# Chapter 14

# Discovering New Antiepileptic Drugs Addressing the Transporter Hypothesis of Refractory Epilepsy: Ligand-Based Approximations

**Manuel Couyoupetrou, Mauricio Di Ianni, Melisa Gantner, Guido Pesce, Roxana Peroni, Alan Talevi, and Luis E. Bruno-Blanch**

## Abstract

About one third of the epileptic patients cannot control their symptoms with antiepileptic drugs, despite the introduction of more than 15 novel therapeutic agents to the market since 1990. The most studied hypothesis to explain the phenomenon of drug resistance in epilepsy maintains that it might be related to regional overactivity of efflux transporters from the ATP-binding cassette (ABC) superfamily at the blood–brain barrier and/or the epileptic foci. Here, we review scientific evidence supporting the transporter hypothesis along with its limitations. We also cover some technical aspects of computational and experimental approaches used for the early detection of substrates of such efflux systems.

**Key words** ABC transporters, Breast cancer resistance protein, CETA, Ligand-based approaches, Meta-classifiers, MDCK–MDR1, P-glycoprotein, Refractory epilepsy, Transport assays

## 1 Introduction

### *1.1 Refractory Epilepsy: Current Explanations*

About 30 % of the epileptic patients suffer from refractory or intractable epilepsy [1], i.e., they fail to achieve seizure freedom through adequate trials of two tolerated appropriately chosen antiepileptic drug (AED) schedules [2]. A clear and universal definition of refractory epilepsy is fundamental to understand the limitations of the neurobiological explanations to refractory epilepsy, which are discussed later in the present chapter. This scenario has not changed substantially despite the introduction of more than 15 AEDs from 1990 to the present [3]. Biological mechanisms underlying drug-resistant epilepsy have not been fully elucidated [4], though there are several hypotheses that explain this phenomenon; among them, the more prominent are the transporter hypothesis [5, 6], the target hypothesis [6, 7], the neural network hypothesis [8], the gene variant hypothesis [9], and the intrinsic severity hypothesis [10].

Alan Talevi and Luisa Rocha (eds.), *Antiepileptic Drug Discovery: Novel Approaches*, Methods in Pharmacology and Toxicology, DOI 10.1007/978-1-4939-6355-3_14, © Springer Science+Business Media New York 2016

The transporter and target hypotheses have been proposed earlier and have thus been more extensively studied. The former states that drug resistance in epilepsy may be a consequence of the local overactivity of ATP-binding cassette (ABC) transporters at the blood–brain barrier (BBB) and/or the epileptic foci. An overview on the evidence and limitations of this hypothesis is provided under the next subsection. The target hypothesis proposes that the reduced sensitivity to AEDs might be linked to acquired modifications in the structure and/or functionality of AED targets. While some years back constitutive alterations of drug transporters or targets were also included within the scope of the transporter and target hypothesis [7, 11], leading experts in the field now categorize intrinsic alterations of drug targets within the gene variant hypothesis [4]. The latter, however, also covers other possible genetic causes of drug resistance, such as polymorphic variants of CYP biotransformation enzymes. The nature of the pharmacokinetic or pharmacodynamic alteration is not trivial since it could have a profound impact on the clinical approach to the drug resistance issue. While genetic causes of pharmacoresistance might currently be detected through simple diagnostic tests even before starting the treatment, acquired modifications linked to the pathophysiology of the disease are more difficult to prove and nowadays still require more invasive procedures (e.g., surgery resection). Recently, the possible role of epigenetics in drug-resistant epilepsy has also been underlined [12].

It has been noted that none of the hypotheses provides a full or universal explanation to nonresponsive patients with epilepsy: a certain hypothesis might be applicable to a particular subgroup of patients, or alternatively, some patients could require multiple hypotheses to explain their refractoriness [4, 11, 13]. It is worth highlighting that the best treatment approach should be highly dependent on the underlying drug resistance mechanisms observed in a given patient.

