In Vitro/In Vivo Correlation for Transporters

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Contents

Abstract

Membrane transporters play one of the key roles in drug disposition and drug-drug interactions. Therefore, one of the prerequisites in the development of new drugs and dosage forms is determination of potential transporter-mediated processes a drug may undergo in the organism. The importance of this subject has been recognized by the regulatory authorities who have issued relevant guidelines on the in vitro and clinical investigations of potential drug-transporter interactions. In vitro transporter studies are designed to determine

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whether a drug is a substrate/inhibitor/inducer of the clinically relevant transporters and to describe the interaction in terms of kinetic parameters, with the final goal to provide information on the clinical outcomes of the observed interactions with transporters. A number of in vitro methodologies have been designed to provide information on drug-transporter interplay, and the most prominent ones will be described in this chapter. Approaches to extrapolate in vitro to in vivo data will also be addressed. However, it should be stressed that knowledge on transporters and transporter-mediated processes is constantly growing, and, as new technologies are emerging, transporter science is making its headway toward the application of advanced in vitro-in vivo-in silico assessment tools. Although being routinely performed for years now, investigation of drug-transporter interactions and in vitro-to-in vivo translational modeling are still evolving, and breakthrough may happen in the following years.

Abbreviations

BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
CAR	Constitutive androstane receptor
CHO	Chinese hamster ovary cell
CYP	Cytochrome P450
HEK293	Human embryonic kidney 293 cells
LLC-PK1	Lewis-lung cancer porcine kidney 1 cells
MATE	Multidrug and toxin extrusion protein
MCT1	Monocarboxylate transporter 1
MDCK	Madin-Darby canine kidney cells
MDR1	Multidrug resistance protein 1
MRP	Multidrug resistance-associated protein
OAT1	Organic anion transporter 1
OATP	Organic-anion-transporting polypeptide
OCT	Organic cation transporter
PEPT1	Peptide transporter 1
P-gp	P-glycoprotein
PXR	Pregnane X receptor

Introduction

Drug bioperformance is a complex phenomenon ruled by a number of factors and processes, including drug interference with membrane transporters. These specific proteins mediate drug transport through physiological barriers, including plasma membranes and membranes of intracellular structures, and therefore influence all ADME properties (absorption, distribution, metabolism, and elimination) of the drug. Consequently, they may impact therapeutic and adverse drug effects. Moreover, due to the intrinsic properties of transporters such as specificity and limited capacity, they play an important role in drug-drug interactions (DDIs) and drug-nutrients interactions. Thereof, it is important to establish reliable means to estimate all these effects.

There are different ways to classify membrane transporters. Based on the direction of the transmembrane transport, they are divided into influx (facilitate entry of a molecule into the cell) and efflux (carry a molecule out of the cytosol) transporters. In addition, transporter-facilitated movement of molecules can be classified as uniport (one-way direction of one molecular entity), symport (the same one-way direction of two different molecular entities), or antiport (transport of different molecular entities in different directions), although some transporters act in bidirectional way. Regarding the mechanism of transport, transporters are differentiated as passive or active. The first group functions in energy-independent manner and carry a substrate molecule down the concentration gradient, whereas the later one consumes energy and enables movement of a substrate against the concentration gradient. This last group comprises ATP-binding cassette (ABC) transporter family, which consists of primary-active transporters (since they use energy directly, commonly from ATP hydrolysis). These are mostly efflux transporters that drive translocation of drugs and endogenous substances from the cell and are therefore predominantly localized on the basolateral membrane, e.g., intestinal epithelium, hepatocytes, renal epithelia, and blood-tissue barrier. On the other hand, secondary-active transporters use ion gradient across the membrane

to pair it with the transport of a substrate molecule. Together with passive transporters, secondary-active transporters form the second large, solute carrier (SLC) transporter family. SLC transporters predominantly modulate influx of nutrients and drugs into the cell. According to the current knowledge, there are more than 400 transporters in these two superfamilies, but not all of them are clinically relevant in terms of affecting drugs ADME properties. To read more about clinically relevant ABC and SLC transporters, refer to the chapter ▶ "Relevance of Transporters in Clinical Studies" by Hagenbuch.

Transporters are ubiquitously distributed in the body, but their absolute abundance across organs and tissues is still puzzling. In recent years, advanced technologies such as mass spectrometry-based techniques and quantitative immunoblotting assays enabled localization and quantitative determination of major transporters. Also, with the progress in pharmacogenomics, understanding of the genes encoding membrane proteins has increased significantly.

An intrinsic property of transporters is their specificity for certain molecular entities. Some transporters have broad substrate and inhibitor specificities, while the others are more specific and interact only with a limited number of molecules with more or less similar structure. For this reason, some transporters lack specific probe substrates and inhibitors for in vitro and in vivo studies. Moreover, drug transport across biological membranes depends upon a variety of factors (e.g., it can be energy-dependent, concentration-dependent, pH-dependent, plasma membrane potential-dependent), requiring careful selection of in vitro setups for determination of transporter substrates and inhibitors.

In 2007, the International Transporter Consortium (ITC) was formed to coordinate and promote activities toward resolving the transporter-related issues in drug development. Alongside, regulatory agencies in the USA, EU, and Japan have issued guidelines on drug interaction studies, including transporter-mediated drug interactions. According to the regulations, transporter interaction studies are required for all new molecular entities (NME), but the choice of assays and transporters to be tested depend upon drug physicochemical and pharmacokinetic (PK) properties. The goals of these guidelines are to (i) define clinically relevant transporters that can impact drug efficacy and safety, (ii) establish standards for in vitro evaluation of transporter-mediated drug interactions, (iii) provide decision trees with "cutoff" values to determine whether it is necessary to conduct clinical transporter interaction studies, and (iv) establish standards for in vivo evaluation of transporter-mediated drug interactions.

From a number of transporters, only some of them need to be evaluated in the prospective in vitro transporter substrate and/or inhibition studies (Table 1). Regulatory opinions regarding major transporters are not fully harmonized, but an all-inclusive list for the European Medicines Agency (EMA), the US Food and Drug Administration (FDA), and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) currently comprises nine transporters: MDR1 (P-gp), BCRP, OCT2, OAT1, OAT3, OATP1B1, OATP1B3, MATE1, and MATE2K, while OCT1, BSEP, and MRP2 are also considered as important. It should be noted that this is an evolving list susceptive to changes in line with the progress in transporter science. In vitro assaying of other transporters may also be necessary if there is evidence that a drug might interact with certain transporter(s). Examples of membrane transporters of emerging importance include OATP2B1, OAT2, OAT4, OCT3, and MRP4.

Results of transporter interaction studies can be useful in different phases of drug discovery. In vitro assessment of potential drug interactions with transporters is important to guide and assure safety during concomitant clinical studies. In vivo studies serve to prove the assumed interactions and provide information on drug treatment and DDIs for labeling. DDI issues are addressed in chapters ▶ "Drug-Drug Interaction Studies" by Stopfer and ▶ "In Vitro/In Vivo Correlation for Drug-Drug Interaction" by Wahlstrom and Wienkers. Also, if the new information on potential drug-transporter interaction arises during the life cycle of a product, additional transporter studies are recommended.

Table 1 Clinically relevant transporters, their localization, and examples of in vitro probe substrates and inhibitors (bold, regulatory recommended; italic, for consideration)

Transporter/ encoding gene	Transport direction	Localization	In vitro substrates	In vitro inhibitors
MDR1 (P-gp)/ ABCB1	Efflux	Enterocytes (luminal membrane) Hepatocytes (canalicular membrane) Renal proximal tubules (luminal membrane) Brain capillary endothelia (luminal membrane) Other tissues (placenta, testes, cornea)	Digoxin *N*-Methyl-quinidine Talinolol	Cyclosporin A GF120918 PSC833 Ketoconazole Verapamil
BCRP/ ABCG2	Efflux	Enterocytes (luminal membrane) Hepatocytes (canalicular membrane) Brain capillary endothelia (luminal membrane) Other tissues (testes, placenta, mammary glands)	Estrone-3-sulfate Prazosin Sulfasalazine	GF120918 Fumitremorgin C Ko134 Ko143
BSEP/ ABCB111	Efflux	Hepatocytes (canalicular membrane) Other tissues (kidney, testes, choroid plexus)	Taurocholate Glycocholate	Rifampicin Cyclosporin A
MRP2/ ABCC2	Efflux	Hepatocytes (canalicular membrane) Enterocytes (luminal membrane) Renal proximal tubules (luminal membrane) Other tissues (placenta, gallbladder, bronchi)	Estradiol-17b-glucuronide Carboxy-dichlorofluorescein Vinblastine	MK-571 PSC-833 Probenecid Cyclosporin A
MATE1/ SLC47A1	Efflux	Renal proximal and distal tubules (luminal membrane) Hepatocytes (canalicular membrane) Other tissues (skeletal muscle, adrenal gland, testes, heart)	Tetraethylammonium (TEA) 1-Methyl-4-phenylpyridinium Metformin	Cimetidine Quinidine Pyrimethamine
MATE2-K/ SLC47A2	Efflux	Renal proximal tubules (luminal membrane)	Tetraethylammonium (TEA) 1-Methyl-4-phenylpyridinium Metformin	Cimetidine Quinidine Pyrimethamine
OATP1B1/ SLCO1B1	Uptake	Hepatocytes (sinusoidal membrane)	Bromosulfophthalein Estrone-3-sulfate Estradiol-17β-glucuronide Pitavastatin Rosuvastatin	Rifampin Cyclosporine A Rifamycin SV Bromosulfophthalein Estropipate
OATP1B3/ SLCO1B3	Uptake	Hepatocytes (sinusoidal membrane) Other tissues (prostate, colon)	Bromosulfophthalein Estradiol-17β-glucuronide Cholecystokinin octapeptide (CCK-8)	Rifampin Cyclosporine A Rifamycin SV Bromosulfophthalein Ursolic acid
OAT1/ SLC22A6	Uptake	Renal proximal tubules (basolateral membrane) Other tissues (placenta)	Para-aminohippurate (PAH) Cidofovir	Probenecid
OAT3/ SLC22A8	Uptake	Renal proximal tubules (basolateral membrane)	Estrone-3-sulfate Cimetidine	Probenecid
OCT1/ SLC22A1	Uptake	Hepatocytes (sinusoidal membrane) Enterocytes (basolateral membrane) Renal proximal tubules (luminal membrane) Other tissues (neurons, heart, skeletal muscle, lung)	1-Methyl-4-phenylpyridinium Tetraethylammonium (TEA) Metformin	Quinidine Quinine Verapamil

(*continued*)

Table 1 (continued)

Transporter/ encoding gene	Transport direction	Localization	In vitro substrates	In vitro inhibitors
OCT2/ SLC22A2	Uptake (primary)	Renal proximal tubules (basolateral membrane) Other tissues (neurons, small intestine, trachea, bronchi, skin, placenta, brain, inner ear)	Tetraethylammonium (TEA) Metformin	Cimetidine Quinidine

This chapter is aimed to provide an overview of the best practices for the in vitro characterization of drug interactions with major clinically relevant transporters and the approaches to link in vitro findings to the expected clinical outcomes. The discussion is focused on the interaction of drugs with transporters involved in the intestinal drug absorption, as well as hepatic and renal elimination, since these transporters received notable attention in recent years. However, the importance of transporters governing drug disposition in other tissues (e.g., brain penetration, pulmonary absorption) should not be disregarded. Interactions with transporters in these tissues might have been neglected in the past, but it is expected that future research will shed more light on this matter.

Disposition-Based Classification Systems to Elucidate Drug-Transporter Interactions

The potential of a drug to interact with specific transporter, in a way that can limit drug absorption and disposition, can roughly be predicted based on the drug physicochemical properties, administration route, elimination route, and knowledge on the types and function of transporters. These basic considerations have been implemented in the concept of classification systems that address the key factors governing drug disposition in humans. Several disposition-based systems have been proposed so far (Fig. 1), based on somewhat different premises, targeting different objectives, and applicable in different phases of drug discovery and development. A major benefit of these systems is that they can facilitate the decision on whether to conduct in vitro and in vivo transporter studies and aid to identify target transporter(s) that should be tested for possible interaction with the investigational drug. It is important to note that these systems are not interchangeable; instead, they should be considered as supplementary.

The Biopharmaceutical Drug Disposition and Classification System (BDDCS) (Wu and Benet 2005) was constructed on the basis of the Biopharmaceutics Classification System (BCS), but in contrast to BCS which considers the key factors limiting oral drug absorption (solubility and permeability), BDDCS is focused on the parameters reflecting drug disposition (solubility and extent of metabolism). In terms of metabolism, drugs are classified as either extensively (>70%) or poorly (<30%) metabolized, since examples of intermediate (30–70%) drug metabolism are generally rare.

