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EXPERT REVIEW

Challenges of Using In Vitro Data for Modeling P-Glycoprotein Efflux in the Blood-Brain Barrier

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ABSTRACT The efficacy of central nervous system (CNS) drugs may be limited by their poor ability to cross the blood-brain barrier (BBB). Transporters, such as p-glycoprotein, may affect the distribution of many drugs into the CNS in conjunction with the restricted paracellular pathway of the BBB. It is therefore important to gain information on unbound drug concentrations in the brain in drug development to ensure sufficient drug exposure from plasma at the target site in the CNS. *In vitro* methods are routinely used in drug development to study passive permeability and p-glycoprotein efflux of new drugs. This review discusses the challenges in the use of *in vitro* data as input parameters in physiologically based pharmacokinetic (PBPK) models of CNS drug disposition of p-glycoprotein substrates. Experience with quinidine demonstrates the variability in *in vitro* parameters of passive permeability and active p-glycoprotein efflux. Further work is needed to generate parameter values that are independent of the model and assay. This is a prerequisite for reliable predictions of drug concentrations in the brain *in vivo*.

KEY WORDS blood-brain barrier · efflux transport · *in vitro—in vivo* extrapolation · p-glycoprotein · physiologically based pharmacokinetic modeling

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ABBREVIATIONS

BBB	blood-brain barrier
BCRP	breast cancer resistance protein
BMEC	brain microvessel endothelial cell
CL_{eff}	clearance related to (p-glycoprotein) efflux
CL_{pass}	clearance related to passive permeability
CNS	central nervous system
CSF	cerebrospinal fluid
$C_{u,brain}$	unbound concentration in the brain interstitial fluid
ER	efflux ratio
$f_{u,brain}$	unbound fraction in brain
ISF	brain interstitial fluid
IVIVC	*in vitro—in vivo* correlation
IVIVE	*in vitro—in vivo* extrapolation
K_m	substrate concentration required for half-maximal transport rate
$K_{p,uu}$	ratio of unbound drug concentrations in brain and plasma
PAMPA	parallel artificial membrane permeability assay
P_{app}	apparent permeability
PBPK	physiologically-based pharmacokinetics
PET	positron emission tomography
P_i	inorganic phosphate
PK	pharmacokinetic(s)
P_{pass}	passive permeability
PS	permeability-surface area product
QSPR	quantitative structure-property relationship
TEER	transendothelial electrical resistance
UWL	unstirred water layer
V_{max}	maximal rate of transport
$V_{u,brain}$	unbound volume of distribution in brain

INTRODUCTION

Proteins for transport of endogenous compounds and xenobiotics are widely expressed in the human body (1). The transporter proteins may influence drug clearance in the

liver and kidney, drug absorption in the gut, and they may limit or enhance drug distribution into various tissues. Drug disposition into the brain is influenced by the transporters in the blood-brain barrier (BBB). The BBB consists of tightly inter-connected endothelial cells in the brain microvessels that have low paracellular permeability and low levels of pinocytic activity (2). BBB transporters include solute carrier family members for regulation of amino acid and glucose transfer, as well as protective efflux transporters of the ATP-binding cassette family (3,4), such as p-glycoprotein (ABCB1 or MDR1) and breast cancer resistance protein (BCRP or ABCG2) (4,5). These efflux transporters will decrease drug concentrations in the brain, whereas influx transporters on the blood side of BBB will increase the uptake clearance of their substrate drugs. P-glycoprotein has been shown to limit brain uptake of drugs: in p-glycoprotein knockout mice increased brain uptake of ivermectin and vinblastine (6), digoxin and cyclosporine A (7), loperamide (8) and certain HIV-1 protease inhibitors (9) has been demonstrated. Even though the impact of p-glycoprotein in humans is less prominent than in the rodents, inhibition of p-glycoprotein by quinidine has been suggested to cause loperamide-induced respiratory depression, a sign of increased brain exposure (10). P-glycoprotein is also considered to be responsible for the lack of central nervous system (CNS) penetration and side-effects of non-sedative antihistamines (11–13). Furthermore, it has been proposed that the efficacy of antidepressants is dependent on the p-glycoprotein function in the BBB (14). In summary, p-glycoprotein recognizes an assortment of diverse substrates (15) and it is expressed in the BBB in mice (16) and humans (4). Therefore, it may be an important player in the BBB permeation of drugs.

Even though there are no specific regulatory guidelines on BBB permeation, both the European Medicines Agency and the Food and Drug Administration have acknowledged the influence of transporters on drug disposition (17,18). They recommend that p-glycoprotein and BCRP mediated transport of investigational drugs should be tested *in vitro*. Further *in vivo* tests are needed in selected cases on drug-drug interactions of inhibitors or inducers of p-glycoprotein with a substrate drug. Transporter-mediated drug-drug interactions in the BBB have, however, recently been shown to have only minor clinical significance (19).

In drug discovery, the BBB presents a dual role: its protective role is beneficial in preventing side-effects of peripheral drugs, but it sets constraints in the development of CNS drugs. Due to the restrictive role of the BBB drug concentrations in plasma may not be directly related to pharmacological effects in the brain (20,21). Unbound drug concentrations in the brain would be more relevant, but their direct measurement is limited to the labor intensive method of brain microdialysis. Drug exposure to the brain can be described by the ratio of unbound drug concentrations in the brain and plasma ($K_{p,uu}$) (22). The relationship between active transport and passive diffusion determines the $K_{p,uu}$ and informs about the relative significance of the active transport. It would be useful to evaluate the permeation of investigational drugs into the brain early in development (23,24). Various *in vitro* and *in vivo* methods have been developed for this purpose and they have been summarized elsewhere (23,24). For example, inhibitors or transporter knockout animals can be used *in vivo* to assess the role of specific transporters in drug disposition.

The paradigm in CNS drug research is to combine *in vitro* data on BBB permeability, active efflux/influx, and binding in plasma/brain with *in vivo* measures to elucidate brain exposure and predict $K_{p,uu}$ (24–26). To facilitate early pharmacokinetic (PK) predictions and selection of compounds for further development, the *in vitro* data could be used as input parameters for physiologically-based pharmacokinetic (PBPK) modeling. In PBPK modeling, the body is represented as organ compartments that are linked to each other by blood flow (27). Unlike traditional compartmental PK models, PBPK models offer a mechanistic approach in pharmacokinetics: they separate drug and system based variables and utilize physiological parameters (e.g. tissue volumes, protein expression) to scale *in vitro* parameters, such as intrinsic organ clearances, to the *in vivo* context (28). In PBPK models drug distribution to the organs may be limited by tissue permeability. This approach may help in understanding the significance of transporters in different barrier-like tissues. Therefore *in vitro—in vivo* extrapolation (IVIVE) of transporters' role has been discussed in the case of hepatic, renal and intestinal drug permeation (29,30). The same approach can be applied for drug distribution to the brain, as transporters may have an important role in drug access to the CNS.

Successful *in vitro* based PBPK modeling would allow early prediction of $K_{p,uu}$ and unbound drug concentrations in the brain, thereby facilitating the optimization of *in vivo* experiments. This approach would also help to understand the impact of individual processes, such as binding and active transport, in drug distribution to the brain. This would be useful in compound selection and lead optimization processes in drug discovery. PBPK modeling allows easy simulation of different dosing regimens, thereby providing predictions of saturation of carrier-mediated transport in the BBB. Theoretical simulations (31–34) have been used to understand concepts of brain pharmacokinetics and top-down PBPK modeling of *in vivo* rat data has been used to study drug distribution to the brain and to predict the human situation (35–40). However, there are only very few publications on *in vitro* based and transporter-related predictions of brainPK (41,42).