### *1.2 Strong and Weak Points of the Transporter Hypothesis*

In eukaryotes, ABC transporters are transmembrane efflux transporters characterized by wide substrate specificity [14, 15]. They are highly expressed in barrier tissues (e.g., blood–brain barrier) and elimination organs (such as the liver and kidneys), restricting the bioavailability of xenobiotics and being consequently involved in multidrug resistance phenomena. They also play a role in the traffic of physiologic compounds (e.g., cholesterol or amyloid beta) and the pathogenesis of diverse disorders [15–17]. Most of the research on ABC transporters has focused on P-glycoprotein (Pgp), the first historically identified member of the superfamily, although the attention of the scientific community now also extends to other members, namely, multidrug resistance proteins (MRPs or ABCCs) and breast cancer resistance protein (BCRP or ABCG2).

Validation of the transporter hypothesis for drug-resistant epilepsy has been achieved at the preclinical level, where drug resistance in animal models of seizure and epilepsy has been reverted by coadministration of ABC transporter inhibitors. In 2001, Potschka and coworkers proved that the levels of carbamazepine in the extracellular fluid of the cerebral cortex could be enhanced through perfusion of the Pgp inhibitor verapamil and the MRP1/2/5 inhibitor probenecid [18]. Next, the same group proved that coadministration of probenecid and phenytoin increased phenytoin anticonvulsant effect in kindled rats [19]. Neither 50 mg/kg probenecid nor 6.25 mg/kg phenytoin exerted significant anticonvulsant effect when given alone. It was argued that such raise in the seizure threshold was unlikely to result from additive effects of the chosen sub-anticonvulsant doses. Similar results were later obtained in the focal pilocarpine model of limbic seizures [20]. These pioneering works had two important limitations: (a) they used weak, unspecific modulators of ABC transporters; and (b) experiments were performed without discriminating between responder and nonresponder animals. The first issue was solved with tariquidar, a third-generation and more specific inhibitor of Pgp [21]. Inhibition of Pgp by tariquidar increased the phenytoin brain-to-plasma ratio. Definitive preclinical proof of concept of the transporter hypothesis was obtained by coadministration of tariquidar to drug-resistant animals with Pgp overactivity [22]. Similar results were observed in the 3-mercaptopropionic acid model of refractory seizures, which is associated with Pgp upregulation at the blood–brain barrier, neurons, and astrocytes [23]. Remarkably, verapamil add-on therapy did not improve seizure control in a study on phenobarbital-resistant dogs, and some animals showed a worsening of seizure control [24], which highlights the potential influence of interspecies variability and the necessity to validate the transporter hypothesis at clinical trials.

Concerning clinical data, substantial evidence shows high expression levels of ABC transporters at the neurovascular unit of nonresponders [25–32]. Most of these studies compare samples from patients with intractable epilepsy subjected to surgical removal of the epileptic focus with samples of human brain from people with no history of seizures. While brain tissue from drug-responsive epileptic patients would be a more adequate control, such samples are unavailable since the invasive procedure to obtain them is unacceptable in responders. This limitation has recently been overcome using positron emission tomography [33, 34] which showed that the plasma-to-brain transport rate constants K1 for [11C]verapamil and (R)-[11C]verapamil tend to be reduced in different brain regions of drug-resistant epileptic patients in comparison with both healthy individuals and seizure-free patients.

Reversal of drug resistance after blocking ABC transporters would constitute definite proof of the transporter hypothesis.

Anecdotal cases of refractory patients who have shown improvement when AEDs were coadministered with verapamil have been reported [35–38], but it is unclear if the observed results could be a consequence of the intrinsic anticonvulsant effects of verapamil and/or other drug interactions of pharmacokinetic nature. More recently, a study was conducted on seven children with drug-resistant epilepsy [39]. They received verapamil as add-on therapy to baseline AED. Three subjects with genetically determined Dravet syndrome showed a partial response to adjunctive verapamil; another patient with the same syndrome but no known mutation showed partial seizure control during 13 months followed by seizure worsening. Two subjects with structural epilepsy and one with Lennox–Gastaut syndrome showed no improvement. In spite of the limited number of patients participating in the study, the results seem in agreement with the idea that some therapeutic interventions might be more effective in certain subgroups of refractory patients. A double-blind, randomized, single-center trial (initial sample size = 22) showed mild benefits of verapamil in comparison to placebo as add-on therapy for refractory epilepsy for a subset of the participants [40]. Randomized controlled multicentered trials and studies addressing the effect of selective inhibitors of Pgp with no intrinsic activity are still needed to obtain definitive clinical evidence for the transporter hypothesis. Regarding a possible association between genetic variants of ABC transporters and drug-resistant epilepsy, the available studies are controversial and sometimes inconclusive; while former meta-analysis failed to establish an association between ABCB1 variants and refractory epilepsy [41], subgroup analysis in more recent ones suggests associations in Caucasian and Asian subjects [42–44].