In view of this system, disposition of BDDCS class 1 drugs (highly soluble, extensively metabolized) is not affected by transporters, and there is no need to test them as substrates in vitro. However, their interaction (induction or inhibition) with transporters may be relevant in transporter-mediated DDI with victim drugs from other classes. BDDCS class 2 drugs (poorly soluble, extensively metabolized) are not affected by uptake transporters on the luminal intestinal membrane, but the effect of efflux transporters might be significant (due to limited solubility of these drugs, intracellular drug concentration might not be high enough to saturate efflux transporters). Consequently, this class is also prone to the effect of uptake and efflux transporters in the liver and

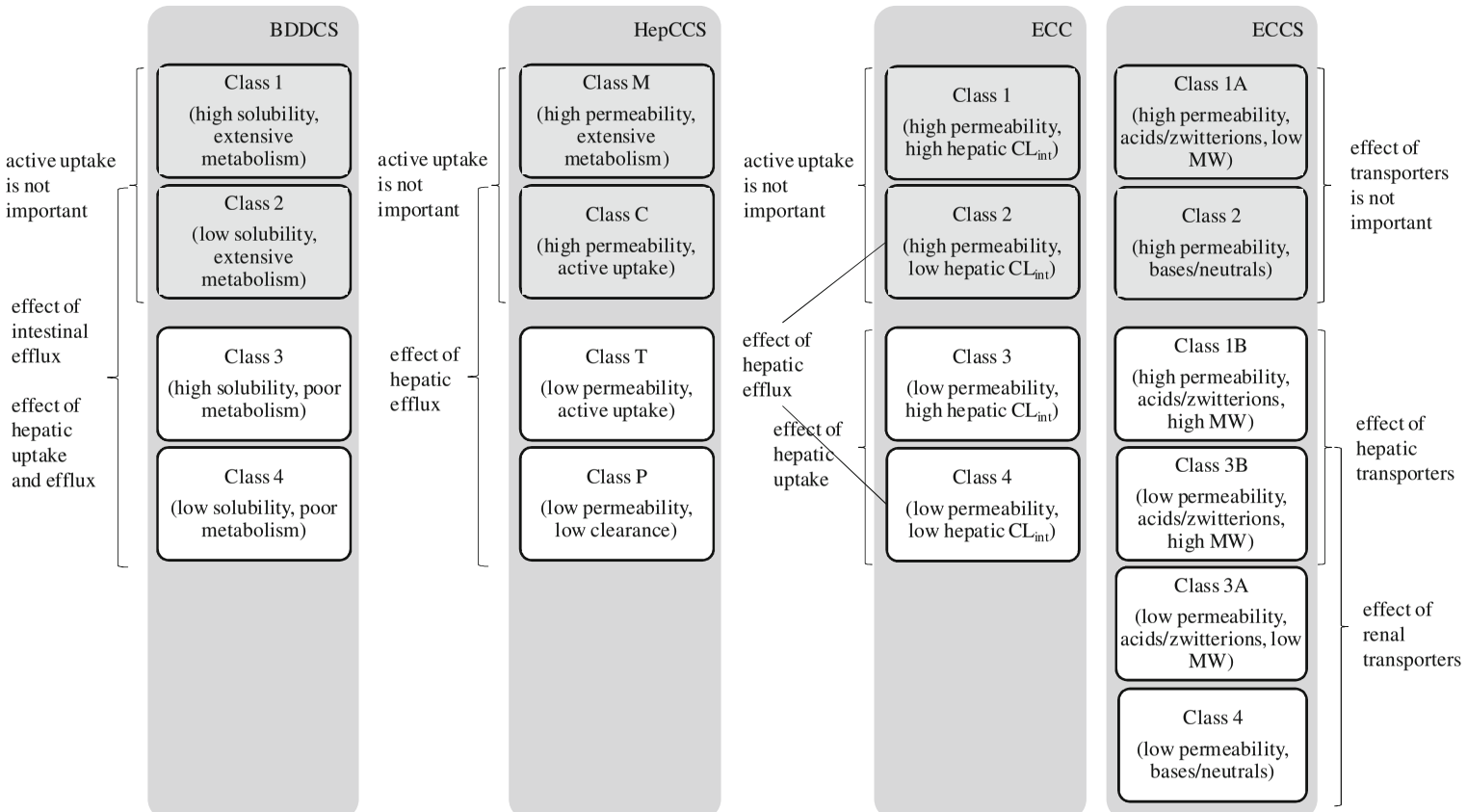

Fig. 1 The role of transporters in the proposed drug disposition and clearance classification systems

brain. Finally, disposition of BDDCS classes 3 and 4 drugs (poorly metabolized) may be affected by interaction with uptake and efflux transporters in different organs, and these drugs are candidates for transporter interaction studies.

A limitation to classify a drug within BDDCS system in the early stage of drug development is that experimental data on the extent of metabolism are not available before phase I clinical trials. An alternative approach is to use in vitro permeability data (e.g., using Caco-2 or MDCK cells) as an indicator of the extent of drug metabolism. It should also be noted that certain unexplained outliers within the proposed BDDCS drug classes have been identified, with class I statins being the most prominent ones.

The hepatic clearance classification system (HepCCS) (Fan et al. 2014) resulted from the efforts to estimate the contribution of hepatic clearance in an early drug discovery phase. The basic idea of this system is that hepatic drug clearance is influenced by a complex interplay of drug passive permeability, active uptake into the hepatocytes, and rate of metabolism and that gradual assessment of the influence of these factors might indicate the rate-determining step in hepatic drug clearance. The suggested classification criteria included passive permeability determined in MDCK cells (with a cutoff value of 5×10^{-6} cm/s) and liability to active uptake transporters (assessed using cryopreserved rat hepatocytes).

According to this system assumption, class M (highly permeable, cleared by hepatic metabolism) and class P (poorly permeable, low clearance) compounds are not prone to interfere with transporters. But, poorly permeable class T compounds interact with hepatic uptake transporters, and suitable in vitro or animal studies should be incorporated in the development pipeline of these drugs. Class T drugs can also be substrates for efflux transporters; however, efflux transport has not received much attention within HepCCS. In addition, it is postulated that, if the drug efflux is negligible, class T parent drug or metabolite might accumulate in hepatocytes, and routine measurements of drug plasma concentration might underestimate actual drug concentration relevant for the assessment of DDI. Class C is a distinct class comprising of highly permeable drugs, but substrates for uptake transporters. Hepatic clearance of class C drugs is not expected to be influenced by the activity of transporters because these drugs perfuse freely across the membranes.

HepCCS classification data can be quite useful because they imply suitability of an in vitro system to predict human in vivo clearance and potential drug-transporter interaction. But the focus of this system is on hepatic drug clearance, while other major clearance mechanisms, such as renal secretion, are not considered.

The extended clearance concept (ECC) (Camenisch et al. 2015) is similar to HepCCS in a way that it takes into account the interplay between drug permeability, (hepatic) metabolism, and biliary excretion and classifies drugs into four groups depending on the rate-determining step for hepatic elimination (passive diffusion, active uptake, or biliary efflux). However, there are differences between HepCCS and ECC, e.g., concerning classification criteria and experimental methods to determine clearance parameters. In particular, ECC classifies drugs based on in vitro determined permeability and metabolism data. In terms of permeability, a drug is classified as highly permeable when passive permeability across sinusoidal hepatocyte membrane ($PS_{inf,passive}$) is much higher than hepatic blood flow ($PS_{inf,passive} \geq 3$-fold Q_h). The extent of metabolism is expressed in terms of hepatic intrinsic clearance, which represents the sum of metabolic clearance and biliary secretion clearance. All of the key classification parameters can be determined experimentally in early phases of drug development.

Similar to HepCCS presumptions, the overall hepatic clearance of highly permeable ECC classes 1 and 2 is not considered to be affected by uptake transporters. But in contrast to HepCCS, highly permeable drugs prone to active uptake have not been classified as a separate group. ECC class 2 drugs have low hepatic clearance, and they can be secreted in bile as unchanged drugs. Therefore, the effect of efflux transporters should be considered. Low permeability ECC classes 3 and 4 are susceptive to the effect of uptake transporters, and these are generally

candidate drugs for in vitro and in vivo uptake transporter interaction studies. In addition, ECC class 4 drugs are potential substrates for efflux transporters, and these interactions should also be investigated.

An inherent drawback of ECC is that it is focused on the parameters describing hepatic drug disposition, and thus the system is informative only of the role of hepatic transporters. The contribution of other (in particular, renal) routes of elimination has also been implied, e.g., for low permeability ECC classes 3 and 4 drugs. However, the role of renal transporters has not been addressed here.

Extended clearance classification system (ECCS) is another framework aimed to identify the predominant drug clearance mechanism (El-Kattan and Varma 2018). It considers several pharmacokinetic processes: active hepatic uptake (predominantly by OATP transporters), hepatobiliary transport (mediated by MRP2, BCRP, and P-gp), hepatic drug metabolism, and renal clearance (mediated by OCT2, OAT1, OAT2, and OAT3). Hepatobiliary efflux is not considered as a rate-limiting step in drug elimination since drug exposure to efflux transporters depends upon the rate of active uptake. Comprehensive analyses of large database of drugs with diverse physicochemical and pharmacokinetic properties implied that predominant clearance pathway of a drug depends upon drug molecular weight (MW), ionization state, and passive permeability. The following cutoff values were proposed for classification: MW of 400 Da, membrane permeability of 5×10^{-6} cm/s determined using MDCK cells, and drug ionization estimated based on pKa value.

According to ECCS framework, acids and zwitterions with high MW (classes 1B and 3B), regardless of permeability, are recognized as potential substrates for hepatic uptake transporters (OATPs), and these drugs should be subjected to appropriate in vitro, and if necessary, clinical transporter interaction studies. According to the ECCS premises, substrates for hepatic uptake transporters also interact with hepatobiliary transport pathway (mediated by MRPs, BCRP, and P-gp), indicating that substrate affinity for these efflux transporters should be assessed in vitro. The major ECCS-envisaged difference between class 1B (highly permeable) and class 3B (poorly permeable) is that the first class refers to drugs that are principally eliminated as metabolites via hepatic route (due to the extensive liver metabolism), while the latter one comprises drugs eliminated unchanged via hepatic (OATP-mediated) and/or renal (OAT1, OAT2, OAT3-mediated) route. Whether a class 3B drug will be cleared predominantly through the bile or urine depends upon the substrate selectivity for OATPs vs. OATs. Therefore, class 3B drugs should also be tested for potential interactions with renal transporters. Two other ECCS classes of low permeability drugs (classes 3A and 4) are typically eliminated via the renal route, and their clearance is affected by renal transporters including uptake transporters on the basolateral membrane (OATs for class 3A and class 4 drugs and OCT2 for class 4 drugs) and efflux transporters on the apical membrane (MATE1/2K and P-gp for class 4 and potentially BCRP, MRP2, and P-gp for class 3A). Therefore, appropriate studies should be conducted to describe ECCS class 3A and 4 drug interactions with uptake and efflux renal transporters. In addition, oral pharmacokinetics of ECCS low permeable drugs (classes 3A/3B/4) is likely influenced by intestinal uptake (OATP2B1, PEPT1, MCT1, etc.) and efflux (P-gp, BCRP, MRP2) transporters, and relevance of these interactions for drug PK and possible DDIs should also be assessed. Highly permeable ECCS classes 1A and 2 are not prone to the effect of transporters, and they are mostly cleared by metabolism.

In Vitro Transporter Studies

In order to assess whether an investigational drug interacts with transporter (as substrate, inhibitor or inducer), the first step is to conduct relevant in vitro studies. Decision trees on whether and when to conduct in vitro and in vivo transporter studies are provided in the relevant guidelines issued by FDA, EMA, and PMDA (EMA 2012; PMDA 2014; FDA 2017).

Several aspects need to be considered when designing in vitro drug-transporter interaction studies. Firstly, the design of the study depends on the investigational objective, e.g., whether the objective is to identify the transporter(s) interacting with the drug, to estimate kinetic parameters that describe drug-transporter interaction, or to provide information to guide concomitant in vivo interaction studies. Also, study design depends upon the type of interaction to be investigated (e.g., transporter-mediated drug uptake or efflux, drug-mediated inhibition or induction of transporter, DDI study, complex transporter-enzyme interaction studies). For each type of study, appropriate in vitro system and experimental procedure need to be defined.

This section provides an overview of the standard tests, experimental conditions, protocols, and probe substrates/inhibitors used for the in vitro assessment of transporter-mediated drug interactions, along with the basic descriptions regarding data analysis and interpretation.

In Vitro Systems

A drug-transporter interaction can be assessed in vitro using various systems, e.g., different cell-based systems, membrane vesicles or artificial systems, depending on the type and objective of the study, and characteristics of the investigational drug (e.g., high or low permeability). When possible, the expression and activity of the transporter in the chosen system should closely resemble the conditions in vivo. Expression of transporters can be confirmed by quantitative polymerase chain reaction (qPCR), immunochemistry, or functional assay. The selected test system and experimental procedure should be validated and well characterized (e.g., source of the cell lines, cell culture conditions, incubation time, buffer pH, concentration of probe substrate/inhibitor, sampling intervals, method for data analysis, cutoff values). The activity of transporters needs to be verified using positive and negative controls with known probe substrates, and the obtained results should comply with the values from literature.