Several commercial software are available for PK predictions (43) and some of them, such as Simcyp Simulator

(Simcyp Ltd, United Kingdom), include specific modules for brain PK. The development of user-friendly software has facilitated PK modeling and eliminated the requirement of advanced skills in mathematics and programming of the user. However, the quality and usefulness of predictions depends on the relevance of *in vitro* input data, model structure and knowledge of the user in making reasonable assumptions in the model. Therefore, this review discusses the feasibility of predicting brain concentrations of p-glycoprotein substrates using *in vitro* and *in situ* data. We chose p-glycoprotein because its influence on drug distribution to the brain has been demonstrated in variouos publications. The issues of this review are applicable also to other efflux proteins.

As a framework, we used the brain PK model of p-glycoprotein efflux of Syvänen *et al.* (34) (Fig. 1). The parameters in this paper include passive permeability, Michaelis-Menten parameters of p-glycoprotein efflux (K_m and V_{max}) and drug binding to the brain tissue. Quinidine, a well-known p-glycoprotein substrate, is used as an example to demonstrate variability in the *in vitro* literature values and the impact of this variability on PK modeling.

BRAIN PHYSIOLOGY AND PHARMACOKINETIC MODEL STRUCTURE

For the purpose of modeling, the complex structure of the CNS must be simplified. One example of such a simplification is the three compartment brain PK model of Syvänen *et al.* (34). This model includes the compartments for plasma, BBB endothelial cells and brain interstitial fluid (Fig. 1). In the model, drug entry into the brain occurs through the BBB and is limited by BBB permeability. The dominance of this route is anticipated due to the dense network of microvessels in the brain providing a large surface area for diffusion (22). Some compounds may enter the brain via cerebrospinal fluid (CSF) that is contained in the brain ventricles. In this case, the drug gains access from the systemic circulation via the blood—cerebrospinal fluid barrier (BCSFB) of the choroid plexus and then further diffuses from the CSF to the brain interstitial fluid (ISF), but this access is limited by the bulk flow of ISF. Therefore, the CSF compartment has been discarded (34).

Syvänen *et al.* (34) generated a semiphysiological model as plasma PK is described using conventional compartmental modeling, but this part may be replaced with a more complex whole body PBPK model to describe plasma concentrations. The BBB endothelial cells are depicted as a separate compartment with a volume of 0.8 $\mu l/g_{brain}$ (44). Passive diffusion across the luminal and abluminal membranes is governed by Fick's law. P-glycoprotein is presented as a luminal efflux obeying Michaelis-Menten kinetics and pumping drugs back into the plasma according to the BBB intracellular concentration. Accumulation and binding of drug in endothelial cells is considered negligible. In plasma and brain instant equilibrium between the unbound and bound drug is assumed. The simulated unbound concentration in the brain reflects the concentration in the ISF and it is calculated using the unbound volume of drug distribution in the brain ($V_{u,brain}$). Next, we discuss the experimental methods that are available for quantification of passive permeability and efflux transport at the BBB as well as drug binding in the brain.

PASSIVE PERMEABILITY

Successful PBPK modeling of drug transport requires reliable estimates of both efflux/influx transport and passive permeability. The combination of these factors determines the net permeation across the BBB. In this review we concentrate on *in vitro* methods of generating input data for the models. However, in the passive permeability section we first describe the *in situ* brain perfusion technique as it is a common point of comparison for *in vitro* BBB permeability. *In silico* predictions of passive permeability are described shortly. If successful, these models would provide a very efficient way of deriving parameter estimates for passive permeability.

In Situ Brain Perfusion

In the *in situ* brain perfusion experiment the drug uptake into the brain of an anesthetized laboratory animal is measured

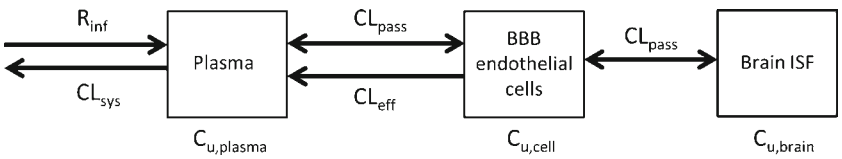

Fig. 1 Scheme of a simple compartmental model of drug entry into the brain, consistent with the model of Syvänen *et al.* (34). The boxes represent the three compartments: plasma, BBB endothelial cells and brain. Parameters related to systemic kinetics: R_{inf} = infusion rate, CL_{sys} = clearance, $C_{u,plasma}$ = unbound concentration in plasma. Parameters related to brain disposition: CL_{pass} = clearance related to passive permeability. Flux by passive diffusion is defined according to Ficks's law as CL_{pass}*(difference of unbound concentrations between compartments). CL_{eff} = clearance related to p-glycoprotein efflux, which is described by Michaelis-Menten kinetics as $V_{maxl}/(K_m+C_{u,cell})$, where V_{max} = maximum rate of transport and K_m = concentration required for half-maximal activity. $C_{u,cell}$ and $C_{u,brain}$ = unbound concentration in endothelial cells and brain, respectively.

over a short period (tens of seconds to minutes) (45). During this time the brain is perfused with drug solution (45). In the original method, the perfusion is performed via a catheter into the external carotid artery. Branch arteries are ligated and cut so that cardiac blood supply is cut off at the beginning of the perfusion and mixing of the perfusion fluid and systemic blood is minimized. After the perfusion, brain tissue is removed and analyzed for its drug content. Due to the short duration of the experiment, drug movement is assumed to take place primarily from the capillary lumen to the brain, although some compounds may show back diffusion (46). Assuming unidirectional uptake, the uptake clearance, K_{in} (or CL_{up}), can be calculated as follows:

$$K_{in} = \frac{X_{brain}}{T} \times \frac{1}{C_{perf}} \quad (1)$$

where X_{brain} is the amount of drug in the brain corrected for residual perfusate in brain vasculature, T is the duration of the perfusion and C_{perf} is the concentration of drug in the perfusate (47). Since K_{in} is dependent on the perfusate flow, it must be converted with the Crone-Renkin equation (Eq. 2) to obtain a flow-corrected clearance, commonly denoted as the permeability-surface area product (PS):

$$PS = -F_{pf} \times \ln\left(1 - \frac{K_{in}}{F_{pf}}\right) \quad (2)$$

where F_{pf} is the cerebral perfusion fluid flow rate during the experiment (45). The PS can be further derived to permeability (cm/s) by dividing PS with the surface area of the BBB. This allows comparisons to *in vitro* apparent permeability values.

The *in situ* brain perfusion method has been optimized for use in rats and mice (45,47). The method has been able to cover a 10,000—fold range of permeability values (45), but calculation of flow corrected permeability values for highly permeable compounds may require extra consideration (48). The method can be used with transporter-knockout animals or inhibitors to study transporter effects (49). The possibility to eliminate transporter effects enables studies on passive permeability if all relevant carrier-mediated pathways are inhibited. Rodent knockout models are available for two important ABC transporters at the BBB: p-glycoprotein (6,50–52) and BCRP (52,53). Although transporter knockout could in principle result in compensatory changes, such as up-regulation of other transporters this has not been seen in knockout mice models of p-glycoprotein and BCRP (54). However, use of the *in situ* brain perfusion method is limited in early phases of drug discovery, since it requires surgical skills and laboratory animals. So far, this method has been used for validation of *in vitro-in vivo* correlations of BBB permeability (see next section).