The main argument against the transporter hypothesis is that not all AEDs are Pgp substrates. Seemingly contradictory evidence exists regarding which AEDs are substrates and which are not [45, 46], but it should be kept in mind that results are highly dependent on the experimental setting, including the type of assay (in vivo, ex vivo, or in vitro, human versus animal models, concentration equilibrium transport assay, or nonequilibrium conditions). Still, it seems safe to say that some AEDs are unlike Pgp substrates. A number of points should be considered to reach a conclusion regarding the assigned category (substrate or non-substrate). Possible interspecies variability in substrate specificity should not be excluded. Bidirectional transport assays in the presence and absence of a selective Pgp inhibitor might lack sensitivity since directional transport might be masked by the contribution of passive diffusion; this is especially true when high concentrations of the test drug are used. The magnitude of this effect depends on the expression levels of the transporter in the cell culture, the substrate-tested concentrations, the drug affinity for the transporter, and the physicochemical features of the test drug, among others

[47]. Starting the assay with equal drug concentrations on both sides of the cell monolayer (concentration equilibrium transport assay, CETA) removes the concentration gradient, avoiding net diffusion and enhancing sensitivity [48].

Even if some available AEDs are not Pgp substrates, does this entirely preclude the validity of the transporter hypothesis? Not really. First, Pgp is one among many other efflux transporters possibly involved in drug-resistant epilepsy. Most of the studies determining the directional transport of AEDs have focused on Pgp; however, some of the AEDs are transported by other members of the ABC superfamily. The role of BCRP in the drug resistance phenomena might have been overlooked: while previous work seemed to suggest that AEDs were not transported by BCRP [49], more recent studies using double-knockout Mdr1a/1b(−/−)/Bcrp(−/−) mice and the CETA model suggest otherwise [50, 51]. Interestingly, proteomic studies have revealed ABCG2 as the transporter with the highest expression levels at the BBB of healthy subjects [52]. Moreover, due to the partial overlapping of the substrate specificity of different ABC transporters (together with reported co-expression and co-localization patterns that point to a cooperative role in the disposition of common substrates) [50–52], the role of a certain ABC transporter might be obscured owing to the function of others, their concerted function, and possible compensatory regulation, thus requiring complex models to study the phenomena. The difficulties to quantify the levels of expression of a given transporter in different regions of the brain of patients who have not been subjected to surgical procedures and the uncertainties regarding the ability of experimental models to reflect the absolute and relative expression levels of the different ABC efflux transporters at the epileptic foci and the BBB contribute to the difficulties to study the influence of a given transporter in the regional AED bioavailability in the brain.

The current definition of refractory epilepsy itself suggests that the transporter hypothesis may hold even if some of the known AEDs are not recognized by ABC transporters. Since the definition indicates that a patient should be considered unresponsive after failure of two well-tolerated and appropriately chosen and used AED trials, the key to the preceding reasoning lies in what is considered an appropriate drug choice. The definition of drug-resistant epilepsy weakens the transporter hypothesis if and only if one of the two appropriate therapeutic interventions was in fact a non-substrate for ABC transporters. At present, in the absence of definitive clinical proof of the transporter hypothesis, clinical guidelines for the management of epilepsy do not recommend to try at least one non-substrate AED; thus, the quality of substrate or non-substrate is presently unrelated to the appropriateness of the intervention. If the transporter hypothesis was validated, then a method for patient selection capable of identifying patients that

may benefit from therapeutic strategies targeting efflux transport will be required; what is more, patient selection should also be considered when designing clinical trials to study the clinical relevance of the transporter-associated resistance [53], excluding other sources of drug resistance as possible confounders.