Commonly used cell-based systems include Caco-2 cells and transfected cell lines such as MDCK, LLC-PK1, CHO, and HEK293. One of the comprehensive reviews on the cell-based in vitro models to predict drug permeability, including transporter-mediated translocation, is provided by Sarmento et al. (2012). Also, some of the most prominent in vitro test systems are addressed in the review of Brouwer et al. (2013).

Caco-2 cells, derived from human colon adenocarcinoma cells, morphologically and functionally resemble intestinal epithelial cells. They express most of the key human intestinal transporters and are generally used to assess overall (passive and active) intestinal permeability of a drug. However, the expression of transporters highly depends upon the origin of cells, number of passage, culture conditions, and seeding density. For transporter studies, the cells are seeded on a semi-porous filter as a monolayer with a density of 2.5×10^5 cells/cm^2 and cultivated in normal serum-containing cell culture medium, e.g., Dulbecco's modified eagle's medium. The cultivation is relatively long (usually 20–30 days), which is one of the limitations of this in vitro system. Viability of the cells needs to be confirmed using, e.g., Trypan blue. Also, integrity of the monolayer needs to be verified by measuring transepithelial electrical resistance (TEER) or paracellular flux of a low permeability compound (e.g., phenol red, ^{14}C-mannitol). Prior to the transport experiments, the cells are washed with transport medium and incubated at 37 °C for 30 min. Afterward, a drug solution is added to one side of the cells – basolateral (B) or apical (A), and the samples are taken from the other side in predetermined time intervals. The amount of drug can be quantified using various methods such as high-performance liquid chromatography (HPLC) or liquid scintillation spectrometry. A major disadvantage of using Caco-2 cells for transporter experiments is high interlaboratory variability in the obtained results. This is mainly caused by variations in the permeability and expression of transporters. Therefore, each laboratory is responsible to check permeability of the cells using proper controls (e.g., propranolol, inulin) and determine the expression of transporters.

MDCK cells are often used to study drug permeability, but the activity of membrane transporters in parental cells is relatively low. The cells are usually seeded in well plates at the concentration of 2.5×10^5 cells/ml, in culture with a suitable medium, e.g., Earle's balanced salt solution supplemented with 10% fetal bovine serum, at 37 °C, in the atmosphere with 95% humidity and 5% CO_2. In general, testing procedure is similar to the one applied for Caco-2 cells, except that MDCK cells require only 3 days of growing before they are ready for permeability experiments.

Transfected (recombinant, overexpressing) cells are suitable to test interaction with a single transporter, although a suitable cell line can also be transfected to host more than one transporter. The choice of the cell line depends upon the type of the assay, e.g., HEK293 and CHO for the expression of uptake transporters or MDCK and LLC-PK1 for efflux transporters. These cells usually require shorter cultivation time (e.g., 3 days for MDCK transfectant cells), while the experimental procedure depends upon the type of study (substrate or inhibition study). If the compound of interest is a potential substrate, incubation usually lasts less than 10 min, and the experiments need to be conducted in vector-free (parental, wild-type) cells as control. The difference in the efflux ratio between the transfected and parental cells indicates transporter-mediated uptake or efflux process. In the inhibition study (when the accumulation of the probe substrate is measured), incubation time is adjusted depending on the compound properties (i.e., permeability) and mechanism of inhibition. In certain cases, time-dependent inhibition may occur, and therefore it is important to use initial uptake rates of the probe substrate when calculating relevant transporter kinetic parameters.

Other cell-based systems for transporter studies include cells used in fluorescent dye-based assays, e.g., calcein assay for P-gp and MRPs transporters or Hoechst 33342 assay for BCRP transporter (Bircsak et al. 2013).

Hepatocyte cultures have been historically used to determine hepatic drug clearance (mostly CYP-mediated), but nowadays they are also used to in vitro assess drug interaction with hepatic transporters. Different hepatocyte cultures of human or animal origin can be used for these purposes, e.g., suspension, plate, and sandwich-cultured hepatocytes (SCH), depending on the type of the study.

Hepatocytes in suspension contain freshly isolated or cryopreserved hepatocytes in the appropriate medium, e.g., William's E medium or Hanks' balanced salt solution. Prior to the experiment, the suspension is preincubated at 37 °C for about 10 min, followed by incubation during predetermined time intervals with different concentrations of the test drug, both at 37 °C and on ice (or at 4 °C). In the subsequent step, hepatocytes are separated from excess substrate using either oil spin or filterplate method. Quantification of the drug in hepatocytes is performed using scintillation counting (for radioactively labeled drug) or liquid chromatography-mass spectrometry (LC-MS). Determination of the uptake transport rate is determined by subtracting the value obtained at 4 °C (corresponds to passive diffusion) from the value obtained at 37 °C (overall drug uptake including active and passive transport). Although widely used, this approach to determine active uptake rate has been criticized due to the fact that membrane fluidity, and consequently, drug passive permeability also changes with temperature (the membrane becomes more rigid), and this may lead to erroneous results. An alternative approach to determine passive transport, in order to subtract it from the total cellular uptake, is incubation with specific transporter inhibitors. But the problem with this approach is the lack of specific inhibitors (Zamek-Gliszczynski et al. 2013).

SCH can be obtained from humans and animals, including genetically modified animals with knockdown of a specific transporter. Hepatocytes are cultured between the two extracellular matrices, and as opposed to hepatocytes in suspension, functionality and polarity of the cells is preserved in this system. From all hepatocyte cultures, only SCH express fully functional canalicular efflux transporters, and therefore they are able to mimic biliary excretory function. Regarding that they also maintain enzyme activity, SCH can be used to study enzyme-transporter interplay and DDI. Experimental protocol with SCH system depends

upon the goal of the study, e.g., different protocols are adopted for studying interaction with uptake transporters or for determination of biliary clearance. Example protocols are described in literature (De Bruyn et al. 2013; Pfeifer et al. 2013; Cantrill and Houston 2017). In general, the isolated hepatocytes are seeded at a density of 1.5×10^5 (rat SCH) or 1.8×10^5 cells/cm^2 (human SCH) on multi-well plates. Following the initial culturing period when the cells attach to the plate, the unattached excess is removed, and the culture is covered with another plate. Depending on the culturing time and conditions, expression and functionality of transporters (and metabolizing enzymes) may vary significantly (De Bruyn et al. 2013).

Membrane vesicles came to use more recently, but they are now available from different sources, and they are mainly used to investigate drug interaction with efflux transporters from the ABC family. Depending on the source, membrane can express various transporters. Inverted (inside-out) membrane vesicles can be derived from insect cells (e.g., baculovirus-infected insect cell lines Sf9 or Sf21, High Five, and Sf+ insect cells), from cDNA-transfected mammalian cell lines (e.g., CHO, HeLa, V79 hamster, HEK293), from tissues (e.g., kidney or liver), or from artificial membranes that express the transporter of interest. Testing with vesicles from transfected cell lines requires the use of vesicles from non-transfected (wild-type) cells because these cells may also possess some background transporter activity. Vesicles derived from tissues are useful for simultaneous investigation of multiple transporters on apical or basolateral membrane, but the procedure requires special care because the preparation may contain vesicles from the opposite membrane expressing transporters that share the investigational drug as a substrate.

Prepared membrane vesicles can be resuspended in hypotonic or isotonic buffer, or freezed, and stored at −80 °C or in the liquid nitrogen. Commercial preparations are also available. A raw vesicle mixture is composed of inside-out vesicles, right-side-out vesicles, and open lamellar membrane fragments. In efflux assays only inside-out vesicles can interact with ATP and transport a substrate into the vesicle, so the raw mixture can be purified to increase the concentration of these vesicles. The experiments are performed by incubating vesicles in a buffer containing different concentrations of the tested substrate (radiolabeled or fluorescence labeled) at 37 °C in the presence of ATP. Parallel testing in the presence of AMP serves as a negative control for passive permeability, while probes with the reference standard serve as a positive control. After predetermined incubation time, active uptake process is terminated by adding the ice-cold solution, and the vesicles are separated by filtration or centrifugation (centrifugation is preferable for highly hydrophobic substances that tend to bind to the filter). Separation of the vesicles should be performed quickly, because the stop solution does not withhold efflux of substrate out of the vesicle. The collected vesicles are then lysed, and the accumulated drug is quantified based on radioactivity, fluorescence, or using LC-MS. One of the benefits of the membrane vesicle systems is that the measured concentration of a substrate or inhibitor represents the unbound concentration which is used to estimate kinetic parameters of drug transport.

Transport assays in membrane vesicles are usually performed in indirect manner (as an inhibition test), whereas the amount of accumulated probe substrate inside the vesicles is measured after incubation with the investigational drug in the presence of ATP. Indirect assay can only show whether the tested drug interacts with transporter (manifested as inhibition of the substrate transport), but it does not provide information on the nature of interaction (whether the drug is inhibitor or competitive substrate). Membrane vesicles can also be used in the direct assay, if the tested drug is available in, e.g., radiolabeled form. In this case, the outcome indicates the nature of the interaction with transporter.

Advances in microfluidics and lab-on-a-chip technologies provided new potential means to study drug disposition, e.g., 3D cell culture platforms that mimic the conditions of renal proximal tubule epithelial cells (Gozalpour and Fenner 2018). These systems are gaining more attention, and they may be more routinely used to study transporter-mediated drug interactions in the future.

Probe Substances

A number of probe substrates and inhibitors can be used in transporter interaction studies, and some of them are listed in Table 1. Examples of well-known probe substrates, inhibitors, and inducers for the key clinically relevant transporters are provided in the FDA online database (https://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm), together with the relevant notes concerning their selectivity. Namely, some of these probe substances are selective substrates or inhibitors of a certain transporter, but some interact with more than one transporter. It should be noted that the FDA list is not a definitive one (nor all-inclusive), and it is occasionally updated. On the other hand, EMA guideline (EMA 2012) does not provide a comprehensive list of probe substances, and instead the manufacturers are instructed to refer to the relevant literature. An example of a good review on probe substrates and inhibitors for major transporters (except for P-gp) has been provided by Momper et al. (2016).

Approaches to Estimate Transporter Kinetic Parameters

In order to understand and quantitatively assess the role of transporters in drug PK and DDIs, drug-transporter interactions need to be characterized in terms of relevant kinetic parameters. Depending on the type of interaction (uptake, efflux, inhibition, induction), different parameters are used to describe transport kinetics, e.g., net transport rate for P-gp and BCRP efflux substrate, inhibition constant (K_i) and/or half-maximal inhibitory concentration (IC_{50}) for transporter inhibitor, and intrinsic clearance for uptake transporter substrate. These parameters can be obtained by different methods, including in vitro assays, preclinical testing in animals (transporter gene-knockout models), and human in vivo studies.

One of the commonly used approaches to analyze kinetics of drug-transporter interactions refers to the employment of conventional static methods. These methods are based on relatively simple relationships and equations, but they imply certain approximations and simplifications of the in vivo conditions, e.g., that only one process is governing drug disposition (active uptake or efflux) while other concomitant processes (passive diffusion, metabolism, intracellular and extracellular binding) are neglected. Such methods can be useful for collecting prior information to guide future interaction studies, but they are not applicable when complex interactions are involved in drug disposition.

On the other hand, transporter kinetic parameters can be assessed using more complex mechanistic compartmental models. These models account for cellular and media compartments, and they take into account parallel processes (active transport, passive diffusion, drug binding, and metabolism) that influence drug concentration in the predefined compartments. Additional advantage of mechanistic models is that transporter kinetic parameters are estimated based on multiple drug concentration data at different time points to account for dynamic changes during, e.g., drug uptake process. Some of the mechanistic compartmental models have been detailed in the review published by ITC group (Zamek-Gliszczynski et al. 2013).

Substrate Studies

According to the regulatory guidelines (EMA 2012; PMDA 2014; FDA 2017), in vitro assessment of a drug potential to act as a substrate for transporter is recommended for several transporters (efflux or influx), localized mostly in the major clearance organs (the gut, liver, and kidneys). Regarding the gut efflux transporters, substrate potential should be investigated for P-gp and BCRP. Substratability studies for hepatic uptake transporters are suggested for OATP1B1 and OATP1B3. In case of renal transporters, substratability for uptake transporters OCT2, OAT1, and OAT3 and efflux transporters MATE1 and MATE2-K should be evaluated. The decision tree to evaluate whether a drug is a substrate of the major transporters is shown in Fig. 2.