In Vitro Cell Methods

There has been interest in using *in vitro* cell models for predicting BBB permeability, but the development of a stable, reproducible, easily cultured model that maintains the morphological and functional properties of the BBB has been difficult (55). Efforts have been made to derive models from brain microvessel endothelial cells (BMECs) of different species, such as rat, mouse, pig, cow and human (55–57). Primary cell cultures require recurrent isolation of fresh cells, as the primary cells lose their BBB characteristics over time. On the other hand, immortalized cell models fail to preserve the cell layer tightness, which is a main feature of the BBB *in vivo* (55). Due to the tight junctions, the BBB has a transendothelial electrical resistance (TEER) of over 1,000 Ωcm^2 (58), whereas the BMECs in cultures show variable values, in the range of tens to hundreds of Ωcm^2 (57,59). A fully functional BBB model should not only enable easy measurement of net permeability, but it should show correct expression of all transporters that are expressed *in vivo* in the BBB. Use of specific inhibitors is needed to dissect the contribution of the carrier-mediated transport from the passive diffusion. This is difficult due to the number of different transporters expressed, and generally poor selectivity of the inhibitors. High concentrations of substrates or inhibitors may cause toxicity to cells when complete transporter inhibition is being sought (18).

Some cell lines from other origin than brain have been considered for use in BBB permeability studies. These include Caco-2 (colorectal adenocarcinoma) (60), MDCK (Madin-Darby canine kidney) (61–63) or LLC-PK1 (porcine kidney) cell lines (7,64). The cell lines can be used also as stable transfectants of p-glycoprotein. These epithelial cell lines are well characterized, more easy to culture than primary BMEC cells, and they show TEER values around 150 Ωcm^2 (LLC-PK1 (5), MDCK (65)) and 800 Ωcm^2 (Caco-2) (65,66). However, the tightness of MDCK cells varies from very high (> 1,000 Ωcm^2) to low (100 Ωcm^2) (67). The cell lines also show some endogenous transporter activity, for example MDCKII (a commonly used strain of MDCK cells) and LLC-PK1 cells show endogenous mRNA expression of canine or porcine forms of mdr1, mrp1 and mrp2 (68–70). To avoid the bias of transporters on passive permeability measurements, a low efflux MDCKII cell line has also been presented (70). The applicability of Caco-2 and MDR1-MDCKII cells as BBB surrogates has recently been assessed by Hellinger at al. (71), illustrating for example the difference between the cuboidal morphology of MDCK cells and the spindle-like brain endothelial cells. The question of possible effects of differing lipid compositions of the MDCKII and BMECs has also been raised (72). These differences may complicate the use of surrogate cell lines, but the significance of this issue is still unclear.

Permeability through cell monolayers is commonly assessed in the two chambered Transwell® system. Drug solution is applied to the apical or basolateral chamber and samples are withdrawn from the receiver side to quantify the amount of permeated drug. Apparent permeability (P_{app}) is calculated using the equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_0 \times S} \quad (3)$$

where dQ/dt is the rate of compound appearing in the receiver chamber, C_0 is the initial concentration in the donor compartment and S is the surface area of the insert. To prove *in vitro—in vivo* correlation (IVIVC) of permeability measurements, permeability data from *in vivo* brain microdialysis studies would be the most relevant point of comparison. Because microdialysis data is available only for a limited number of compounds, correlations are commonly performed against *in situ* brain perfusion data. Such *in vitro—in situ* correlations have been studied using several cell models: MDCK (61), MDR1-MDCKII (73), rat BMECs (74), porcine BMECs (75), bovine BMECs (60,76,77) and an immortalized human brain endothelial cell line (hCMEC/D3) (78). The reported correlations have been fairly good in small compound sets (up to $R=0.95$, $n=13$ (60), but in the larger sets of Polli *et al.* (61) ($n=22$) and Summerfield *et al.* (73) ($n=50$), correlations were only found for a subset of hydrophilic compounds. Interestingly, *in vitro* permeability showed a 100-fold range, whereas *in situ* permeability had more than a 1,000-fold range. These data suggests that in spite of the correlations, the magnitudes of permeability vary between *in vitro* and *in situ* models.

Some of the discrepancies between *in situ* and *in vitro* experiments may be due to the differences in transporter activity. In addition, there is an inherent difference between *in vivo* and *in vitro* conditions, which should be kept in mind (79). Whereas very lipophilic compounds may accumulate in the cell monolayer instead of passing into the receiver chamber *in vitro*, the ability of brain tissue to bind drugs *in vivo* deters this accumulation behavior (73). Therefore binding in brain may be one reason for disparity between *in situ* and *in vitro* results. Sampling practices are also different: *in vitro* only the receiver buffer is usually sampled, but the drug analysis *in situ* from the 'donor compartment' may reflect also the drug in the endothelial cells, unless capillary depletion (80) or other corrections are implemented. The *in vitro* permeability range can be limited by the leakiness of the cell lines and experimental conditions that have influence on the unstirred water layer (UWL) (81). The UWL may limit the measured permeability especially for highly permeable compounds (82). Depending on cell line and/or experimental setup, *in vitro* permeability values for the same compound can differ by as much as one order of magnitude (83). Also, the apparent permeability for the monolayer should be corrected for the resistance of the filter; otherwise permeability of lipophilic drugs may be underestimated (59,75). Finally, the permeability values *in situ* are influenced by the BBB surface area in calculations, but the estimates for BBB surface area range from 100 cm^2/g to 240 cm^2/g (48,60,73,77,81,84,85). This increases uncertainty in the values and hampers the *in vitro—in situ* comparisons.

Parallel Artificial Membrane Permeability Assay

The parallel artificial membrane permeability assay (PAMPA) is an *in vitro* method that is used to estimate passive permeability of compounds through lipid membranes (86). It measures the permeation of compounds on a 96-well plate through a phospholipid impregnated filter that mimics a cellular barrier. Although originally described as an intestinal absorption model, the composition has been modified for BBB permeability predictions by using, for example, porcine brain lipid as the phospholipid component (termed PAMPA-BBB) (87). PAMPA has been used to predict passive BBB permeability and to classify drugs as CNS positive or negative (72,87–89). Nevertheless, the quantitative equivalence of PAMPA with *in vivo* BBB permeability remains to be elucidated as many studies only compare PAMPA to *in vitro* cell assays (62,90,91) and not to *in situ*/*in vivo* data. Di *et al.* (72) reported correlation of $R^2=0.47$ for 37 compounds between PAMPA and *in situ* brain perfusion permeability, where transporter effects were claimed to be minor due to high drug concentrations in the perfusing solution. Improved correlation between PAMPA and *in situ* permeability of 85 weak bases has also been reported, but this required extensive mathematical treatment of data (92).

The rationale of the PAMPA method is based on the rate-limiting role of lipid membranes in passive BBB permeability. The paracellular route is assumed to be only a minor contributor in passive BBB permeation. PAMPA is also attractive, because it is a high throughput method that is mainly limited by availability of suitable fast analysis methods (86) and the throughput may be further increased by assaying multiple compounds at once in a cassette approach (90). However, despite being a fairly straightforward method, the results can be influenced by experimental factors (e.g. the UWL, membrane composition) (87,89,90,92,93). Correction for these effects may require additional experimental and calculation steps (94,95).

In Silico QSPR Approaches

The need to screen large compound libraries has led to an interest in using computational quantitative structure-property relationship (QSPR) models that would predict the BBB transport of the compounds based on molecular structure without experiments. Most published QSPR models of brain distribution

are based on experimental K_p data (also known as LogBB), denoting the ratio of total concentrations in the brain and plasma. As this parameter describes drug distribution rather than the permeability rate, these models are not discussed here and readers are referred other reviews (96,97). There are nevertheless a few publications that present QSPR models of BBB permeability based on *in situ* brain perfusion data (98–101). A linear free energy relationship (LFER) model based on Abraham's solvation parameters (102) was developed by Gratton *et al.* (98) and revisited by Abraham (99) with an extended dataset (n=30). Liu *et al.* (100) conducted a stepwise multivariate linear regression on 23 compounds lacking significant active transport, which resulted in a model based on $logD_{7.4}$, van der Waals surface area of basic atoms and topological polar surface area. These parameters are also often used to predict oral drug absorption (103). LogP and hydrogen bond formation properties were also among the important descriptors in the QSPR model of Lanevskij *et al.* (101). This study involved a large dataset (more than 150 observed values), but unfortunately the data was diverse in terms of experimental methods and species.

