestinal segment and *GIS S.A* is the gastrointestinal segments surface area.

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To elucidate functional protein abundance for certain transporters is testing, especially the MRP transporter protein family, due to substrates interacting with multiple transporters making it challenging to obtain kinetic data specific to a particular transporter isoform [40]. At present, the use of expression scaling factors assumes a direct relationship between the levels of protein and activity of the transporter. This may be true for ATP-dependent transporters but there are potentially confounding factors. One such factor is based on structure–activity relationships. It is possible that there is a requirement to consider that BCRP is structurally a half transporter comprising six membrane spanning domains and one nucleotide binding domain, whereas the transporters from the same superfamily as BCRP, MDR1 and MRP2, comprise two repeating halves of six membrane spanning domains and two nucleotide binding domains [130]. Evidence from expression systems suggests that BCRP functionally requires a homodimeric structure to confer activity [131]. Therefore, simply quantitating the membrane abundance of BCRP by techniques that cannot distinguish between monomeric and dimeric forms in the membrane, may not inform us of the relationship between expression and activity.

The composition of the lipid environment in which the protein is embedded has been shown to modulate its activity. Romsicki and Sharom hypothesized that the composition of the lipid bilayer plays a role in the partitioning of MDR1 substrates into the membrane and subsequent interaction with MDR1 [132]. Results from this study and the study by Meier and co-workers showed the intimate relationship between membrane phospholipids and the phospholipid composition of membrane and the binding affinity of verapamil and vinblastine to their primary binding site on MDR1 [132,133]. Furthermore, in MDR1 reconstituted proteoliposomes, the measurement of ATPase activity, by determination of the released inorganic phosphate as an indirect measure of MDR1 activity, demonstrated a difference in ATPase activity in response to changing verapamil concentration when the acyl chain composition within the lipid membrane is altered [132]. It was postulated that the lipid environment modulates the binding of verapamil to its high affinity binding site [132]. *In vivo* it is known that there is heterogeneity in microvillus membrane composition between various intestinal segments in rats where cholesterol, cholesterol/phospholipid ratio and the saturation of acyl chains are different when comparing proximal and distal regions [134]. In the future, studies to distinguish the effects of heterogeneity in membrane composition in various intestinal regions using *ex vivo* or *in situ* techniques in intestinal tissues could lead to the development of scaling factors to account for differences in intrinsic transporter activity in various regions of the intestine.

Due to the prevalence in the use of over-expressing systems to study xenobiotic transporter proteins, it is important to consider the effects of protein over-expression on transporter kinetics. It has been demonstrated that the kinetic activity ($CL_{int,T}$) of vinblastine in MDCK-II-MDR1 cells was approximately 2-fold lower compared with Caco-2 cells, yet there was a greater immunoblot determined expression and efflux ratio in MDCK-II-MDR1 cells [47]. In the same study, kinetic dissociation constants (K_i) are also different between Caco-2 and MDCK-II-MDR1 with generally lower inhibition potencies in MDCK-II-MDR1 for 10 MDR1 substrates, with the exception of GF 120918. For over-expressing systems of SLCs such as PEPT1 or OATs whose activity relies on counterions and their gradient across the membrane [18,135], the simple REF approach may also not be appropriate. Therefore, these complexities are likely to require attention if relevant mechanistic scaling factors are to be attained.

Currently there are sparse data linking abundance to function for human transport proteins. The efflux ratio of the MDR1 substrate digoxin was investigated with reference to MDR1 abundances in Caco-2 cells aged 10- and 29-days old grown on filters. MDR1 expression was 2.2-fold higher in 29-day-old cells compared with 10 day old cells with digoxin efflux ratio reciprocally increased [114]. A similar trend of protein abundance linking to activity was also observed within the same study for *N*-methylquinidine uptake into HEK293 inside-out membrane vesicles expressing MDR1 [114]. These data are in agreement with earlier reports for MDR1 [59,136]. In addition to the relatively simple expression–activity relationships demonstrated in these studies, there may be added complexities as shown for quinidine bi-directional transport in Caco-2 monolayers, from previously unpublished data (Figure 5). The figure describes the concentration-dependence of the