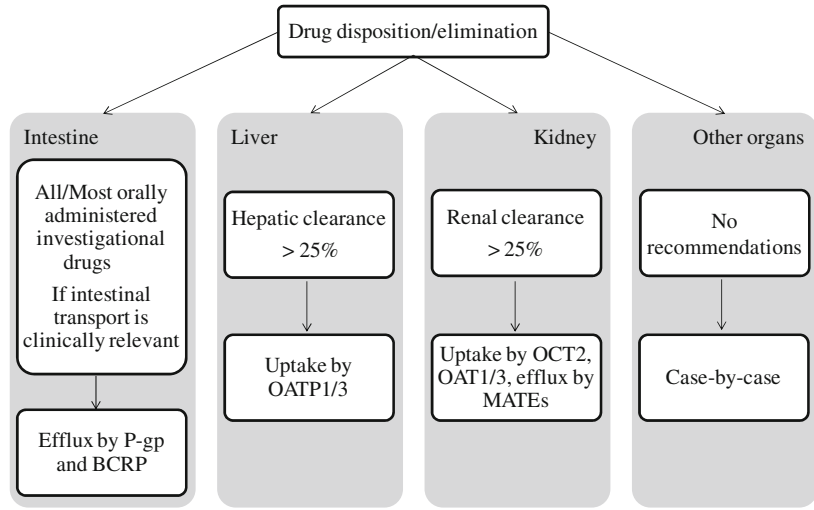

Fig. 2 Recommendations on the investigation of a drug substrate potential for major transporters

In vitro substrate studies are performed in the presence/absence of the known inhibitors. Inhibitors should be selected based on likelihood of co-administration with the tested drug, and a general suggestion is to start with selective inhibitors (if available).

Substrates for P-gp and BCRP Efflux Transporters

In cases when intestinal wall secretion exceeds 25% of the total clearance for orally administered drug, in vitro testing should be performed to identify the transporter involved and to qualitatively or semiquantitatively describe the interaction. The involvement of gut secretion is assessed when intestinal drug transport is clinically relevant (e.g., for oral, nasal, or inhalation delivery route), and it can be estimated based on drug PK and mass balance studies. In practice, substrate potential for P-gp and BCRP efflux transporters is assessed for all (or most) orally administered investigational drugs (Fig. 2).

Standard systems for determination of whether a drug is a substrate for P-gp or BCRP efflux transporters include Caco-2 cells, but other cell cultures (e.g., MDCK, LLC-PK1) or membrane vesicles overexpressing the transporter of interest can also be used. To confirm adequate expression of transporters, experiments with known P-gp or BCRP probe substrates are performed as positive control.

Routinely used cell-based test to identify substrates for efflux transporters is bidirectional transport assay. The test system comprises two compartments (donor and acceptor) divided by a cell monolayer. The conditions are usually kept the same in both compartments (pH 7.4). Transport of a drug (expressed as apparent permeability coefficient, P_{app}) is determined in both directions (B–A and A–B) using the following equation:

$$P_{app} = \frac{(dQ/dt) \times V}{A \times C_0} \quad (1)$$

Here, dQ/dt is the permeability rate, C_0 the initial concentration in donor compartment, V the volume of receiver compartment, and A surface area of the monolayer.

Based on these values, the efflux ratio (R_E) is calculated as:

$$R_E = \frac{P_{app}\ (B - A)}{P_{app}(A - B)} \quad (2)$$

In cases when transfected cell lines are used, efflux ratio is calculated for both transfected (R_T) and wild-type cells (R_W), and the resulting ratio is expressed as:

$$R_E = \frac{R_T}{R_W} \quad (3)$$

R_E value higher than 2 indicates that a drug is a substrate for the tested efflux transporter. In contrast, R_E around 1 implies that there is minimal or no drug efflux. Low R_E values, i.e., $R_E < 0.5$, suggest the involvement of uptake transporters.

Bidirectional transport assay should be performed with various concentrations of the test drug (at least four different concentrations) covering the range of clinically relevant concentrations (concentration at the luminal intestinal membrane), except when tested concentration of a drug is limited by its solubility or cytotoxicity. Also, transport rate should be linear over the employed concentration range and under the selected experimental conditions.

To confirm that investigational drug is a substrate for the efflux transporter of interest, additional study with P-gp or BCRP inhibitor should be performed. If possible, a selective inhibitor should be used, at concentration of at least ten times its K_i value. At least 50% reduction in R_E value in the presence of the inhibitor in comparison with R_E in the absence of inhibitor or R_E equal to unity in the presence of inhibitor confirms that a drug is a substrate for the tested transporter. In such a case, additional in vivo interaction studies should be considered. Other cutoff values may also be used if they are adequately justified and validated with known drug substrates. The problem with the identification of P-gp substrates with the addition of inhibitor lies with the fact that no specific P-gp inhibitor have been identified so far. Therefore, when using Caco-2 cells, which express various transporters, the study should be performed with two or more P-gp inhibitors to insulate specific contribution of this transporter.

Inverted membrane vesicles can also be used to identify drug substrates for efflux transporters. Here the drug-binding site of the transporter is located at the outer membrane surface, so the transported substrate is accumulated within the vesicles. Vesicles are subsequently filtered, and the amount of drug is quantified using a suitable method (as noted before). Transport activity is determined by subtracting the amount of drug accumulated in control vesicles incubated with AMP from the transporter-overexpressing vesicles incubated with ATP.

Substrate uptake rates determined in membrane vesicles are calculated based on mg protein, so they represent relative values in relation to the specific vesicle system. In order to calculate absolute values, i.e., Michaelis-Menten constant (K_m), a range of substrate concentrations should be tested. The ITC group (Brouwer et al. 2013) suggests to test at least seven substrate concentrations, whereas the highest one should correspond to at least 90% of the maximum transport velocity, along with a control (zero concentration) probe. Subsequently, kinetic parameters can be obtained from the Michaelis-Menten equation (Eq. 4) by applying regression analysis:

$$V = \frac{V_{max} \times [S]}{K_m + [S]} + CL_{passive} \times [S] \quad (4)$$

In Eq. 4, V is the uptake rate, V_{max} the maximum uptake rate, $CL_{passive}$ passive diffusion clearance, and [S] substrate concentration.

Membrane vesicle-based test may not be appropriate for highly permeable lipophilic compounds because extensive passive diffusion in both directions may overrun the efflux process (i.e., highly permeable drugs may escape the vesicle via passive diffusion through the lipid bilayer). An alternative way to evaluate whether a drug is a substrate for efflux transporters is ATPase assay, which is especially useful for highly permeable drugs. The test is performed on transporter-rich membranes obtained from mammalian cells or baculovirus-insect cells. The activity of transporter is reflected in changes in ATPase activity, which can be estimated by quantifying the amount of the generated inorganic phosphate. This test is useful in identifying highly permeable drug substrates for ABC transporters, but since this is an indirect assay, it is recommended to confirm the results by an additional assay.

Substrates for SLC Transporters

In vitro uptake studies are routinely used to investigate whether a drug is a substrate for the key transporters from the SLC family, namely, OATP transporters in hepatocytes and OCT, OAT, and MATE transporters in renal tubules. According to the regulatory recommendations, OATP-mediated hepatic uptake studies should be performed when hepatic transformation is major or one of the major routes of drug transformation, i.e., when hepatic metabolism or biliary secretion contributes to more than 25% of the total drug clearance (Fig. 2) or when uptake of a drug into the liver is clinically important (e.g., to exhibit pharmacological effect). In a similar manner, uptake by renal transporters (OCT, OAT, and MATE) should be assessed when renal secretion exceeds 25% of the total drug clearance and when in vivo renal clearance (in preclinical species) is higher than glomerular filtration rate (GFR). Assuming there is no reabsorption of a drug, active renal secretion can be estimated based on renal clearance (CLr), GFR, and unbound fraction of drug in plasma ($f_{u,p}$) using Eq. 5. In addition, renal clearance mechanisms should also be elucidated for drugs that exhibit pharmacological or toxic effect in kidneys.

$$\text{Active renal secretion} = \text{CLr} - \left(f_{u,p} \times \text{GFR}\right) \tag{5}$$

Commonly used in vitro systems for the assessment of OATP-mediated transport include hepatocyte cultures and transfected cell lines (CHO, HEK293, MDCK). Substrates for renal transporters (OCT, OAT, and MATE) are usually assayed on transfected cell lines (CHO, HEK293, MDCK), although MATE-mediated uptake can also be investigated in membrane vesicles. A note herein is that, when estimating MATE substrates, pH in the test system should be adjusted because activity of this transporter is affected by proton gradient.

When conducting an uptake study, a substrate can rapidly accumulate inside the cell or vesicle forcing the uptake process to deviate from linearity. For this reason, it is important to select early time points (in the linear phase) to estimate the uptake rate and calculate relevant kinetic parameters. The initial uptake rate can be estimated using linear or dynamic regression analysis. Active uptake clearance (CL_{uptake}) is then determined using Eq. 6, based on the data from the initial linear uptake phase:

$$CL_{uptake} = \frac{\text{Amount}_{t2} - \text{Amount}_{t1}}{(t2 - t1) \times C_{medium}} \tag{6}$$

In Eq. 6, Amount_{t1} and Amount_{t2} are cumulative amounts of drug over time t1 and t2, respectively, and C_{medium} concentration of drug in medium.

In addition, percentage of the active uptake (in relation to passive diffusion) can be estimated from the slope of the initial uptake phase determined in the presence and absence of a known inhibitor:

$$\%\text{Active uptake} = \left(1 - \frac{\text{Slope with inhibitor}}{\text{Slope without inhibitor}}\right) \times 100 \tag{7}$$

When a drug is identified as a substrate for the uptake transporter, kinetic parameters (K_m, V_{max}, CL_{int}) are calculated to characterize the transport process. Uptake kinetic parameters can be estimated using Eq. 4 and regression analysis of the experimental data. Then active uptake clearance (or unbound active uptake clearance, $CL_{active,u}$) can be expressed as:

$$CL_{active,u} = \frac{V_{max}}{K_m} \tag{8}$$

whereas the total unbound uptake clearance of a drug ($CL_{uptake,u}$) is the sum of passive ($CL_{passive,u}$) and active clearances ($CL_{active,u}$):

$$CL_{uptake,u} = CL_{passive,u} + CL_{active,u} \tag{9}$$

A drug is considered as OATB substrate if uptake ratio, in terms of drug uptake in transfected cells in comparison to drug uptake in empty vector-transfectant cells, is equal to or higher than 2.

In addition, the test should be performed with a selective inhibitor of the transporter of interest (e.g., rifampin for OATP1B1/OATP1B3), at concentration of at least ten times its K_i or IC_{50} value. This test confirms that a drug is a substrate for the tested transporter if drug uptake in the presence of selective inhibitor is reduced for at least 50% in comparison to the uptake in the absence of inhibitor. Other cutoff values may also be used if properly justified and validated with known probe substrates.

Hepatobiliary Transport

Drug transport from the blood, through the hepatocytes, into the bile requires the activity of both uptake transporters located at the basolateral (sinusoidal) membrane of hepatocytes and efflux transporters on the canalicular hepatocyte membrane. Therefore, this kind of transport cannot be assessed in cell systems expressing only uptake or efflux transporters. Up to now, the most suitable in vitro assay to assess the interplay between uptake and efflux hepatocyte transporters and estimate biliary clearance of a drug has been SCH assay (Nakakariya et al. 2012; De Bruyn et al. 2013; Yang et al. 2016). Based on SCH-generated data, it is possible to calculate biliary efflux clearance and biliary excretion index (BEI) of a drug using the following equations (Liu et al. 1999a):

$$CL_{bile,app} = \frac{Amount_{cell+bile} - Amount_{cell}}{t \times C_{medium}} \quad (10)$$

$$CL_{bile,int} = \frac{Amount_{cell+bile} - Amount_{cell}}{t \times C_{cell}} \quad (11)$$

$$BEI = \frac{Amount_{cell+bile} - Amount_{cell}}{Amount_{cell+bile}} \times 100\% \quad (12)$$

$$C_{cell} = \frac{Amount_{cell}}{V_{intracell}} \quad (13)$$

Depending on whether the drug concentration in medium (C_{medium}) or drug intracellular concentration (C_{cell}) is used, in vitro apparent biliary clearance from medium to bile ($CL_{bile,app}$) and in vitro intrinsic biliary clearance from hepatocytes to bile ($CL_{bile,int}$) can be estimated, respectively. Here, C_{medium} is the initial drug concentration in the incubation medium, C_{cell} is concentration of a drug in hepatocytes which can be calculated using Eq. 13, and t is the incubation time. $V_{intracell}$ in Eq. 13 is the volume of intracellular space per mg protein. $Amount_{cell}$ is the amount of drug accumulated in the cells, and $Amount_{cell+bile}$ is the sum of drug amount in the cells and bile canaliculi. These values can be estimated experimentally by modulating opening of tight junctions to bile canaliculi using incubation media with and without Ca^{2+}/Mg^{2+}. The principle is described within the patented B-Clear® technology (Liu et al. 1999b). BEI is a measure of drug accumulation in the bile and serves as a qualitative indicator of biliary excretion of drug.