The QSPR models have shown some good correlations between predicted and experimental permeability values. However, they can be criticized based on the lack of validation, small datasets, limited chemical space in the training set, and/or quality problems in the permeability data. It is encouraging that the predicted values seem to mainly reflect passive permeability properties as actively transported compounds are clear outliers (100). The fair success of the models indicates that a QSPR model may be applicable for early permeability prediction and selection of compounds from chemical libraries. QSPR models are only reliable in chemical space covered by the training set. This should be kept in mind when making predictions for novel compounds.

PARAMETERS TO DESCRIBE P-GLYCOPROTEIN EFFLUX

The complexity of the p-glycoprotein binding site(s) and its function complicates the assessment and description of its interaction with drugs. It is thought that p-glycoprotein works by taking up its substrates from the cell membrane and extruding them into the extracellular medium (the vacuum cleaner mode) or by transferring them from the cytosolic membrane leaflet into the outer leaflet (the flippase mechanism) (104). Multiple binding sites of substrates and modulators have been suggested based on drug-drug interactions in *in vitro* experiments (105,106), which is also supported by the x-ray structure of murine p-glycoprotein (107).

A common way of quantifying p-glycoprotein efflux *in vitro* is by assaying the P_{app} of compounds across p-glycoprotein expressing cell monolayers at a single concentration in both apical to basolateral ($P_{app,A-B}$) and basolateral to apical direction ($P_{app,B-A}$) and further calculating the efflux ratio (ER), defined as:

$$ER = \frac{P_{app,B-A}}{P_{app,A-B}} \tag{4}$$

Compounds showing ER > 2.0 are usually classified as p-glycoprotein substrates (108,109). *In vitro* ER has recently been used to reconstruct *in vivo* $K_{p,uu}$ values in mice for 11 common p-glycoprotein substrate drugs (110). However, ER results from the interplay of active and passive transport and, for example, passive paracellular diffusion may be over-emphasized in too leaky *in vitro* cell models. ER may also be insensitive to high permeability compounds, such as verapamil, a well-known p-glycoprotein substrate that commonly shows ER below 2.0 in monolayers (64,108,111). Also importantly, ER does not take into account the saturable nature of efflux. To include concentration dependency, p-glycoprotein-mediated transport, like enzyme-substrate interactions, may be described using Michaelis-Menten kinetics (1) and clearance related to p-glycoprotein efflux (CL_{eff}) is described as:

$$CL_{eff} = \frac{V_{max}}{K_m + C} \tag{5}$$

where V_{max} is the maximum transport rate, K_m is the substrate concentration required for half-maximal transport and C is the unbound concentration of substrate at the binding site. However, unlike water-soluble enzymes, that interact with drugs in the aqueous phase, p-glycoprotein interaction can take place only if the substrate permeates into the membrane. This complicates the estimation of intrinsic V_{max}, K_m and the concentration at the binding site (112). Further complication arises from the fact that some compounds show inhibition rather than saturation at high concentrations in *in vitro* assays. For these cases, a modified Michaelis-Menten equation, with one high affinity activating binding site and one low affinity inhibitory site, has been suggested (113). Also other mathematical models for p-glycoprotein efflux have been presented (114–116). The next sections will concentrate on methods used to quantify the Michaelis-Menten parameters of p-glycoprotein efflux. Although they can be used to compare or categorize compounds (108), the challenge is to further optimize these assays to derive reliable values for the parameters.

Assays Using Membrane Vesicles

Drug-transporter interactions can be studied in membrane vesicle preparations that are derived from cells expressing the transporter of interest. Although transporter enriched membrane vesicles can be made from different types of cells, they are most commonly prepared using baculovirus infected *Spodoptera frugiperda* (Sf9) insect cells (117). In those preparations, some membranes will be in the inside-out conformation

having the inner leaflet facing the buffer outwards (Fig. 2). In these vesicles substrates of efflux transporters (e.g. p-glycoprotein) will be pumped into, rather than out of the vesicles. Only the inside-out vesicles will show active transport as only those transporters will have their ATP binding site accessible to the ATP in the buffer.

The use of vesicles has been rationalized by assuming that, due to the conformation, the measurable concentration (i.e. the buffer concentration) represents the binding site concentration better than the drug concentration in the buffer of whole cell assays. However, as hydrophobic substrates seem to access the p-glycoprotein binding site from inside the membrane (104), buffer concentration may misrepresent binding site concentration even in the inverted conformation. Consequently, experimental factors affecting drug partitioning into the membrane such as pH conditions may alter results (118). The level of cholesterol, which is lower in insect than in mammalian cells (119,120), may also affect the apparent K_m through changes in partitioning and possibly p-glycoprotein function (121,122).

Membrane vesicles are commonly used in two different assay systems to study the efflux kinetics of a substrate drug (117). One method is the ATPase activity assay, which is based on the ATP dependency of active transport, where activation of the transporter leads to ATP hydrolysis and liberation of inorganic phosphate (P_i) (123). When test compounds are incubated with membrane vesicles, the amount of liberated P_i can be measured with appropriate colorimetric assays. When this assay is performed over a concentration range of the test compound the K_m of the compound can be calculated. The ease of detection and possibility for high

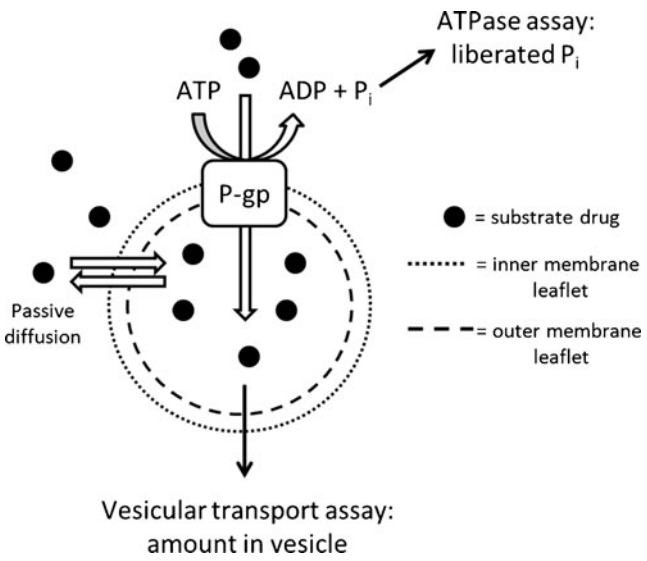

Fig. 2 Scheme of the principles of the efflux studies in inverted membrane vesicles. The ATPase assay measures the P_i liberated by p-glycoprotein during substrate transport, whereas the vesicular transport assay measures the amount of substrate accumulated into vesicles. P-gp = p-glycoprotein, ATP = adenosine triphosphate, ADP = adenosine diphosphate, P_i = inorganic phosphate.

throughput is a significant asset of the ATPase assay. However, the ATPase assay is an indirect assay as it measures the maximal rate of P_i liberation and not actual transport. Therefore, unless the amount of released P_i per transported drug molecule is known, the V_{max} from this assay cannot be used in modeling. In addition, contrary to ER measurements in cell monolayers, the ATPase assay may show insensitivity for low permeability compounds (108).

The vesicular transport assay measures the ATP-dependent uptake of substrates from buffer into the inside-out vesicles (124). Unlike the ATPase assay, this assay can be used to calculate both the K_m and V_{max}, because the transport of substrate into the vesicles is measured (Fig. 2). V_{max} must, however, be scaled based on the transporter expression, taking also into account the portion of the vesicles that is in the active, inside-out conformation (117). In comparison to the ATPase assay, analytics in the vesicular transport assay is more demanding as a specific and sensitive analysis method, such as mass spectrometry, is needed to detect the substrate in the vesicles. For p-glycoprotein, the assay has been used successfully with low permeability substrates, such as vinblastine and colchicine (124,125). However, the direct vesicular transport assay is less suited to medium or highly permeable compounds, because high passive diffusion allows them to escape from inside the vesicles (117) and, therefore, it may have limited use for many p-glycoprotein substrates, which are often hydrophobic (104) and thus show high passive permeability.