### 1.3 Therapeutic Approaches to Transporter-Mediated Refractory Epilepsy

There are a number of possible therapeutic solutions that are being explored in relation to the transporter hypothesis. Inhibition of ABC transporters by adding on transporter blockers has already been studied as a possible therapeutic solution to multidrug resistance in cancer, though clinical trials have so far been disappointing ([13, 15, 53] and references therein) due to safety issues. The reader should bear in mind the physiologic role of ABC transporters as a general detoxification mechanism and their involvement in the traffic of endogenous substrates, which discourages the use of add-on inhibitors in the context of long-term therapeutic interventions (such as the ones used in epilepsy). The potential effects of such inhibitors in the pharmacokinetics of other drugs should also be considered in a polymedication scenario owing to the high probability of adverse drug interactions. Moderate or weak inhibitors of ABC transporters emerge as possible solutions; so do therapeutic agents directed to the signaling cascade that regulates the transporter expression [53]. A deep review on such approaches can be found in the excellent articles by Potschka and Luna-Munguia [53, 54].

The use of a "Trojan horse" approach to deliver therapeutic levels of the ABC transporter substrates to the epileptic focus, avoiding the recognition of the efflux pumps, could also be mentioned. This type of intervention encompasses prodrug design and particulate delivery systems (mainly, pharmaceutical nanocarriers) ([55, 56]; the reader may also refer to the special chapter on this subject, in this same volume). Finally, the design of novel AEDs which are not recognized by ABC transporters constitutes an interesting but presently overlooked alternative solution. In the following sections, we will describe some protocols directed to the early in silico and in vitro identification of substrates of ABC transporters in the frame of AED discovery programs.

## 2 In Silico Identification of Substrates for ABC Transporters

The general procedure to build ligand-based computational models has already been discussed by Talevi and Bruno-Blanch in another chapter of this volume. Similarly, the use of target-based approximations for the early recognition of Pgp substrates through homology modeling and subsequent virtual screening has been discussed by Palestro and Gavernet. Thus, we will only discuss under this

section particular aspects of ligand-based approximations aimed to in silico early recognition of ABC transporter substrates, with emphasis on the suitability of meta-classifiers for this purpose. Naturally, the general objective here is to discard substrates of ABC transporters; that is, ABC efflux pumps will be regarded as antitargets, i.e., *a biological target that causes undesirable effects when interacting with a drug*. Note that unlike common screening campaigns, where the compounds that interact with a given biological target are retained for further studies, the compounds interacting with an antitarget will not progress to additional testing.

We suggest starting any protocol to identify potential new treatments for efflux transporter-associated refractory epilepsy with high-throughput cost-efficient in silico screening tools and then gradually advance to computationally demanding models with lower throughput, reserving in vitro and in vivo models to the last stages of the screening. This cascade "in silico and in vitro first, in vivo later" approximation is not only cost-efficient (experimental tests are always more expensive than in silico experiments) but is also in good agreement with the 3R's bioethical principle, since it helps replacing and reducing animal testing as much as possible.

The reader may choose between developing its own in-house in silico model and resorting to any of the (many) models reported previously (see, for example, the models reviewed in references [57–59]). Some model developers offer their models online or in software packages, either freely or commercially. See, for example, Biozyne (http://pgp.biozyne.com/, last assessed January 2016) and Althotas Virtual Laboratory (http://pgp.althotas.com/, last assessed January 2016) [60, 61]. Some models can be reproduced from literature reports provided that you can access the required software tools. If you decide to use models developed by someone else for your predictions, it might be a good idea to examine the original papers in which such models are described, in order to assess the suitability of the procedures that have been used for model building and possible limitations of the approach. Do not rely blindly in computational models whose details have not been disclosed. Note that very frequently reported models related to ABC transporters are based on unbalanced training sets in which substrates significantly outnumber non-substrates, resulting in possible bias toward the prediction of the dominant category. On the other hand, many of the reported models have been derived from congeneric series of molecules, severely restricting their chemical space coverage. Finally, models to predict interaction with ABC transporters are seldom complemented by experimental validation of the results. As a general rule, when using models related to ABC transporters developed by other modelers, you should try posing the following questions: Have the models been derived from a balanced training set? Have the models been