Another method to calculate biliary clearance of a drug (CL_{bile}) was proposed by Cantrill and Houston (2017) (Eq. 14), whereas hepatocyte-to-media unbound concentration ratio (Kp_u) is used to reflect drug partition between hepatocytes and external medium and hence the contribution of active uptake process (Eq. 15):

$$CL_{bile} = \frac{Amount_{cell+bile} - Amount_{cell}}{t \times (Kp_u \times C_{medium})} \quad (14)$$

$$Kp_u = \frac{CL_{uptake}}{CL_{passive}} \quad (15)$$

In vitro obtained hepatic drug clearance values are usually expressed in µl/min/mg protein, and for further scaling to in vivo estimates, these values should be converted to ml/h/kg using the available data on liver weight and protein content in liver tissue of the species of interest. The converted values can also be used as inputs in physiologically based pharmacokinetic (PBPK) models to predict PK of a drug in humans or in preclinical species.

Inhibition Studies

Inhibitors affect transporter activity by non-competitive binding to the receptor site, thus hindering other drugs to interact with the transporter, or they can obstruct the process that generates the required energy for active transport (e.g., P-gp inhibitors that block ATP hydrolysis).

According to the regulatory guidelines (EMA 2012; PMDA 2014; FDA 2017), the potential of a drug to inhibit a transporter should be accessed for transporters that are known to be involved in clinically relevant drug interactions. There are certain differences between the regulatory documents, but the inclusive list of recommended target transporters comprises P-gp, BCRP, OATP1B1, OATP1B3, OCT2, OAT1, OAT3, MATEs, OCT1, and BSEP.

An inhibition study can be performed as unidirectional or bidirectional transport assay with an annotation that monolayer cell cultures are not suitable for efflux inhibition tests because there is no accepted method to calculate K_i value. Preferable method to estimate drug inhibition potential for an efflux transporter includes testing with membrane vesicles. Also, due to the pronounced variability regarding P-gp inhibition parameters between laboratories, EMA (2012) suggests to use at least two systems to test a drug inhibition potential for this transporter. If justified, this approach can be used for other transporters as well. An alternative to detect a drug inhibition effect on P-gp and MRP1 transporters is to use whole cell-type calcein assay. This is an indirect type of test that measures fluorescence of free calcein trapped inside the cell due to the inhibition of P-gp and MRP1 activity. The test is based on the fact that hydrophobic calcein ester (calcein AM), added to the cell culture, can be actively transported out of the cell. But if efflux transporters are blocked, hydrolyzed calcein derivative (fluorescent) will accumulate within the cell. So, if fluorescence in the presence of different concentrations of the investigational drug increases, this indicates that a drug is an inhibitor of the tested transporter (Glavinas et al. 2011).

The inhibitory potential of a drug is assessed in the presence of a known probe substrate for the transporter of interest. Since inhibitory potential of a drug can be substrate-specific, the best option for the in vitro study is to use the same probe substrate as in the prospective clinical study. If this is not an option, a preferred in vitro probe substrate is the one that generates lower IC_{50} value for the known inhibitors (to minimize the chance for false-negative results). Also, it should be noted that investigation of a drug inhibition potential for OATP1B1/OATP1B3 transporters requires an additional preincubation step for at least 30 min to detect possible time-dependent inhibition which could reduce a drug IC_{50} value, e.g., as observed for cyclosporine (Gertz et al. 2013).

The test should be performed with a range of substrate concentrations (at or preferably below its K_m, taking care that the transport is linear over the employed concentration range) and the range of concentration for the investigational drug, starting from the highest concentration (higher than clinically expected at the site of interaction) to potentiate inhibitory effect, but not to exceed drug solubility and cytotoxic concentrations.

Clinically relevant concentration of the tested inhibitor (I) depends upon the localization of the interacting transporter:

I. Intestinal luminal concentration (I_{gut}) for luminal intestinal transporters (P-gp, BCRP):

$$I_{gut} = \frac{\text{Dose}}{250\ \text{ml}} \tag{16}$$

II. Maximal unbound hepatic inlet concentration ($I_{u,in,max}$) for hepatic uptake transporters (OATP1B):

$$I_{u,in,max} = f_{u,p} \times \left(\frac{C_{max} + F_a \times F_g \times k_a \times \text{Dose}}{Q_h \times R_{bp}} \right) \tag{17}$$

In Eq. 17, F_a is fraction of drug absorbed, F_g fraction of drug that escapes the intestine unchanged, k_a absorption rate constant, Q_h hepatic blood flow, and R_{bp} blood-to-plasma concentration ratio. Certain approximations

can be made when F_a, F_g, and k_a are unknown, and then a worst-case scenario is assumed with $F_a \times F_g = 1$ and $k_a = 0.1$. Also, $f_{u,p} = 1\%$ can be used when experimentally determined value is lower than 1% (due to uncertainties in the measurements).

III. Maximal unbound plasma concentration ($I_{max,u}$) for renal transporters (OAT, OCT, MATE):

$$I_u = C_{max,u} \tag{18}$$

If a drug shows inhibition effect, the test is repeated with additional drug concentrations to calculate IC_{50} and K_i values. According to EMA (2012), it is preferable to use K_i to assess a drug potential to inhibit a transporter. IC_{50} is suggested only as an alternative when K_i cannot be determined, if linear conditions are maintained, and there is no time-dependent inhibition.

IC_{50} can be estimated using several methods (Balimane et al. 2008; Volpe et al. 2014). In general, the calculated P_{app} values for a probe substrate in the presence of different inhibitor concentrations are plotted against inhibitor concentrations, and the obtained curve is fitted by regression analysis to determine the inhibitor concentration that corresponds to 50% reduction in the probe substrate P_{app}. The final value is usually expressed as a mean for at least three separate measurements. There are also different methods to calculate K_i. One of the approaches is to convert IC_{50} to K_i using the following equation:

$$K_i = \frac{IC_{50}}{1 + \frac{S}{K_m}} \qquad (S - \text{substrate concentration}) \tag{19}$$

Several aspects need to be considered when determining IC_{50} and K_i values, as highlighted by the ITC group (Brouwer et al. 2013). One thing is that the obtained values may vary depending on the method employed, and this may complicate comparison of the results obtained in different laboratories. Moreover, IC_{50} does not provide information on the type of inhibition (competitive, noncompetitive, uncompetitive). Also, in case of competitive inhibition, IC_{50} depends upon the substrate concentration (in oppose to K_i). This annotation should be taken into account if IC_{50} is used for in vitro-in vivo extrapolation (IVIVE). IC_{50} is generally determined with a single probe substrate concentration, and when the substrate concentration is far below K_m, IC_{50} will be equivalent to K_i value. On the other hand, substrate concentration above its K_m (>50% K_m) leads to erroneous K_i estimates based on Eq. 19.

Another approach to analyze inhibition data refers to the use of Dixon plots (distinguishes between competitive and noncompetitive or uncompetitive inhibition) or, e.g., "quotient velocity plot" (distinguishes between all types of inhibition, including competitive, noncompetitive, and mixed-type inhibition) (Yoshino and Murakami 2009). These graphical methods enable determination of the inhibition type and K_i which can be considered as a more robust parameter than IC_{50}.

In vitro determined IC_{50} and K_i values are subsequently used to estimate a drug potential to in vivo inhibit transporters of interest, which will be discussed in section "In Vitro/In Vivo Extrapolation."

Induction Studies

Although it is known that certain drugs may induce membrane transporters, up to date there are no official recommendations on how to conduct in vitro transporter induction studies. The only suggestion given so far concerns investigation of potential P-gp inducers in cases when an investigational drug is identified as CYP inducer (due to similar mechanism of inducing CYP enzymes and P-gp transporter by interacting with PXR and CAR nuclear receptors). In case a drug is a CYP inducer, an induction study should be conducted using the same assumptions and test conditions as suggested for the investigation of CYP induction potential.

Interaction with Metabolites

Metabolites are hydrophilic in nature and usually not able to passively cross lipid membrane barrier, so they require active transport. Inhibition of these processes may lead to accumulation of potentially toxic or active metabolites in tissues, which can lead to potentially dangerous clinical consequences. For this reason, investigation of metabolite-transporter interaction should be included in drug development pipeline. Regulatory recommendation is that metabolite should be tested as a substrate for transporter(s) when a metabolite contributes to $\geq$50% of the total drug activity (EMA 2012; FDA 2017). Inhibition potential of metabolites is usually assessed in vivo, along with the parent drug. But in the cases when parent drug is not identified as inhibitor, in vitro inhibition studies with metabolites should still be performed when a metabolite is less polar than the parent drug and exposure to metabolite exceeds 25% of parent drug exposure or when a metabolite is more polar than the parent drug and exposure to metabolite exceeds 100% of parent drug exposure (FDA 2017).

Still, these studies can only be planned in later stages of drug development, after the metabolites have been identified in the relevant in vivo studies. Additionally, in silico quantitative structure-activity relationship (QSAR) may contribute to early investigation of the presumed metabolites of NDE and their elimination routes.

Critical Assessment of In Vitro Methods

In vitro studies are, with no doubt, important part of the overall assessment of transporters' effect on drug absorption, disposition, and DDIs. However, almost all of these studies have certain limitations, depending on the in vitro system and type of the study.

Most of the suggested probe substrates and inhibitors are interacting with multiple transporters, meaning that they usually lack sensitivity (e.g., rosuvastatin is suggested as OATP1B1 substrate, but it is also a substrate for, e.g., BCRP, MRP2, P-gp, OAT3; metformin is substrate for OCT2, but it also interacts with MATE1/MATE2 and OCT1). The criteria for the selection of appropriate probe substrates and inhibitors are also lacking, except for P-gp, and need to be established in the near future.

Additional matter to consider in the in vitro studies is whether qualitative information on the involvement of a single transporter in drug absorption and disposition is enough to decide upon the need to conduct clinical interaction studies. Namely, neglecting the role of multiple transporters on drug interactions may lead to false-positive (implying unnecessary clinical studies) or false-negative results (when necessary clinical testing is left out). Also, for drugs whose PK is influenced by the combined effect of transporter (s) and metabolic enzymes, a standard transporter in vitro assay might not be able to indicate clinical relevance of the tested transporter for a particular drug. The use of multiple tests with different systems might be the most accurate option to predict the transporter-mediated drug interactions.

As noted above, for certain types of transporter studies, various in vitro test systems and methods can be used, so different laboratories can obtain different results. And comparison of such results can be challenging. Even with a single method, and the same type of material, the results may vary (Cantrill and Houston 2017).

In case of cell-based studies, certain drugs cannot be tested in clinically relevant concentration ranges because of the limited solubility or high cytotoxicity. Organic solvents might be added to increase drug solubility, but in limited concentration (less than 1% vol/vol) due to potential effect on cell integrity and transporter activity. As for cytotoxic drugs, an alternative option is testing in membrane vesicle-based systems.

Nonspecific binding to the system components (cells, apparatus) is also a potential problem because it may reduce effective drug concentration. Therefore, the concomitant mass balance tests (percent recovery) are routinely done to evaluate nonspecific binding. Percent recovery is expressed as the sum amount of substrate remaining in donor and acceptor compartments at the end of test in relation to the initial substrate amount in the donor compartment. The test is

considered valid if percent recovery is more than 70%. A drug can also bind to membrane vesicles, and therefore its binding affinity should be checked prior to experiments. Moreover, a drug can bind to the filter used to separate membrane, and this phenomenon may be obviated by using nonreactive filter materials or by preincubation of filter with excess unlabeled substrate (Brouwer et al. 2013).

Membrane vesicles assays have many advantages (suitable system for different types of transporter studies, can express various proteins, the estimated unbound substrate concentration is useful to calculate relevant kinetic parameters), but they have some drawbacks. One of them concerns lipophilic compounds that may penetrate the membrane by passive diffusion or bind to the membrane, indicating false-negative results in drug-transporter interaction studies. In these cases, alternative tests (e.g., cell monolayers) are preferable to study transporter interactions.

As for suspended hepatocyte cultures, their functionality may be altered due to cryopreservation and membrane leakage (Yang et al. 2016). In addition, possible bias in cells polarity may happen during isolation of the cells.

When it comes to the use of SCH, one of the limitations observed in rat SCH is downregulation of uptake transporters. However, this has not been an issue with human hepatocytes when cultured under appropriate conditions (Kotani et al. 2011). Reduced expression of uptake transporters in hepatocytes can further limit concentration of a drug substrate available for efflux transporters and lead to underprediction of in vitro obtained biliary efflux values. In order to predict relevant in vivo values based on these data, scaling factors (SFs) have to be used. But the problem is that these factors are drug- and transporter-specific, and evaluation of the predictive value of such in vitro data is difficult (Cantrill and Houston 2017).

Looking at the type of study, certain approximations are often made in the estimation of kinetic parameters describing transporter-mediated drug permeability in a bidirectional in vitro assay. This particularly concerns K_m value which is assumed to be similar in both directions if the experimental conditions (buffer pH) on both sides are the same. However, some observations indicate that K_m estimates can be direction-dependent (Harwood et al. 2013).

General problem with the inhibition studies is that decreased transport of probe substrate in the presence of the investigational drug does not provide information on the nature of the interaction (investigational drug can be either inhibitor or competitive substrate). However, subsequent substrate studies can indicate whether a drug is a substrate. For this reason, substrate and inhibition tests should be performed in combination.