Transcellular Monolayer Experiments

In addition to assessment of ER, permeability measurements from cell monolayers have been used to determine the Michaelis-Menten parameters for p-glycoprotein efflux (126–132). Unlike ER, which is based on permeability at a single concentration, V_{max} and K_m are solved by fitting permeability values determined over a concentration range. Different mathematical models for analyzing *in vitro* permeability data have been developed (133). The standard equation is consistent with a single barrier model (Fig. 3), where cellular space is excluded and initial buffer concentration is related to efflux activity.

$$J_{A-B} = P_{app,pass} \times S \times C_0 - \frac{V_{max} \times C_0^r}{K_m^r + C_0^r} \quad (6)$$

where J_{A-B} is the measured flux in the apical to basolateral direction, $P_{app,pass}$ is the passive permeability, S is the surface area, C_0 is the initial concentration in the apical chamber and r is the Hill coefficient (optional) (130). The drawback of this model is that it does not take into account the real drug concentration at the p-glycoprotein binding site, inside the lipid bilayer, but uses the drug concentration in the buffer

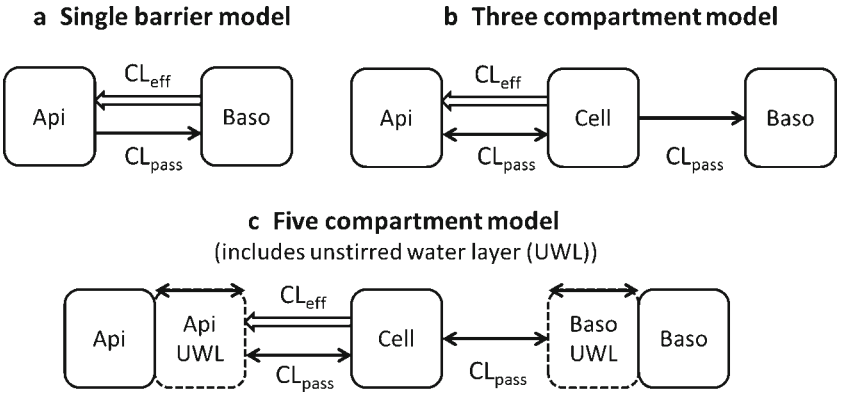

Fig. 3 Schemes of models used to describe monolayer permeability data in the apical to basolateral direction and calculate P_{app}, V_{max} and K_m. CL_{pass} = clearance related to passive permeability, CL_{eff} = clearance related to p-glycoprotein efflux, which obeys Michaelis-Menten kinetics (Eq. 5). Api and Baso refer to the apical and basolateral chambers, respectively. Note that in a. CL_{eff}, is in fact related to the apical concentrations (see Eq. 6), contrary to the other two models, where it is related to intracellular concentrations. Schemes a, b and c based on references (130), (134) and (131) respectively.

(129). These two concentrations may be different, particularly in the case of low permeability compounds. Due to this, although constant by definition, K_m values calculated with Eq. 6 show dependency on p-glycoprotein expression level, which has been explained by high efflux that decreases intracellular drug concentration (129,130). Although the actual binding site concentration of the substrate drug is difficult to estimate, even consideration of intracellular concentrations in fitting seems to improve the stability of K_m estimates in different cell lines (131,132,134). V_{max} estimates between the single-barrier and three compartment model seem to show less variation than K_m (i.e. less than two fold) (134). Nevertheless, V_{max} is dependent on the expression of the transporter and this needs to be taken into account in modeling and IVIVE. Schematic representations of three models that have been used for calculations are shown in Fig. 3. Other models that take into account the asymmetry of the apical and basal membranes or include binding to these membranes have also been presented (132,135). Although currently demonstrated with only a few compounds, the improved robustness of estimates supports the use and further development of these comprehensive models to achieve reliable estimates for the intrinsic parameter values. This is also supported by theoretical considerations and simulations (136,137).

It should be noted that assay conditions may affect the measured permeability values of the drug. For example, the UWL or direction of transport, can distort the kinetic parameters of efflux, if these factors are not correctly taken into account in the calculations (129,131,138–140). As these experiments are commonly performed with transfected cell lines (such as LLC-MDR1 and MDR1-MDCKII) that express several endogenous transporters, expression of these other transporters may obscure determination of Michaelis-Menten parameters for a single transporter. Standardization with the native cell line may not always help, since transfection can sometimes alter the expression of endogenous transporters (69,70). In addition to a complex endogenous background, monolayer experiments have lower throughput and require longer preparation than Sf9 membrane vesicles. Typically, it takes at least several days to reach confluence and form the tight cell layer that is needed in the experiments.

BINDING IN BRAIN

Non-specific drug binding in brain tissue can be evaluated *in vitro* by measuring the unbound fraction ($f_{u,brain}$) in brain homogenates with equilibrium dialysis (141) or by determining the apparent unbound volume of distribution in the brain ($V_{u,brain}$). $V_{u,brain}$ can be assessed *in vivo* in brain microdialysis studies (142), but to facilitate the use of this parameter in drug discovery, an *in vitro* method using 300–400 μm thick brain slices has been developed and optimized (143–145). Importantly $V_{u,brain}$ relates the total concentration of drug in the brain (A_{brain}) to the pharmacologically relevant, unbound concentration in the brain ISF, $C_{u,brain}$ (145):

$$C_{u,brain} = \frac{A_{brain}}{V_{u,brain}} \tag{7}$$

$V_{u,brain}$ and $f_{u,brain}$ are often used in a similar manner, as assuming a density of 1 g/ml for brain tissue, $1/f_{u,brain}$ should in principle equal $V_{u,brain}$ (22). Nevertheless, this is not the case for drugs that are unevenly distributed between extra- and intracellular spaces, because homogenization destroys distinct cellular spaces. To examine the significance of this, the difference between brain homogenate and brain slice data has been compared (144,146,147). Even though the overall correlation between the methods was good ($R^2 = 0.78$, $n = 56$), the homogenate method consistently underestimated the intracellular accumulation of the drugs (147). Therefore, the slice

method may be preferable. However, a correction for the accumulation of ionizable drugs, based on pH partitioning, can be applied to convert homogenate based $f_{u,brain}$ to $V_{u,brain}$ values (147). The drawback of slice based $V_{u,brain}$ determinations is that very lipophilic compounds require long incubation times, but these can be shortened by decreasing the ratio of buffer to slice volume (145). Throughput of brain slice as well as homogenate methods can be increased by pooling compounds (145,148).

DISCUSSION

The importance of transporters on the disposition of drugs is now well recognized, especially regarding the entry of drugs into the brain (1,109). It is the complex interplay of passive and transporter mediated processes that govern the brain exposure of drugs (22,34) and, therefore, transporter effects should be studied in drug development. Achieving reliable predictions of p-glycoprotein efflux in the BBB is important in the development of CNS drugs to ensure adequate entry into the brain. Transporter IVIVE and PBPK modeling is used to predict drug absorption and systemic exposure. This approach may have additional value in CNS drug development, because measurement of drug concentrations in the brain *in vivo* is challenging and expensive. The methods covered in this review are commonly used in drug development to study efflux or to screen drugs for further development. The main advantages and disadvantages of the methods of studying passive permeability and efflux parameters are summarized in Figs. 4 and 5. It is important that the measured parameters give accurate and reliable information and they are utilized to their full potential. This could mean their use for PBPK simulations and not limiting their use to categorization of drug candidates. Recent reviews on IVIVE of transporters in intestinal PBPK simulations and predictions of drug-drug interactions have pointed out the need for reliable estimates of intrinsic kinetic parameters, as method dependent values may give misleading information (30,149). We agree with this view and highlight these issues using quinidine, a well-characterized p-glycoprotein substrate, as an example and discuss the findings for quinidine in conjunction with the general findings in literature.