inferred from a chemically diverse training set? Is it possible for you to estimate whether a predicted compound belongs to the applicability domain of the model? Have the models been appropriately validated using at least external validation procedures? If your answer to any of this questions is no or if you do not know the answer, avoid using the correspondent model(s). As a final advice on this matter, try to review the quality of the biological data used for training purposes. Section 3 will discuss some advice for in vitro permeability assays that could be used as reference. As discussed in the chapter by Talevi and Bruno-Blanch in this same volume, there are some robust modeling approaches (namely, classification models and ensemble learning) that can help mitigating noise related to dubious or heterogeneous experimental data. Finally, note that accuracy metrics reported in the literature for a given model often correspond to a single score cutoff value. If you are able to build a receiving operating characteristic (ROC) curve, you may even optimize the score threshold value according to your own scenario, opting for a cutoff value that favors specificity or sensitivity depending on your particular needs [62]. From the work by Truchon and Bayly, it is suggested to study the behavior of a given virtual screening/ranking method by seeding a relatively small number of known hits among a large number of decoys [63]. Through analytical work and proper statistical simulation methods, these authors demonstrated that the standard deviations in different metrics used to assess ranking methods tend to converge in such conditions, simultaneously removing a possible "saturation effect." In brief, do not excessively rely in conclusions regarding ranking metrics when they have been drawn from a limited-size test sample; instead, conduct your own evaluation through a pilot screening campaign dispersing a small sample of known hits among a large number of decoys: you might find the directory of useful decoys valuable for this purpose [64].

If you choose to build your own model(s) to predict affinity for ABC transporters, you must assume similar considerations. The key point here is that, owing to the polyspecificity that characterizes ABC transporters and the high interlab variability associated with experimental data, predicting whether a substance is or is not transported by a given member of the ABC superfamily is particularly challenging. Back in 2007, based on the high variability of Pgp experimental affinity data, Zhang and colleagues estimated the upper bound of accuracy for Pgp models in 85% [65], which is quite low compared with the accuracy achieved in the frame of other modeling problems. With some exceptions that overcome that theoretic upper bound (see, for instance, [66]), most of the reported models on ABC transporters display an overall accuracy similar to 80%. Typically, modeling efforts rely on biological data and chemical datasets compiled from literature: classification models can be used to alleviate the noise associated with such heterogeneous experimental data and large interlaboratory variability [67]; as Polanski et al.

affirm, extensive data independence implies qualitative rather than quantitative solutions [68].

The intrinsic difficulty of predicting affinity for ABC transporters has led many researches in the field to contemplate more flexible techniques such as nonlinear models [59] and more robust approximations such as ensemble learning/consensus QSAR or locally weighted methods [58, 69–75]. Also note that there is some evidence that ensemble learning could reduce the necessity of applicability domain estimation, assuring wider coverage of the chemical space [76]. Despite a very large number of models and algorithms for the computer-aided recognition of substrates for ABC transporters have been reported, very few have been applied in the specific field of drug discovery for refractory epilepsy, including the models by Di Ianni et al. [74] which have been used either alone or combined with docking protocols, as described by Palestro and Gavernet in this same volume.

Finally, when compiling training data, interspecies differences and other sources of variability (e.g., expression systems, genetic variants) must be considered.

## 3 In Vitro Permeability Assay to Identify ABC Transporter Substrates

In vitro models for the prediction of drug transport across biological barriers include cell cultures that reproduce physiological characteristics of a variety of barriers, such as the intestine and the blood–brain barrier. As previously insinuated, one of the main limitations of such systems is the high variability in permeability estimations, which makes difficult the comparison and combination of data from different laboratories and demands careful validation and continuous suitability demonstration: proper standardization of preexperimental, experimental, and post-experimental factors helps in reducing intra- and interlaboratory variability [77–79]. In the conclusion of their recent and remarkable study assessing the influence of a diversity of factors (mainly, days between seeding and experiment, passage number, coating, and data analysis approach) on variability and permeability, Oltra-Noguera and coworkers conclude that a similar study should be undertaken in each laboratory to evaluate the influence of protocol variables on the cell monolayer properties, in order to standardize the conditions and set acceptance criteria [77]. Caco-2, MDCK, and MDCK Pgp-transfected clone (MDCK–MDR1) cell lines are the most frequently used to determine in vitro permeability values and characterize drug transport mechanisms. Among them, we are currently using MDCK–MDR1 cells to study if AED candidates are or are not Pgp substrates. MDCK–MDR1 constitute a fast maturation model; note that MDCK–MDR1 lines with low values of transepithelial resistance (TEER) have also been used as blood–

brain barrier model [80, 81]. Back to the study of Oltra-Noguera, MDCK–MDR1 showed constant CV among passages, protocols, and experimental conditions, but permeability values were affected by all the studied conditions mentioned previously, indicating that for this cell line, standardization of experimental conditions is particularly critical to obtain comparable results between different laboratories [77].