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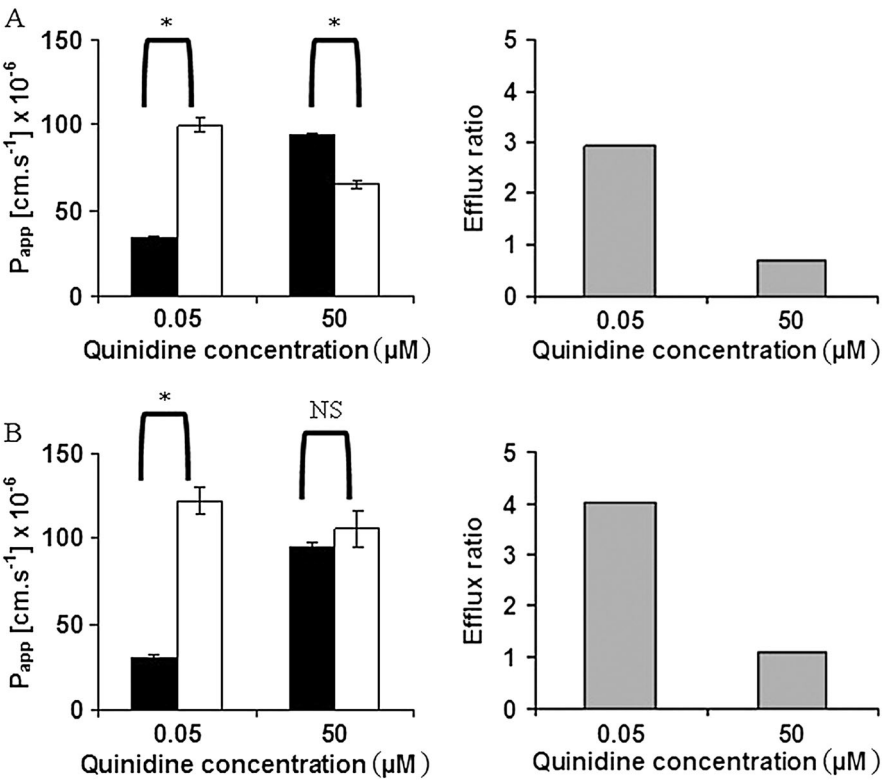

Figure 5. Bidirectional transport of quinidine across Caco-2 monolayers after (A) 16 and (B) 24 days in culture. The apparent permeability coefficient (P_{app}) in the apical-to-basolateral direction (black bars) increased and P_{app} in the basolateral-to-apical direction (white bars) decreased as the concentration increased from 0.05 to 50 μM. The buffer pH was always kept at 7.4. Each bar indicates mean ± SD ($n \geq 3$). NS, not significant. *$p < 0.01$ comparing P_{app} in A-to-B and B-to-A transport direction

bi-directional transport of quinidine in 16 and 24 day old grown Caco-2 monolayers as indicated by increasing efflux ratios obtained across the 24-day-old Caco-2 monolayers, when comparing experiments performed at the same initial donor concentration, which was in agreement with the higher MDR1 mRNA expression measured in these older cells. However, the lower permeability in the B-to-A compared with the A-to-B transport direction across the 16 day Caco-2 monolayers at the higher initial donor concentration may indicate the role of a non-proton gradient-dependent active apical influx transporter or basolateral efflux transporter involvement, which potentially counteracts the effect of MDR1 in these cells. Reports on the relationship between BCRP and MRP2 protein abundance and corresponding function are presently lacking. Work using a combination of *in vivo* knockout mouse studies and filter grown LLC-PK_1 cell monolayers transfected with mouse mdr1a, identified, that for compounds that are transported solely via mdr1a, integrating efflux function (in the form of *in vitro* mdr1a efflux ratio) together with a REF value, to correct for absolute expression difference between the mouse blood capillaries and the *in vitro* system, provides reasonably good estimates for *in vivo* brain: plasma ratios [116].