Quinidine was chosen as an example drug due to the availability of *in vitro* data on p-glycoprotein kinetics. We collected K_m, V_{max} and passive permeability (P_{pass}) values from *in vitro* studies with MDR1-MDCKII and Caco-2 cells (Table I). Table I shows the variation in the parameter values. The most notable outlier in the data is the 5 compartment model V_{max} and P_{pass}. We also searched the literature for reports on the passive permeability of quinidine based either on inhibitors, low expression of transporters or functional knockout in different *in situ, in vitro* and *in silico* systems (Table II). In an attempt to evaluate the kind of *in vivo* estimates of steady-state brain exposure these parameters would give, we used Eq. 8 below, modified from Eq. 14 in Syvänen *et al.* (34), to calculate $K_{p,uu}$ at binding site concentrations well below K_m:

$$K_{p,uu} = \frac{CL_{in}}{CL_{out}} = \frac{CL_{pass}}{CL_{pass} + CL_{eff}} = \frac{CL_{pass}}{CL_{pass} + \frac{V_{max}}{K_m}} \quad (8)$$

where CL_{in} and CL_{out} are the inward and outward clearances to the brain. This $K_{p,uu}$ value describes the worst case scenario, where efflux is working at full capacity. As V_{max} is reported here in proportion to surface area, P_{pass} may be used in Eq. 8 in place of CL_{pass}, which is equal to P_{pass} multiplied by surface area.

Passive Permeability

The correct determination of passive permeability for the simulations is very important as it not only affects equilibration time, but also $K_{p,uu}$ (34) is affected. The P_{pass} range in Table I is roughly five-fold (excluding the 5 compartment data), and there is little difference in this parameter between Caco-2 and MDR1-MDCKII cells. The permeability values are also in the same range as the additionally collected values of passive permeability, although these show a wider, 10-fold, variability (Table II). The *in vitro* permeability tends to be lower than that measured *in situ,* which may be partly explained by the fact that the UWL has been shown to decrease *in vitro* measurements of quinidine permeability (129,131). These errors arising from UWL effects may be larger for more lipophilic compounds (82). Permeability in mdr1a-/- knockout mice was slightly higher than values from the rat using an inhibitor, although in general, correlation (R^2=0.898, n=21) between rat and mouse BBB permeability suggests that species differences are small (46).

The challenge of quantitative IVIVC of permeability measurements still remains. The IVIVC link is still lacking for permeability measurements in the cell monolayers and PAMPA. This is complicated by the poor availability of *in vivo* microdialysis data, as validation using *in situ* perfusion data should be regarded with caution, due to the experimental differences (73). All in all, care should be taken in all measurements to ensure that no transporters or experimental factors are biasing the measured passive permeability and that assay conditions are relevant to the *in vivo* environment (79). Optimized PAMPA and QSPR models could be considered for generating data. However, as of yet QSPR seems more applicable for quick screening, as errors above two-fold are not uncommon, even in the successful models (100).

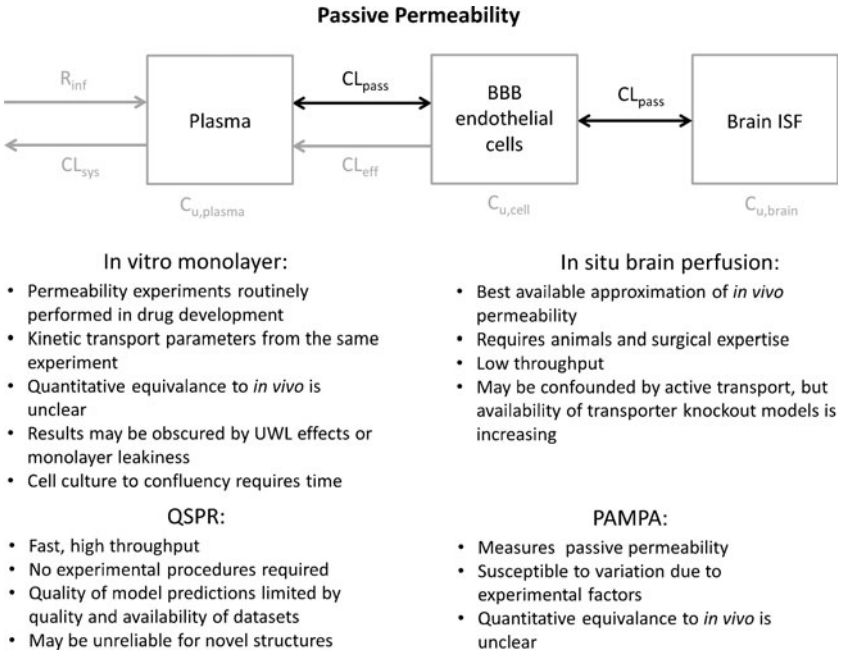

Fig. 4 Summary of the advantages and disadvantages of different methods of measuring passive permeability. See legend of Fig. I for description of abbreviations.

Efflux Kinetics

Estimating intrinsic kinetic parameters of active transport, for example p-glycoprotein mediated efflux, is perhaps the greatest obstacle in current BBB modeling. Both K_m and V_{max} have their own challenges. Table I indicates the low variability in K_m between both cell lines and between fittings achieved using the 3 and 5 compartment models that relate p-glycoprotein efflux to intracellular concentrations, discussed in section "Transcellular Monolayer Experiments". Similar more stable predictions of K_m were also achieved for verapamil and vinblastine with the 3 compartment model compared with the single-barrier model by Tachibana *et al.* (134).

As K_m values can also be generated using the inverted membrane vesicles, which could improve throughput and reduce costs, we collected K_m values of quinidine also from ATPase assays. These reported K_m values varied between 5.42 and 13.7 μM (62,64,128), a similar range as the K_m values from monolayer experiments in MDR1-MDCKII cells or Caco-2 monolayers (3.1–27 μM) based on the single-barrier model (129–131). The values are clearly higher from the more robust estimate of roughly 0.3 μM, questioning the notion that binding site concentrations are well described by buffer concentrations in assays using inverted membranes vesicles. Unfortunately, we did not find any reports on K_m values of quinidine from the vesicular transport assays.

The system-dependency of V_{max} is due to the expression levels of transporter and its correction using scaling is an important step in the translation of *in vitro* results to the *in vivo* environment. In Table I we see that V_{max} values from Caco-2 cells are lower than from MDR1-MDCKII cells. This can be explained by the lower p-glycoprotein expression in Caco-2 cells based on Western blots (130). However, the recently published absolute proteomic data for Caco-2 (150) and MDR1-MDCKII (70,151) indicate that p-glycoprotein expression may be more prominent in Caco-2 cells (30). This example highlights the complexity of scaling and the discrepancies arising between scaling methods, issues

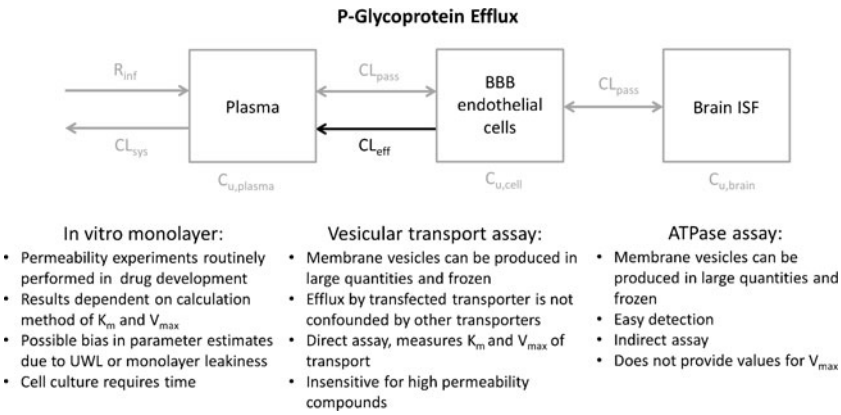

Fig. 5 Summary of the advantages and disadvantages of different methods of determining K_m and V_{max} of efflux. See legend of Fig. I for description of abbreviations.