Since the more relevant transporters possibly contributing to drug-resistant epilepsy are preferentially expressed in the apical membrane of the cells, the efflux (or transport) ratio can be generally used to define whether a given drug is or is not a substrate from an ABC transporter. Briefly, using a permeability chamber in which a donor and a receiver compartment are separated by the cell monolayer growing on a polycarbonate membrane, apparent permeability coefficients (Papp) are calculated in both apical-to-basolateral and basolateral-to-apical directions; the efflux ratio is defined as the apparent permeability obtained in the basolateral-to-apical direction divided by those obtained in the apical-to-basolateral direction. If the only drug transport mechanism is diffusion, then no significant difference between both permeability coefficients is expected, and the efflux ratio will be similar to 1; in contrast, the movement of a substrate for an efflux transporter preferentially expressed in the apical membrane will be restricted in the apical-to-basolateral direction, and the efflux ratio will tend to be larger than 1. Assuming sink conditions (negligible drug concentration in acceptor versus donor compartment, i.e., acceptor concentration <10% of donor concentrations), Fick's first law may be used to compute the correspondent Papp as follows:

$$\frac{\mathrm{d}Q}{\mathrm{d}t} = \mathrm{Papp} \times S \times \mathrm{Co}$$

where dQ/dt is the appearance rate of drug in the receiver side, calculated using linear regression of amounts in the receiver chamber versus time, $S$ is the surface area of the monolayer, and Co is the initial drug concentration in the donor compartment. Since after starting the experiment the concentration in the donor compartment is not really Co but constantly changes with time, the precedent equation can be corrected by replacing Co by Cd, the concentration in the donor compartment at each sample time (which can be simply calculated from the difference between the initial drug amount in the donor compartment and the drug amount in the receiver compartment at each sample time, assuming biotransformation within the cells can be neglected) [82]. If the sink condition is not verified, one should resort to non-sink equations, which are valid both in sink and non-sink conditions [77]. Note that concentrations should be corrected to consider the dilution effect related to media replenishment. Ideally, the mass balance should be checked at the end of the experiment by determining the amount of drug in cell membranes and inside the cells,

though the percentage of compound retained in the cell compartment is often negligible [77]. Note that a given cell line will express more than one drug transporter. Thus, the efflux ratio should be repeated in the presence of a specific inhibitor of the transporter being studied. Provided that the test drug is actually a substrate of the (anti)targeted transporter, such experiment should remove the influence of the (anti)targeted transporter returning the efflux ratio to around 1.

According to the results of Oltra-Noguera and collaborators, the best conditions to study compounds subjected to active efflux include using an intermediate or late passage number, polycarbonate filter without collagen coating, and sink corrected equations [77]. Within our group the following experimental conditions are observed: MDCK II–MDR1 cells are grown in 25 $cm^2$ culture flasks using DMEM with 10% fetal bovine serum, 1% l-glutamine, 1% nonessential amino acids, and penicillin and streptomycin at 37 °C in 5% atmosphere. Cells are split twice a week at 70–80% confluence in a ratio of 1:20 or 1:30 using trypsin–EDTA solution (0.25%). All transport assays were done with cells from passages 19 to 43. Cells were kept at 37 °C in 5% $CO_2$. The cells are seeded in 6-well Costar Snapwell plates with polycarbonate membrane inserts at a density of 50,000 cells per insert (1.12 $cm^2$) and grown for 4 days in culture medium. The medium is replaced every day. The apical media volume is 0.5 ml, and the basal volume is 2 ml. Integrity of the cell monolayers is determined by measuring the TEER using an epithelial voltammeter (Millicell-ERS, Millipore Corporation); normal TEER in MDRK and MDCK II–MDR1 cells is about 190 Ω $cm^2$ [83]. In addition, the integrity is also checked using atenolol (which is transported by the paracellular pathway). The Papp of atenolol across MDCK II–MDR1 cell monolayers in these conditions is typically $1–5 \times 10^{-7}$ cm/s. The expression of Pgp is checked by Western blot analysis and by transport assay with trimethoprim, a substrate for Pgp [84]. On the day of the experiment, culture medium is removed, and cells are washed three times with media transport (HBSS, Hanks' balance salt solution, pH 7.4, Gibco-BRL). The filter inserts containing the cell monolayers are placed in an Ussing chamber and kept at 37 °C and under constant gassing with carbogen. Test compounds are added to the donor side (4 ml for the apical and basal chamber). At 20, 40, 60, 80, 100, and 120 min, samples (400 μl) are taken from the acceptor compartment followed by the addition of 400 μl of transport media. For the inhibition experiments, cell monolayers are incubated with amiodarone chlorhydrate (50 μM) [85] for 1 h in apical and basolateral compartments before adding the test compound. A schematic representation of the device is shown in Fig. 1.