In Vitro/In Vivo Extrapolation

Quantitative prediction of the influence of transporters on drug disposition, tissue exposure, and DDIs is challenging. Based on the current knowledge and proposed theories on drug-transporter interaction mechanisms, several approaches to link in vitro data to the expected in vivo outcomes (in vitro-in vivo correlation (IVIVC) or IVIVE) have been suggested, and they will be described in this section.

ITC group and regulatory agencies have provided recommendation on whether to conduct follow-up clinical transporter studies, whereas the decision is made based on in vitro results and IVIVE. To make a decision, data from multiple in vitro assays are needed, along with supportive data from preclinical species and first-in-human studies (if available). Other data such as patient population, drug therapeutic index, safety profile, and therapeutic indications indicating likely co-administration of other drugs that are substrates or inhibitors for the same transporter pathways should also be considered when planning in vivo interaction studies. To exemplify, if in vitro experiments suggest that a drug is a substrate for renal tubule transporters, this finding should be confirmed in the in vivo study. Moreover, if the investigational drug is likely to be co-administered with a drug that is a known substrate or inhibitor of OCT, OAT, or MATE transporters, potential

interaction should be assessed before phase III (preferably before phase II) clinical trials.

In vitro obtained K_m and V_{max} values are preferable transporter kinetic parameters to be used for IVIVE. Clearance value can also be considered, but it should be kept in mind that this parameter does not take into account saturable kinetics of drug transport. A major problem when using in vitro kinetic data for IVIVE is that these values depend upon the applied method, so the experimentally obtained values may vary considerably.

As for the in vivo parameters, drug plasma levels have been traditionally used for IVIVE to assess the interaction of transporters in vivo. However, there are cases when plasma levels do not follow changes in drug tissue exposure, and in these cases, IVIVE may lead to erroneous estimates regarding the interactions with transporters. Technological achievements in imaging techniques may help to overcome these issues. Namely, imaging techniques such as positron-emission tomography, single-photon emission computed tomography, magnetic resonance imaging, etc. can provide valuable information on drug-transporter interactions in vivo, including tracking of dynamic changes in tissue drug concentration and estimation of critical transporter-related kinetic parameters. These data can eventually be correlated to the in vitro findings.

Due to the differences in the expression and activity of transporters in the in vitro systems and in vivo environment, quantitative prediction of transporter-mediated drug PK, tissue exposure, and DDI based on in vitro data requires the use of scaling factors. A scaling factor is defined by dividing fitted in vivo clearance value with the in vivo clearance predicted based on in vitro measurements. One of the suggested approaches is to estimate the transporter-specific scaling factor as the average (geometric mean) factor for different drug substrates, assuming that in vitro measurements for different drugs used to calibrate the SF are done in the same system (Jones et al. 2012). Another approach concerns estimation of the compound-specific SF (Poirier et al. 2009). This empirical value can be obtained by fitting with the animal PK data, and then the same factor can be used to predict human PK based on preclinical data (e.g., in human hepatocytes).

An ideal approach to correlate in vitro to in vivo data is to take into account the effect of all the important transporters enrolled in the disposition of a drug. Such a complex in vitro system that accounts for the effect of all the transporters in different tissues does not exist, nor is likely to be established. SCH model can be used to gain knowledge on the involvement of multiple transporters in liver. Alternatively, multiple assays can be combined (e.g., cell-based systems, membrane vesicles, substrate and inhibition studies) to assess the contribution of each pathway separately.

In general practice, IVIVE approaches for transporter-mediated drug processes can be regarded as static or dynamic. Static approaches are based on drug clearance data (e.g., "Qgut model" for intestinal bioavailability, "well-stirred model," and "extended clearance model" for hepatic drug clearance, "well-stirred renal model") and generally do not take into account changes in drug (substrate or inhibitor) concentration during dosing interval. Also, plasma or luminal concentrations are often used as a substitute for intracellular drug concentration. Moreover, this approach may be suitable to evaluate the effect of a single transporter, but assessing the interaction of multiple transporters or enzyme-transporter interplay is rather difficult. Although this approach has limitations, it is widely used in the initial phase of transporter studies to provide preliminary information on possible drug-transporter interaction before conducting additional in silico and/or in vivo studies. On the other hand, dynamic approaches usually refer to the application of more complex PBPK simulations and modeling tools that can track changes in drug concentration in plasma and tissues as a result of parallel processes a drug undergoes in the organism, including interaction with multiple transporters and/or enzymes. Basic principles of both approaches will be described in the following text.

IVIVE for Oral Absorption

Drugs that are substrates for intestinal absorption may display dose-dependent nonlinear pharmacokinetics, whereas increased drug concentrations (exceeding saturation capacity of transporter) lead to nonproportional decrease in drug absorption (for apical influx transporters) and, in opposite, increase in drug absorption (for apical efflux transporters). For drugs that are substrates for both apical influx and efflux transporters (e.g., quinidine), the situation can be more complicated. Although it is not a rule that substrates for intestinal transporters will exhibit erratic absorption, in vitro screening of possible interactions with intestinal transporters in early drug development phase, and concomitant IVIVE, may help to detect problems with poor oral drug bioavailability.

The influence of transporter interference on drug absorption depends upon its expression in the intestinal tissue and activity for a certain drug (expressed as K_m). In order to establish correlation between in vitro and in vivo data for drugs that are substrates for intestinal transporters, several aspects need to be considered. First, the in vitro component of IVIVC comprises both drug-related and system-related parameters. Drug-related parameters are addressed in the expression for in vitro intestinal drug clearance:

$$CL_{int,active} = \frac{V_{max}}{K_m + C} \tag{20}$$

where $CL_{int,active}$ is drug intrinsic clearance due to active transport and C unbound drug concentration at the transporter binding site.

Furthermore, by applying the concept of the Q_{gut} model, whereas "drug flow" through the enterocytes (Q_{gut}) encompasses both perfusion and permeability drug transport (Eq. 21), intestinal drug bioavailability (F_g) can be predicted based on drug permeability and intrinsic clearance ($CL_{int,gut}$) in the enterocytes (Eq. 22).

$$Q_{gut} = \frac{CL_{perm} \times Q_{ent}}{CL_{perm} + Q_{ent}} \tag{21}$$

$$F_g = \frac{Q_{gut}}{Q_{gut} + \left(f_{u,gut} + CL_{int,gut}\right)} \tag{22}$$

Here, Q_{ent} is blood flow through the enterocytes, CL_{perm} drug clearance defining permeability through the enterocytes, and $f_{u,gut}$ the fraction of unbound drug in the enterocytes.

This model seems to be well predictive for drugs with relatively high F_g ($F_g > 0.5$), but not for drugs with high intestinal clearance because saturation phenomena and nonlinear processes are not taken into account (Zamek-Gliszczynski et al. 2013).

As for the system-related parameters, it has been shown that drug-transporter kinetic determinants also depend on the experimental system (e.g., direction-dependent K_m value in certain systems, V_{max} value dependent on the expression of transporter in the system) and employed conditions. To overcome these issues, more complex multi-compartment mathematical models have been proposed to estimate intrinsic kinetic parameters of active drug transport (Harwood et al. 2013).

IVIVE for Hepatobiliary Transport

Several successful attempts to establish a relationship between in vitro and in vivo biliary clearance data have been reported in literature and among them (Nakakariya et al. 2012). Still, the predictive power of the proposed extrapolation methods is hard to determine, since available data on the rate and extent of biliary elimination of drugs from clinical studies are rather limited.

I. One of the approaches to estimate in vivo biliary clearance of a drug is scaling of in vitro obtained values (Eq. 23). But the problem with this approach is that scaling factors seem to be both drug- and transporter-specific (Cantrill and Houston 2017). Also, the overall intrinsic clearance in vivo for OATP substrates can be underestimated if hepatic uptake intrinsic clearance from SCH or plated hepatocytes is used for prediction and very large scaling factor have to be used to bridge the discrepancy (Li et al. 2014):

$$CL_{bile,int,pred} = CL_{bile,int,in\ vitro} \times SF \quad (23)$$

In Eq. 23, $CL_{bile,int,pred}$ is the predicted in vivo intrinsic biliary clearance and $CL_{bile,int,in\ vitro}$ intrinsic biliary clearance determined in vitro.

II. On the other hand, biliary clearance of a drug can be estimated based on a well-stirred hepatic model. To estimate drug intrinsic biliary clearance in vivo ($CL_{bile,int,in\ vivo}$) from in vivo clearance data ($CL_{bile,in\ vivo}$), the following equation applies:

$$CL_{bile,int,in\ vivo} = \frac{Q_p \times CL_{bile,in\ vivo}}{Q_p - CL_{bile,in\ vivo}} \quad (24)$$

where Q_p represents liver blood flow.

This equation can be modified to account solely for the fraction of drug unbound in plasma (Fukuda et al. 2008):

$$CL_{bile,int,in\ vivo} = \frac{Q_p \times CL_{bile,in\ vivo}}{Q_p - \frac{CL_{bile,in\ vivo}}{R_{bp}}} \times \frac{1}{f_{u,p}} \quad (25)$$

where R_{bp} is blood-to-plasma concentration ratio.

In a similar manner, biliary clearance of a drug in vivo can be estimated using in vitro data:

$$CL_{bile,app,pred} = \frac{Q_p \times CL_{bile,app,in\ vitro}}{Q_p + CL_{bile,app,in\ vitro}} \quad (26)$$

Or if the fraction of inbound drug is taken into account:

$$CL_{bile,app,pred} = \frac{Q_p \times f_{u,p} \times CL_{bile,app,in\ vitro}}{Q_p + f_{u,p} \times CL_{bile,app,in\ vitro}} \quad (27)$$

In Eqs. 26 and 27, $CL_{bile,app,pred}$ is the predicted in vivo apparent biliary clearance and $CL_{bile,app,in\ vitro}$ apparent biliary clearance determined in vitro.

There are speculations about whether it is better to use $CL_{bile,app,in\ vitro}$ or $CL_{bile,int,in\ vitro}$ to predict biliary clearance of a drug in vivo, but generally the data go in favor of intrinsic clearance values (Nakakariya et al. 2012). Also, the correlation is improved if unbound plasma concentration is used in the estimation of in vivo values (Fukuda et al. 2008).

Further modification of the method to estimate drug biliary clearance in vivo was proposed by Li et al. (2010), who introduced the so-called g factor that incorporates quantitative data on the amount of hepatobiliary transporters (MRP2, BCRP, and BSEP) in rat SCH:

$$CL_{bile,int,pred,g} = \frac{Q_p \times g \times CL_{bile,int,pred}}{Q_p + g \times CL_{bile,int,pred}} \quad (28)$$

III. The third approach to estimate hepatic drug clearance in vivo is based on the extended clearance concept which takes into account multiple transports and/or metabolic processes when predicting overall drug hepatic intrinsic clearance. Transporter-enzyme-mediated overall hepatic intrinsic clearance ($CL_{int,H}$) is mathematically described as:

$$\begin{aligned} CL_{int,H} &= \left(CL_{int,met} + CL_{int,bile}\right) \\ &\quad \times \left(\frac{PS_{uptake}}{PS_{efflux} + CL_{int,met} + CL_{int,bile}}\right) \\ &= \left(CL_{int,met} + CL_{int,bile}\right) \times K_{p,uu} \end{aligned} \quad (29)$$

where:

$$PS_{uptake} = PS_{active} + PS_{passive} \quad (30)$$

$$PS_{efflux} = PS_{basal\text{-}active} + PS_{passive} \quad (31)$$

In Eqs. 29, 30, and 31, $CL_{int,met}$ is metabolic intrinsic clearance, $CL_{int,bile}$ biliary intrinsic clearance, PS_{active} active uptake clearance, $PS_{basal\text{-}active}$ active efflux, $PS_{passive}$ passive diffusion clearance on either direction across the sinusoidal membrane,

and $K_{p,uu}$ the unbound drug concentration in the liver relative to plasma at steady state. $K_{p,uu}$ is difficult to measure in vivo, and an alternative in vitro approach is to calculate ratio of drug uptake (in human hepatocyte suspension) at 37 °C and on ice (when $PS_{passive} \gg (CL_{int,met} + CL_{int,bile})$), as described in Eqs. 32 and 33. Different methods to estimate $K_{p,uu}$ are described in the review of Shitara et al. (2013).

$$K_{p,uu} = \frac{PS_{active} + PS_{passive}}{PS_{passive}} = \frac{PS_{uptake}}{PS_{passive}} \quad (32)$$

$$K_{p,uu} = \frac{K_p(37°C)}{K_p(\text{on ice})} \quad (33)$$

In cases when hepatic uptake is the rate-determining step for drug elimination ($CL_{int,met} + CL_{int,bile} \gg PS_{passive}$ and $PS_{efflux} = 0$), the overall hepatic drug clearance can be expressed as:

$$CL_{int,H} = \left(PS_{active} + PS_{passive}\right) \quad (34)$$

Izumi et al. (2017) evaluated different IVIVE approaches for the prediction of human hepatic clearance of OATP substrates and suggested that the best approach, that yielded reasonable correlation with the overall hepatic intrinsic clearance in vivo, is based on the in vitro parameter that describes the rate-determining step for drug hepatic elimination, i.e., uptake intrinsic clearance (PS_{uptake}) while neglecting hepatic metabolism. In addition, it was shown that introducing $K_{p,uu}$ value to account for the difference between drug concentration in hepatocytes and plasma improves correlation between the predicted and in vivo observed clearance values. PS_{uptake} can be estimated from the initial slope of linear equation that illustrates changes in the ratio of hepatocytes to buffer drug concentration during time in relation to the ratio of drug exposure and concentration in the buffer, as shown in Eq. 35:

$$\frac{X_H}{C_{buffer}} = PS_{uptake} \times \frac{AUC_{(0-t),buffer}}{C_{buffer}} + V_0 \quad (35)$$

In Eq. 35, X_H is the amount of drug in hepatocytes, C_{buffer} concentration of drug in the incubation buffer, $AUC_{(0-t),buffer}$ area under the drug concentration-time curve in the incubation buffer, and V_0 initial distribution volume that represents instantaneous drug adsorption to the surface of hepatocytes.