To obtain kinetic data in human intestinal *ex vivo* tissues is a considerably greater challenge than in *in vitro* systems as there are often limited supplies of macroscopically normal fresh human intestinal tissue to quantify abundance and substrate kinetics. Ussing chamber studies are undertaken to elucidate drug transport and to assess regional differences in human functional expression [137,138]. Bi-directional permeability experiments in Ussing chambers are routinely performed with $n = 2$ to 3

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samples from a single donor where a subsequent efflux ratio can be determined. In conjunction with permeability assays, the measurement of transporter abundance could identify if correlations exist between abundance and efflux ratio. A limitation of this procedure is that performing kinetic experiments is demanding as sufficient tissue is required to carry out multiple time and concentration sampling bi-directionally, ideally in duplicate or triplicate similar to *in vitro* assays [51]. This requires a large number of Ussing chambers, mucosal tissue and technical assistance. However, kinetic determinations are achievable [42].

Human intestinal membrane vesicle preparations provide a tool to define substrate kinetics, where the relationship between abundance and activity can be characterized [123,139]. The orientation of vesicles requires consideration depending on the transporter under study. The binding sites of ATP-dependent efflux transporters are thought to be located on the cytoplasmic inner membrane leaflet [52,140]. To expose these binding sites to the external substrate containing buffer, inside-out vesicles are required, and hence it is necessary to determine the proportion of the vesicles that are inside-out versus right-side-out [141], since it is expected that there is a mix of vesicles orientations, which should be corrected when comparing uptake activity and transporter abundance. The substrate binds to an externally exposed binding site(s) for transportation into the vesicle and subsequent quantitation of uptake. A complication leading to erroneous kinetic conclusions is the risk of violating the system's 'sink conditions' due to the relatively small intravesicular volume leading to passive flux out of the vesicle and equilibration taking place, therefore short sampling time points and substrates with low (CL_{PD}) are more appropriate. Finally, the effects of buffer-osmolality on drug flux within *in vitro* systems including cell monolayers grown on filters and vesicle assays require consideration in order to limit their potential effects on flux, which may confound the delineation of the 'intrinsic' active and passive processes acting on the compound [142,143].

Further investigations on absolute abundances and the relation of abundance to function, i.e. k_{CAT} determinations, are required to develop robust scaling factors to enhance predictive outcome, while the impact of perturbations on abundances and activity within a system could also be elucidated as has recently been demonstrated for CYP450 enzymes [105] and is pertinent to modelling of *in vitro* transporter data. Determining the absolute abundance of the transporters driving active processes within the *in vitro* system will allow determination of the k_{CAT} in a similar manner to that performed for *in vitro* metabolism assays, thus permitting mechanistic scaling via the generation of functionally relevant scaling factors for transporters for specific *in vitro* and *in vivo* systems. It will also be important to identify covariate relationships between transporters and their functional synergy, together with the frequency in which they occur in a population to allow those individual's to be represented accurately within population-based modelling and simulation.

The PBPK intestinal transporter studies published to date have neither applied an absolute abundance scalar (the concentration of the transporter normalized to the mass of total protein within the membrane) nor ISEF-T, which would account for absolute abundance as well as activity. Therefore, the relationship between the abundance of different transporter isoforms in a tissue is not distinguishable in absolute terms. From a functional perspective, this can lead to, at best, a semi-mechanistic IVIVE-PBPK framework for gauging the impact of transporters on drug disposition. It is anticipated in the future that development of models based on absolute abundance and the relationship between abundance and function at a particular intestinal site and for particular *in vitro* systems by developing ISEFs-T, will lead to a more mechanistic approach to transporter modelling using the IVIVE approach [16,72]. Incorporation of a membrane protein scalar together with the absolute transporter abundances and their variability permits the definition of transporter protein abundance to whole segments of intestine with relevance to PBPK models (Figure 2). The inter-individual variability in abundance is derived from the variability in the membrane protein scalar, the differences in each individual's anatomical intestinal surface area and the inter-individual variability in the transporter protein absolute abundance in a given intestinal segment, a schematic for this population approach is provided in Figure 6.