Table I K_m, V_{max} and P_{pass} Values of Quinidine from Kinetic Studies in Cell Monolayers and $K_{p,uu}$ Values Calculated Based on These Parameters

Cell line	Model used on *in vitro* data [a]	K_m (μM)	V_{max} (fmol/s/cm^2)	P_{pass} (*10^{-6} cm/s)	Calculated [b] $K_{p,uu}$	Reference
MDR1-MDCKII	Single-barrier	13	920	72	0.50	(131)
		16.4	220.8	14 [c]	0.51	(130)
		27	1,550	51	0.47	(129)
	3 compartment	0.339	291	34	0.038	(134)
	5 compartment	0.23	5,670	1,970 [d]	0.074	(131)
Caco-2	Single-barrier	9	470	69	0.57	(131)
		1.69	12.9	14 [c]	0.65	(130)
		3.1	164	53	0.50	(129)
	3 compartment	0.23	17	28.2	0.28	(134)
	5 compartment	0.28	1,970	1,970 [d]	0.22	(131)

Note! The parameters from references (130,134) are based on the same *in vitro* data

[a] For schematic representation of the mathematical models used to calculate the parameters from *in vitro* data, see Fig. 3 and for more details refer to the cited references

[b] $K_{p,uu}$ was calculated using Eq. 8 and describes the equilibrium state at concentrations well below K_m at the transporter drug binding site

[c] Permeability value estimated from Fig. 4 in Shirasaka *et al.* (130)

[d] Includes permeability of both ionized and unionized species assuming pKa of 8.51 (131)

which have been recently covered in detail by Harwood *et al.* (30). In short, despite advances in measuring absolute protein expression with mass spectrometric methods, accurate scaling factors can only be defined when further research has elucidated the confounding factors in proteomic analysis and scaling. Results may be biased due to biological factors, sample treatment or methodological issues such as membrane extraction.

Table II Reported Values of Quinidine Permeability from Studies Using *In Silico*, *In Vitro* (apical to basolateral direction) or *In Situ* Methods

Method	P_{app} [a] (*10^{-6} cm/s)	Reference
QSPR	63	(100)
PAMPA	12.9	(62)
PAMPA	28.8	(90)
MDCK	8.0	(62)
MDCK + inhibitors [b]	10.9–13.3	(162)
MDR1-MDCK + inhibitors [b]	11.5–12.6	(162)
LLC-PK1	18.8	(173)
LLC-PK1	57.2	(110)
Rat *in situ* perfusion + inhibitors [b]	26.5–33.1	(162)
Rat *in situ* perfusion + GF-120918	22.0	(174)
Rat *in situ* perfusion + CsA	47.0	(63)
mdr1a-/- knockout mouse *in situ* perfusion	74.3	(175)
mdr1a-/- knockout mouse *in situ* perfusion	102	(176)

Permeability is assumed to be mainly passive based on inhibition, knockout or low expression of p-glycoprotein

[a] All *in situ* results are flow corrected and transformed into permeability values using BBB surface area of 100 cm^2/g_{brain} (177) to enable comparison

[b] Inhibitors used were GF-120918, cyclosporine A (CsA) and PSC 833 (162)

An important missing *in vitro - in vivo* link for transporters is also the relationship between protein expression and transporter activity, which would eliminate the assumption that all expressed protein is functional. Proteomic analysis of p-glycoprotein expression in MDR1-MDCKII cells resulted in the values of 1.32 (151) and 1.90 fmol/μg protein for p-glycoprotein (70). Expression of murine p-glycoprotein in mdr1a transfected LLC-PK1 cells is reported to be higher, 15.2 fmol/μg protein (110). This is close to the expression level in mouse brain endothelial cells (16,54). In the case of MDR1-MDCKII cells, however, the difference to *in vivo* mouse would be almost 10-fold, highlighting the significance of scaling. In addition to mouse, proteomic data of BBB transporters is currently available for monkey (152) and human (4) (Table III), and the increase in this kind of data will hopefully aid in bridging the gap between *in vitro* and *in vivo* systems (153). A successful example of IVIVE based on p-glycoprotein proteomic data from the BBB has already been published (110). It should however be kept in mind that these scaling factors assume that transporter expression is uniform throughout the BBB, although this might not be the case for p-glycoprotein (154).

Predicting *In Vivo* $K_{p,uu}$

The $K_{p,uu}$ values calculated with the reported K_m, V_{max} and P_{pass} values according to Eq. 8 are around 0.5 for the single-barrier fittings, but clearly lower for the 3 and 5 compartment fittings (0.038 and 0.074 for MDRI-MDCKII and 0.22 and 0.28 for Caco-2) (Table I). An *in vivo* $K_{p,uu}$ value of 0.17 has been reported for quinidine in rats after continuous infusion approaching steady-state using microdialysis to record brain concentrations (155). Without any scaling of V_{max}

Table III Quantitative Protein Expression of P-glycoprotein in Isolated Brain Microvessels, Cell Lines or Vesicle Preparations

	P-glycoprotein Expression Level (fmol/μg protein)	Reference
Mouse Brain Microvessels	14.1 [a]	(16)
Chinese Adult Monkey Brain Microvessels	5.68 [a]	(152)
Human Brain Microvessels	6.06	(4)
	3.98	(178)
hCMEC/D3	3.87	(179)
Caco-2	4.00 (at 10 days) 7.89 (at 29 days)	(150)
LLC-MDR1	15.2	(110)
MDR1-MDCKII	1.91	(70)
	1.32	(151)
MDR1-Sf9 membranes	73.41 [b]	(151)
HEK-MDR1 membrane vesicles [c]	8.15 32.0	(150)

[a] Average of measurements using two different peptides

[b] Average of two lots

[c] Results from two different batches

or knowledge of the degree of saturation of efflux in the *in vivo* situation, the single-barrier $K_{p,uu}$ values seem to underestimate the influence of efflux. However, there may be substrate specific species differences in p-glycoprotein function, although some results have been contradictory in this respect (62,156–161). Therefore, comparison of *in vitro* results for human p-glycoprotein to rat *in vivo* data may be uninformative.

What is interesting to note is that the *in vitro* data Shirasaka *et al.* (130) performed single-barrier analysis on, is the same as the data Tachibana *et al.* (134) used for their 3 compartment modeling. Therefore one would expect to reach a similar equilibrium with parameters from both fittings if the values really reflect independent, intrinsic parameter values. Unfortunately this was not the case. For example, from the Caco-2 data, the single-barrier parameters predict $K_{p,uu}$ of 0.65 and the three compartment model yields a $K_{p,uu}$ of 0.28. It is also of interest to note that despite higher expression of p-glycoprotein in MDR1-MDCKII cells and differences in V_{max} and K_m values, calculated $K_{p,uu}$ values are quite similar for all the single-barrier data. Differences may also rise in terms of equilibration rates, particularly the compounds with low passive permeability and low efflux will result in slow equilibration. For example the equilibration time will be much longer using MDR1-MDCKII single-barrier data from Shirasaka *et al.* (130) than the models of Heikkinen *et al.* (131) or Korjamo *et al.* (129). This may be important when examining *in vivo* exposure after a single dose or in other non-steady state situations.