PS_{uptake} determined in vitro at 37 °C can be transposed to the corresponding in vivo value (using hepatocellularity for the human liver and relevant SFs). However, PS_{uptake} values obtained in human hepatocyte suspensions are subjected to inter-batch variability, so using batch-specific SF can improve the predictions (Izumi et al. 2017).

IVIVE for Renal Clearance

In vitro-in vivo prediction methods for transporter-mediated renal drug clearance have not been well established, and the confidence in the prediction is rather low. Namely, data on transporter-mediated renal drug clearance is difficult to obtain in vitro, due to current inability to design a system that will reproduce physiology and functionality of nephron. Also, there are no available methods to assess renal drug clearance in humans directly.

Renal clearance of drugs (CL_{renal}) is comprised of three processes (glomerular filtration, active tubular secretion, and tubular reabsorption), which is represented in the following equation:

$$CL_{renal} = \left(f_{u,b} \times GFR + CL_{sec}\right) \times \left(1 - F_{reabs}\right) \quad (36)$$

where CL_{sec} is renal secretory clearance and F_{reabs} fraction of reabsorbed drug.

If the well-stirred renal model is assumed, secretory clearance can be expressed as:

$$CL_{sec} = Q_r \times \frac{f_{u,b} \times CL_{int,sec}}{Q_r + f_{u,b} \times CL_{int,sec}} \quad (37)$$

Here Q_r is the renal blood flow and $CL_{int,sec}$ intrinsic secretion clearance which depends upon the activity of uptake and efflux transporters as illustrated in the following equation:

$$CL_{int,sec} = \frac{PS_{influx,b} \times PS_{efflux,a}}{PS_{influx,b} + PS_{efflux,a}} \quad (38)$$

In Eq. 38, $PS_{influx,b}$, and $PS_{efflux,a}$ represent relevant influx and efflux intrinsic clearances through basolateral (b) and apical (a) membranes of proximal tubules.

A common approach to estimate renal active clearance of drugs includes empirical assessment based on GFR and scaling of animal data. However, allometric scaling based on animal data may not be the best option because of the variations in transporters expression and activity between species. Also, there is a risk of upregulation or downregulation of other transporters in transporter-knockout animals which may tangle interpretation of the results. An additional option is to use SFs to translate in vitro (e.g., from kidney slices) to in vivo data. Several studies demonstrated that clearance data obtained in kidney slices can correlate to the in vivo clearance values for some drugs, but SFs needed to be applied (Watanabe et al. 2011). Alternative approach to predict renal drug clearance in humans is to use PBPK modeling, but this approach also has some drawbacks, including uncertainties regarding the expression levels of renal transporters.

IVIVE for Inhibition Studies

In case of transporter inhibition studies, in vitro-generated K_i or IC_{50} values for the investigational drug are correlated to the in vivo drug concentration (intestinal, liver, or plasma concentration, depending on the localization of the transporter of interest), and the outcome is used to estimate potential clinically significant interactions (Fig. 3).

The criteria to evaluate a drug inhibition potential are not harmonized between the leading regulatory authorities, and in addition recently proposed FDA draft guideline (FDA 2017) introduced certain changes in comparison to the FDA guideline from 2012 (FDA 2012). In summary, the following criteria and cutoff values are recommended, depending on the regulatory document:

I. Orally administered investigational drug has a potential to inhibit P-gp or BCRP transporters in vivo if:
   $I_{gut}/IC_{50} \geq 10$ (FDA 2017)
   $I_1/IC_{50} \geq 0.1$ or $I_{gut}/IC_{50} \geq 10$ (FDA 2012; PMDA 2014)
   $I_{1u}/(K_i$ or $IC_{50}) \geq 0.02$ or $I_{gut}/(K_i$ or $IC_{50}) \geq 10$ (EMA 2012)
   Here I_1 and I_{1u} are total (unbound and bound) and unbound systemic concentrations of the inhibitor, respectively.
   Comparison of the prediction performance of different criteria indicated superiority of the criterion suggested in the novel FDA draft guideline (Zhou et al. 2016). However, one clear disadvantage of using IC_{50} value for the assessment of P-gp inhibition potential of a drug is that it can vary considerably between the laboratories, which may lead to inconsistency in the interpretation of the results. To minimize false results, it is advisable to run inhibition studies with a number of well-known inhibitors (Zamek-Gliszczynski et al. 2013).
II. Investigational drug has a potential to inhibit OATP1B1/OATP1B3 transporters in vivo if:
   $R = 1 + I_{u,in,max}/IC_{50} \geq 1.1$ (FDA 2017)
   $C_{max}/(K_i$ or $IC_{50}) \geq 0.1$ and $R \geq 1.25$ (FDA 2012)
   $R \geq 1.04$ (EMA 2012)
   $R \geq 1.25$ (PMDA 2014)
   where R represents the ratio of victim drug AUC in the presence and absence of inhibitor (investigational drug). Again, criterion indicated in the novel FDA draft guidance seems to be the most appropriate (Vaidyanathan et al. 2016). As in the case of P-gp inhibition, IC_{50} values also show large variability

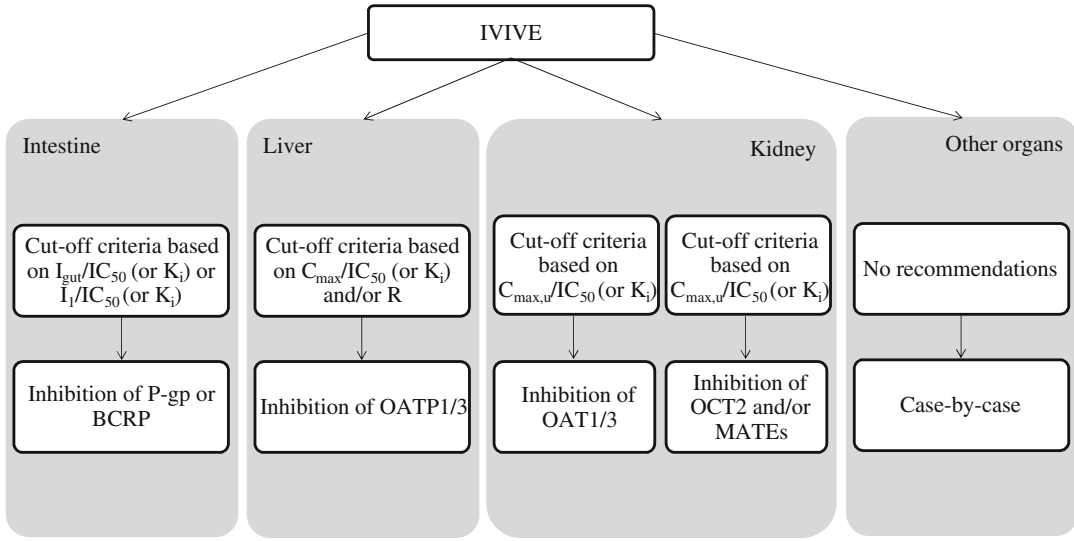

Fig. 3 Recommendations on the in vivo investigation of a drug inhibition potential for major transporters based on in vitro data

depending on the substrate drug and experimental conditions (e.g., with or without preincubation step), and this may influence the validity of the suggested cutoff criteria. Additional problem with the proposed R criteria is that it treats hepatic drug transport solely as OATP-mediated process and neglects the contribution of passive diffusion and/or efflux and hepatic drug metabolism. This often leads to overestimation of the contribution of active hepatic uptake. A way to overpass this limitation is to use relative activity factors (RAFs) when estimating contribution of a specific transporter in the overall hepatic uptake based on the in vitro data.

III. Investigational drug has a potential to inhibit OAT transporters in vivo if:
$C_{max,u}/IC_{50} \geq 0.1$ (FDA 2012, 2017)
$C_{max,u}/(K_i \text{ or } IC_{50}) \geq 0.02$ (EMA 2012)
$C_{max,u}/IC_{50} \geq 0.25$ (PMDA 2014)

According to the results of Dong et al. (2016), the most suitable criterion for OAT transporters appear to be the one proposed by FDA and PMDA.

IV. Investigational drug has a potential to inhibit OCT2 and/or MATE transporters in vivo if:
$C_{max,u}/IC_{50} \geq 0.1$ (OCT2) or $C_{max,u}/IC_{50} \geq 0.02$ (MATE) (FDA 2017)
$C_{max,u}/IC_{50} \geq 0.1$ (OCT2) (FDA 2012)
$C_{max,u}/(K_i \text{ or } IC_{50}) \geq 0.02$ (OCT2) or $C_{max,u}/(K_i \text{ or } IC_{50}) \geq 0.02$ (MATEs) (EMA 2012)
$C_{max,u}/IC_{50} \geq 0.25$ (OCT2) or $C_{max,u}/IC_{50} \geq 0.25$ (MATEs) (PMDA 2014)

Based on the findings of Dong et al. (2016), it seems the best to apply different cutoffs for OCT2 and MATEs, as proposed in the new FDA guideline. In each of the abovementioned cases, other cutoff values may also be considered if properly justified using an in vitro system calibrated with known inhibitors and non-inhibitors. But to highlight again, for each investigational drug and interacting transporter, the final decision on whether to conduct clinical DDI studies will depend on the therapeutic indication of the investigational drug and likeliness of co-medication with drugs that are known substrates for a particular transporter.

PBPK Modeling

In vivo systems are quite complex, and their correct representation in the vitro environment is not feasible. Therefore, in silico tools that represent some of the complexity of the in vivo conditions provide a valuable mean to assess bioperformance

of drugs and elucidate contribution of different mechanisms on drug pharmacokinetics.

PBPK models are physiologically based mathematical models that use a series of differential equations to simulate drug transit and bioperformance in the body. These models usually comprise a set of compartments (organs, tissues, and their substructures) linked by the vascular system. They integrate various physiological data (e.g., characteristics of different cells and spatial structures, blood flow rates) and are able to simulate numerous physiological and biochemical processes including transporter-mediated drug disposition and enzyme-mediated metabolism. PBPK models go even beyond feasible experiments since they are able to test mechanical hypotheses and assess parameters that are difficult or impossible to measure in vivo. Also, simulation and modeling may indicate the involvement of processes and mechanisms affecting drug bioperformance that have not been identified in vitro or in vivo.

Some of the commercially available software packages for PBPK modeling have integrated data on the localization and expression levels of certain human transporters. Data on some animal transporters are also provided. If information on the suspected transporter is not included in the software database, these data have to be input manually, and they should preferably be experimentally obtained. Relative expression of a transporter in organs and tissues can be determined by traditional methods such as immunoblotting and RNA-based methods. More recently, advances in quantitative proteomics enabled determination of absolute transporter abundances in human tissues and commonly used in vitro systems. But these data should be used with caution because obtained expression levels can vary depending on, e.g., age, gender, disease state, and especially drug history, along with specificities related to the applied methodology.

PBPK modeling tools have been accepted by the regulatory authorities in the USA and EU, and nowadays labeling information for several marketed drugs include information on potential DDIs based solely on in silico modeling results. But, these examples are mostly related to simulations regarding the influence of metabolic enzymes on DDIs. As yet, PBPK modeling has not been exploited much to support regulatory submission that address issues related to transporter-mediated interactions, and examples of such submissions are rather scarce. Some of the examples have been listed by Pan et al. (2016) and include the employment of PBPK modeling to understand the role of (i) P-gp on intestinal absorption and possible DDIs of naloxegol and ceritinib as P-gp substrates and ibrutinib as P-gp inhibitor and (ii) hepatic OATP on possible DDIs with simeprevir as OATP substrate.