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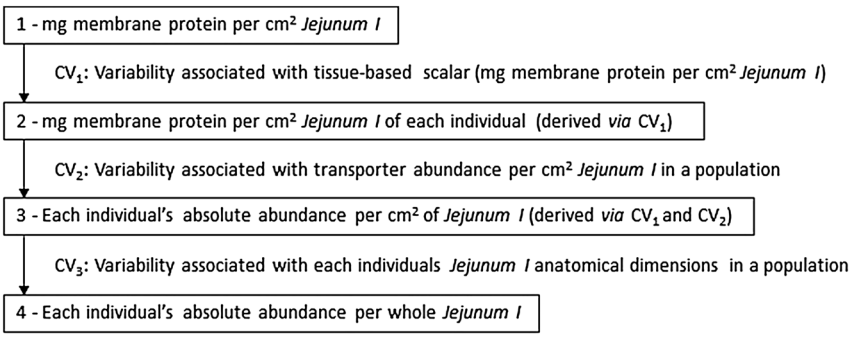

Figure 6. A schematic providing an overview of the scaling process and derivation of inter-individual variability for the absolute abundance of the intestinal transporters in the Jejunum I segment

Future Perspectives and Conclusion

With the enhancing role of mechanistic IVIVE PBPK models to predict pharmacokinetic outcomes, including a role of transporters in drug development and regulatory science, there is also a clear demand and requirement to further develop and populate these models with high quality physiological data necessary accurately to describe the *in vivo* system (Caucasian, Japanese, renal impaired, cirrhosis, healthy volunteer, paediatric, geriatric etc.), and to characterize the *in vitro* systems employed to describe the mechanisms influencing drug disposition. There is a coordinated effort within the pharmaceutical industry and in academia to enhance this knowledge utilizing methodologies to determine absolute protein abundances and to translate these absolute abundance data to relevant activity data in biological samples. However, there are significantly more data required in human intestine, liver, kidney, lung and the blood–brain barrier using quantitative techniques to enable robust functionally relevant IVIVE scaling for transporters. *In vitro* cell systems require characterization with regard to their absolute transporter abundances, which will enable definition of expression-based scaling factors to bridge the gap between the *in vitro* and *in vivo* systems. The abundance of transporters in relation to mRNA gene expression and function, using kinetic analysis, is anticipated. Further validations of the different techniques employed and the endpoint abundances are required by comparison of matching samples to assist in elucidating any methodological bias. To accompany abundance–activity data, tissue-based transporter scaling factors are critical to permit scaling to whole tissues or regions of tissue using well established biochemical techniques. It is imperative that any useful scalars are not compound-specific but rather system-specific and that the inclusion of drug transporter data in models is not biased by the systems in which they were derived. Therefore, the use of *in vitro* models that harness *in vitro* drug concentration–time course data can simulate drug concentrations in each compartment and lead to a greater accuracy in kinetic estimations. PBPK modelling in conjunction with mechanistic absorption models and reliable *in vitro* data and system-specific scalars on metabolizing enzymes and transporters are useful tools to elucidate the complex relationship and relative importance of these metabolizing enzymes and transporters in drug disposition and toxicity.

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Conflict of Interest

MD Harwood, S. Neuhoff and A. Rostami-Hodjegan are employees of, and or shareholders in Simcyp Ltd. The Simcyp® simulator is freely available,

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following completion of the training workshop, to approved members of academic institutions and other non-for profit organizations for research or teaching purposes.

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