As ER values are also used to predict the influence of efflux, we collected ER values for quinidine to show that this parameter shows variability too. Values of 11.4 (90), 16.1 (62), 27.2 (108) and even up to 338 (63) have been reported in MDR1-transfected MDCK cells and 2.56 and 5.99 in Caco-2 and LLC-MDR1 cells, respectively (64). ER can be viewed as the reciprocal of $K_{p,uu}$ and in this view, the $K_{p,uu}$ values from the 3 and 5 compartment models are also closer to the *in vitro* ER values than the single-barrier parameters. Whereas $K_{p,uu}$ calculated with Eq. 8 reflects equilibrium only at concentrations well below K_m, using ER to simulate *in vivo* concentrations may give erroneous results if the simulated concentrations are very different from the concentration used in the *in vitro* assay as the saturation state of efflux may be different.

The variation in reported parameter values is the result of system variability (e.g. p-glycoprotein expression in cell line and monolayer tightness), possible variability in assay conditions (e.g. shaking or sampling time) and the mathematical model used to resolve the parameters. It is important to realize that although the more stable K_m value might be seen as a step towards the real intrinsic values, the same has not been achieved for V_{max} and P_{pass}. This is exemplified by comparing V_{max} and P_{pass} values between the 3 and 5 compartment fits. Both of these values are significantly higher for the 5 compartment than the 3 compartment fit, despite similar calculated $K_{p,uu}$ values. P_{pass} and V_{max} are not independent and, therefore, transferring the data to different systems should be regarded with caution. For example, we explored the possibility of combining permeability measured *in situ* in rats using p-glycoprotein inhibitor PSC 833 (33.1 $*10^{-6}$ cm/s (162)) with the *in vitro* K_m and V_{max} in Table I for $K_{p,uu}$ predictions. Although change in $K_{p,uu}$ was moderate for most cases, unsurprisingly much lower $K_{p,uu}$ values (around 50-fold lower than with *in vitro* P_{pass}) were predicted for the 5 compartment data as the original *in vitro* permeability is almost 60 times higher.

Additional Considerations

Although the main focus of this article has been on passive permeability and kinetic parameters of efflux, a few additional considerations concerning PBPK modeling of brain concentrations may be highlighted. Since unbound concentrations in the brain may be considered as the important link to pharmacodynamics effects in the CNS (21), incorporation of binding in the brain is essential in the models. Based on literature data, it seems that drug binding in brain tissue can be measured reasonably well *in vitro* with brain slices. As $V_{u,brain}$ does not determine the unbound steady-state concentration, although it affects the time required to reach steady-state (34), some variability may be tolerated. Although determination of $V_{u,brain}$ uses isolated brain tissue from animals, it is not necessarily a limitation in extrapolation to humans, as binding measured in brain homogenates seems to be fairly independent of species as

shown by excellent correlation and quantitative similarity of $f_{u,brain}$ (n=47) between 6 species (including human) and 2 rat strains (163). Similarity across species has been reported by others with smaller datasets (148,164). Coming back to the quinidine example, rat $f_{u,brain}$ values of 0.0392 (146), 0.0364 (155) and 0.09 (147) have been reported for quinidine. Results from mouse brain tissue are similar (71,165). However, in rat brain slices quinidine $V_{u,brain}$ has been measured at 38 ml/g_{brain} (147), which is slightly higher than expected based on $f_{u,brain}$, but unsurprising as quinidine is known to sequestrate into lysosomes (131).

One of the current challenges of brain PK modeling is the lack of *in vivo* data for validation, as plasma concentrations cannot be used to validate these models. Experimental methods that can be used to generate concentration-time data include brain microdialysis and imaging techniques, most commonly positron emission tomography (PET). Although sometimes used, CSF samples provide data of only limited value as deviations between CSF and brain concentrations may occur depending on properties of the drug (166,167). Microdialysis is the only currently available method of measuring unbound brain concentrations *in vivo* over a time period, but it is rarely applicable to humans due to its invasiveness. The method is sensitive to experimental factors and may be unsuited for very lipophilic compounds due to adsorption (for review on microdialysis, see (168)). In contrast, PET imaging is non-invasive and applicable also to humans. The disadvantage of PET is that it requires radiolabeling of drugs and only measures the radioactivity of total drug in the brain (169,170). Concentration data, therefore, requires conversion to unbound values (171) and may additionally be confounded by radiolabeled metabolites being formed in the body (169). Despite these drawbacks, PET is a key link in enabling the development of models for human predictions and studies of interspecies differences and scaling.

Lastly, this review has concentrated on the generation of *in vitro* input data and its limitations, but it is important to remember that also the structure of the model and the processes included in it are critical to the success of predictions. In this sense, the model of Syvänen *et al.* (34) is likely to require further development for *in vivo* predictions to accommodate the complexity of the CNS. Although p-glycoprotein has been considered here as the sole transporter significantly affecting brain exposure, the influence of other transporters at the BBB should not be forgotten. The reality may be far more complex with substrates of p-glycoprotein also interacting with BCRP or other transporters, that have yet to be studied in detail, leading to surprising additive effects (29).

Future Perspectives

High hopes have been presented for the future of PBPK modeling (172). More studies are still required to help recognize the factors that cause obscured parameter values instead of intrinsic ones. This has now been studied *in vitro* but these advancements in understanding have not yet been extended to successful *in vivo* predictions. In coming years we shall hopefully see an increasing number of reports on brain PBPK models with careful IVIVE using proteomic data, which will help to clarify the issues related to extrapolation. This requires improvements in *in vitro* investigations, and increased availability of *in vivo* data for validation. Quinidine is an exception in that its interaction with p-glycoprotein has been extensively explored *in vitro* and *in vivo*. A key factor for future IVIVE is the scaling of V_{max}, which will require knowledge of p-glycoprotein expression in both cell lines and in *in vivo* tissue, in this case the blood-brain barrier. Without this scaling it will be difficult to recognize and rule out other system related effects on *in vitro* parameters.

Before we can obtain independent *in vitro* parameter values, we would encourage researches to use the same model structure (preferably including cellular space) for processing *in vitro* data to resolve parameter values and *in vivo* simulations, to avoid distortion of relationships between calculated parameters caused by moving them from one system to another. Although we present other possible methods for studying passive permeability and V_{max}/K_m of efflux besides monolayer studies, we regard combining parameter values from different sources with caution, due to the current difficulty of controlling assay specific and data handling effects. These methods should not, however, be forgotten as they may give additional information and be useful in other aspects beside model input. For example the vesicle assays may aid in revealing mechanistic aspects of transport as they have already done regarding the understanding of binding sites. As understanding increases, the use of these methods may be reconsidered to generate parameters with higher throughput than cellular studies.

With the uncertainty that is still present in the generation of *in vitro* parameters, PBPK modeling of brain exposure is likely to be more useful later on in drug development than for early predictions. Although it may not be feasible at this stage to use PBPK predictions as a way to replace some preclinical studies, the simulations may help optimize experimental studies. As confidence in models increases, they will hopefully aid in disclosing the significance of active transport for developmental drug compounds. PBPK simulations of different situations may also aid in answering questions about possible drug-drug interactions, nonlinearity of kinetics due to transporter saturation and effects of interindividual variability and disease state not only at the level of the BBB, but also in other organs.

CONCLUSIONS

Principles of *in vitro—in vivo* extrapolation of efflux are well described in current literature. However, based on the quinidine

example and recent discussion in the literature, work is still needed to develop *in vitro* methods to obtain accurate and independent parameter estimates of passive permeability, K_m and V_{max} for PBPK modeling. Although some progress has been made, currently used fitting methods of *in vitro* data do not produce intrinsic parameter values that can be reliably moved from one system to another. More examples are needed where *in vitro* parameters are scaled and used successfully in PBPK simulations of *in vivo* situations. For this to yield valuable information, scaling of V_{max} according to transporter protein expression is a key factor as well as the increase in high-quality *in vivo* data for validation of the BBB model.

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