Tissue Models

In a PBPK model, each tissue is displayed as a series of separate compartments. A common tissue structure is composed of vascular, intracellular, and extracellular compartment. The intestine, liver, and kidney (and other organs such as the lungs, brain) models are usually more complex. Drug partitioning between extra- and intracellular space depends on passive diffusion through the cellular membrane and active transit mediated by membrane transporters (influx and/or efflux). In line with this, tissues can be treated as perfusion-limited (drug transfer is limited by blood flow rate through the tissue) or permeability-limited (drug transport is a saturable process partly governed by the expression and activity of membrane transporters, besides passive diffusion) (Fig. 4). Passive diffusion is characterized by the PSt factor (permeability-surface area for the given tissue) (Eq. 39), while carrier-mediated transport is described by Michaelis-Menten equation and transporter-specific kinetic parameters (K_m and V_{max}). The PSt for each tissue can be scaled from the PSt for the liver, using the cell volume (V_{cell}) in each tissue (Eq. 40). Liver PSt can be estimated from drug passive permeability determined in hepatocyte culture. K_m and V_{max} values are usually determined in the in vitro experiments, and, based on the assay type, they can be scaled to the relevant in vivo values. In vitro intrinsic clearance can also be scaled to the relevant in vivo value. Alternatively, K_m and V_{max} might be obtained by fitting PBPK results to the in vivo

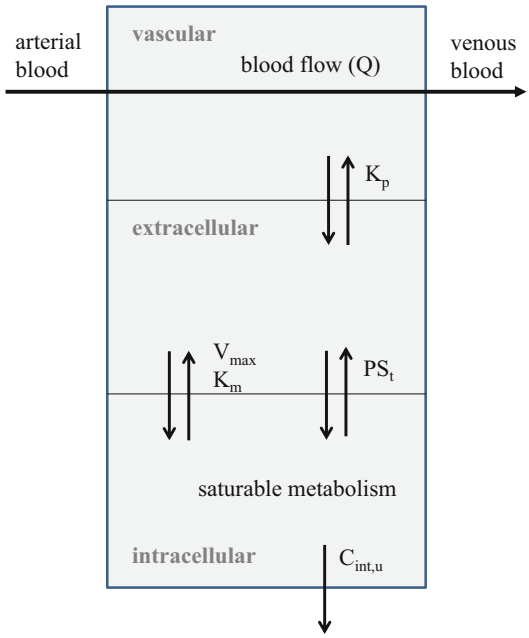

Fig. 4 Schematic representation of permeability-limited tissue in the PBPK model

PK data, when in vivo data are available (after clinical studies).

$$PS_t = P_{app} \times S \qquad (S - \text{exchange surface area}) \tag{39}$$

$$PS_{t(\text{tissue1})} = PS_{t(\text{tissue2})} \times \frac{V_{cell(\text{tissue1})}}{V_{cell(\text{tissue2})}} \tag{40}$$

Knowledge of the expression levels of transporters in different tissues is necessary to enable adequate scaling from in vitro and preclinical studies to human data. Namely, if relative expression levels of a transporter (relative amount in each compartment/tissue compared to its V_{max} measurement environment) are known, then in vitro V_{max} can be scaled to the in vivo value. In order to do so, knowledge on the expression of transporter in the in vitro system used for V_{max} determination is also required (e.g., if hepatocytes are used, it can be assumed that the expression level of transporter in vitro is equal to its expression level in the liver). If the expression level in vitro is not known, the obtained V_{max} needs fitting to the in vivo data.

To extrapolate in vitro K_m to the in vivo value, it is necessary to know relevant drug concentration in vitro (e.g., unbound intracellular concentration for the efflux transporters). Herein, unbound concentration of drug in a tissue depends upon unbound drug concentration in plasma, plasma/tissue partitioning, tissue binding, cellular membrane permeability, and degradation via cellular metabolic pathways. There are several methods to estimate drug partitioning into tissues (expressed as K_p value), but the choice of equations depends upon the processes a drug is assumed to undergo in the organism. For instance, lipophilic drugs with high passive permeability will most likely pass cellular membranes by simple passive diffusion, and distribution into body tissues will be perfusion-limited. In these cases, K_p can be calculated from drug physicochemical properties (e.g., molecular weight, lipophilicity, dissociation constant(s), blood-to-plasma concentration ratio, fraction unbound in plasma and tissue) (Kuepfer et al. 2016).

A combined equation that defines drug entry into extracellular tissue compartment, considering three-compartment tissue model (vascular-extracellular-intracellular), and combination of passive diffusion, active transport, and tissue metabolism are given in a following form:

$$\begin{aligned}\left(V_{ec} + \frac{V_v \times R_{bp}}{K_p}\right) \times \frac{dC_{ec}}{dt} &= Q_T \times \left(C_{art} - \frac{C_{ec} \times R_{bp}}{K_p}\right) - PS_t \\ &\times \left(C_{ec,u} - C_{ic,u}\right) - \sum_{i=1}^{nInfTr} \frac{V^i_{max} \times C_{ec,u}}{K^i_m + C_{ec,u}} \\ &+ \sum_{j=1}^{nEffTr} \frac{V^j_{max} \times C_{ic,u}}{K^j_m + C_{ic,u}}\end{aligned} \tag{41}$$

In a similar manner, change in intracellular drug concentration over time can be expressed as:

$$V_{ic} \times \frac{dC_{ic}}{dt} = PS_t \times \left(C_{ec,u} - C_{ic,u}\right) + \sum_{i=1}^{nInfTr} \frac{V_{max}^{i} \times C_{ec,u}}{K_m^{i} + C_{ec,u}} - \sum_{j=1}^{nEffTr} \frac{V_{max}^{j} \times C_{ic,u}}{K_m^{j} + C_{ic,u}} - \sum_{k=1}^{nEnz} \frac{V_{max}^{k} \times C_{ic,u}}{K_m^{k} + C_{ic,u}} - \left(CL_{ic,u} \times C_{ic,u}\right) \quad (42)$$

In Eqs. 41 and 42, C_{ec} and C_{ic} are drug concentrations in extracellular and intracellular compartment, respectively ($C_{ec,u}$ and $C_{ic,u}$ relevant unbound drug concentrations); V_{ec} and V_{ic} volumes of extracellular and intracellular compartment, respectively; V_v volume of vascular compartment; Q_T tissue blood flow; C_{art} arterial blood concentration; $CL_{ic,u}$ unbound intrinsic clearance in tissue; and nInfTr, nEffTr, and nEnz numbers of influx transporters, efflux transporters, and enzymes, respectively.

Decision on whether to use perfusion- or permeability-limited model for a certain organ might be difficult to make, since both passive and active diffusion contribute to the overall drug transport. With PBPK modeling, it might be the best to test different hypotheses and analyze the outcomes, to eventually choose the most suitable drug-specific model.

As noted before, PBPK models for certain organs/tissues can be more specific to capture the complexity of physiological structure and processes.

In novel PBPK models, gastrointestinal tract (GIT) is represented as a series of separated compartments defined by a number of physiological parameters, including the expression of some major transporters and metabolic enzymes. Based on the relevant drug-related and physiological parameters, a series of differential equations is used to describe drug transport, dissolution, and absorption in various segments of the GIT.

Kidney model comprises an additional kidney tubule compartment to account for drug active and passive secretion and reabsorption, in addition to filtration and metabolic clearance. Kidney filtration can be estimated based on GFR, while the other transport processes (perfusion- or permeability-limited) can be simulated based on the input data from in vitro or animal studies (as for other tissues).

Hepatic PBPK models include the additional gall bladder compartment to account for biliary excretion of a drug. The simulated processes include emptying of the gallbladder over a defined period of time and reabsorption of a drug (enterohepatic circulation model). Thereof, the necessary input kinetic parameters regarding hepatic drug transport include intrinsic passive diffusion clearance ($CL_{int,pass}$), intrinsic uptake clearance ($CL_{int,uptake}$), and intrinsic biliary clearance ($CL_{int,bile}$), while basolateral efflux is usually neglected. Input hepatic clearance values for PBPK are usually obtained using SCH, and in this case, linear kinetics is assumed. An alternative is to use K_m and V_{max} values to simulate nonlinear hepatic drug clearance. These values can be input directly in a PBPK model or transformed into in vivo hepatic clearance based on a well-stirred hepatic model (described in "In Vitro/In Vivo Extrapolation" section).

There are different approaches to scale transporter-mediated intrinsic clearance obtained in vitro to the relevant in vivo parameters, some of them reviewed by Yang et al. (2016).

I. Simple scaling based on physiological parameters (e.g., liver weight and number of hepatocytes per g liver) often lead to overprediction or underprediction of the relevant in vivo values, and therefore, transporter (or compound)-specific SFs should be used. These empirical SFs can be estimated by comparing in vitro data to the estimated in vivo clearance from intravenous data (human or animal).
II. Another way to estimate in vivo clearance values from in vitro data is based on the quantitative data on transporter(s) expression/abundance.

In addition, relative expression factor (REF) and RAF were proposed as correction factors to

bridge the gap between data parameters obtained in different systems (e.g., human hepatocytes vs. recombinant systems or in vitro transport clearance vs. in vivo secretory clearance) and facilitate scaling of in vitro to in vivo data. These factors were initially introduced for metabolic enzymes, but the same concept has been applied to transporters. RAF refers to the difference in activity between the in vitro and in vivo system (based on intrinsic drug clearance in the tissue or V_{max}), while REF describes the difference in transporter expression between in vitro and in vivo system (based on relative mRNA or protein quantification). Absolute scaling factor that describes the difference in "functional transporter expression" (i.e., transporter expression based on absolute protein quantitation and activity) between in vitro and in vivo system can also be used, but kinetic data that link transporter expression with its function are scarce.

There are numerous examples of successful application of PBPK modeling approach to estimate the impact of transporters on drug disposition and DDIs. PubMed database search on terms "PBPK" and "transporters," performed on August 28, 2018, retrieved 133 results, whereas more than 60% of these publications emerged in the last 5 years.

Critical Assessment of IVIVC Methods

Despite the widespread use of IVIVE and in silico predictions to project ADME behavior of drugs, there are still many challenges associated with translation of in vitro to in vivo data. One of them is lack of precise data on transporter expression and activity in the in vitro systems, preclinical species, and humans. Furthermore, ethnic variability and polymorphism in the transporter-encoding genes may cause variations in transporter expression in different tissues and, consequently, distinct effects on a drug PK and DDIs. Statins are a typical example for the influence of transporter (OATP1B1) polymorphism on drug exposure to central and peripheral tissues, i.e., increased exposure to peripheral tissues may lead to myopathy. Prediction of drug-transporter interactions for patients with organ impairment or for specific population groups (pediatrics, geriatrics, pregnant women) is also challenging. It is well known that the expression and activity of transporters changes during aging and in certain physiological and pathological states. But due to the ethical reason, in it is not always feasible to assess the impact of transporters on drug disposition in vivo. Here, PBPK modeling might be a helpful alternative.

Another issue regarding IVIVE is related to the use of blood or plasma drug concentrations as a substitute for the unbound drug concentration in tissues to assess transporter-mediated drug interaction in vivo, which may be a reason for false results, e.g., if DDI is reflected on drug tissue concentration, but not on plasma concentration. In these cases, PBPK modeling may be a useful alternative to in vivo studies to estimate drug tissue concentration. Another available option is to determine drug distribution in different organs and tissues using imaging techniques such as positron-emission tomography.

PBPK modeling undoubtedly offers numerous advantages in addressing a wide range of PK issues. But, no matter how advanced, PBPK models are not self-sufficient, and they require input and/or validation data from in vitro and in vivo studies. Namely, due to the gaps in our current knowledge about human physiology and transporter expression and function, modeling strategy is often driven by assumptions. Also, there are issues regarding the validity of the in vitro data used as inputs or the conformance of the applied modeling approach. This has been elaborated in the review of Yang et al. (2016). In brief, scaling of transporter-mediated in vitro clearance values (or relevant K_m and V_{max} values), based on physiological parameters, may lead to underestimation or overestimation of the in vivo clearance values. In fact, most of the published examples regarding PBPK modeling of transporters' effect relay on the use of scaling factors to bridge the differences in the expression and activity of transporters between in vitro systems and preclinical species and humans. But as mentioned before, these values are compound-specific, system-specific, and species-specific, and their introduction

in a PBPK model brings some distrust in the prediction results.

But despite these limitations, it is rational to believe that new knowledge, and consequently, updates in PBPK models (e.g., regarding model components and organ substructure), will enhance predictability of these tools and lead to increased confidence in in silico results and more wider use of modeling tools.

Concluding Remarks

In recent years, considerable scientific progress, advanced technologies, and new knowledge have enabled deeper understanding of the role of transporter in drug disposition and DDIs. Huge efforts of a number of scientists, research groups, working parties, and regulatory organizations resulted in established methods for in vitro and in vivo assessment of transporter-mediated drug interactions. In addition, development of predictive mathematical models enabled translation of in vitro to in vivo data through the means of IVIVC/IVIVE. As a step upward, in silico PBPK models provided an integrative in vitro-in vivo-in silico platform to mechanistically explain drug interference with transporters. However, transporter science is evolving, and information presented in this chapter, including in vitro setups, decision criteria, IVIVE methods, etc., reflects current thinking on specific transporter issues, which may change when more transporter-related data become available.

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