A critical point in transport assays is the AED concentrations used in these studies [48]; the role of drug transporters may be concealed by the contribution of passive diffusion, especially when

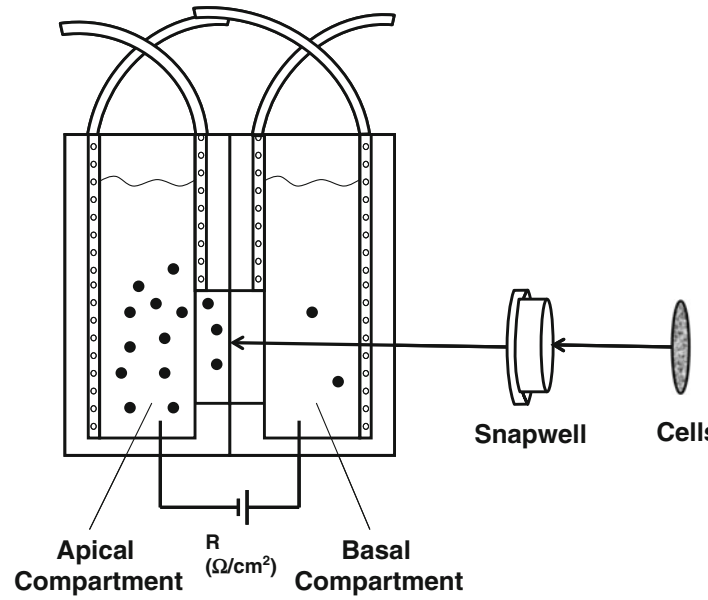


**Fig. 1** Diffusion chamber used in MDCK II–MDR1 permeability studies

highly permeable compounds (which is the case for most AEDs) and/or weak substrates are assayed. This effect could be more pronounced at high concentrations, since the concentration gradient is the driving force for passive diffusion. It is advised to test therapeutically relevant drug concentrations [48]. Note that free drug levels in plasma seldom exceed micromolar concentrations and brain levels are usually even smaller. Thus, the use of as low concentrations as possible is suggested. However, the lower limit of the possible concentration range to be used depends on the sensitivity of the analytical detection method used. HPLC MS/MS methods usually fulfill the requirements. Naturally, the analytical methods should be properly validated. Pay close attention to the fact that some ABC transporter substrates such as verapamil display a biphasic behavior depending on the concentration [48, 86]; thus, using a concentration range covering from low nanomolar to micromolar concentrations is highly preferred to using a single concentration, when possible. If due to sensitivity issues of the available analytical method the lowest concentrations of that range are precluded, try to use at least therapeutically relevant concentrations; the use of as low concentrations as possible could help reduce the impact of passive diffusion on the study results. Take into consideration that in general expression levels of the transporter at the monolayer will not represent local expression levels at epileptogenic regions in the brain and that ABC transporters display stereospecificity for some substrates.

Alternatively, using identical initial concentrations at both sides of the monolayer (apical and basolateral compartment) is an

elegant solution to remove the passive diffusion component, increasing the assay sensitivity (i.e., CETA) [48]. In such case, only the net direction of the transport should be analyzed. In the absence of mediated transport, no net drug movement should be registered. In contrast, if the drug acts as a substrate for an efflux transporter expressed at the apical membrane, a net movement in the basolateral-to-apical direction should take place.

## 4 Ex Vivo Permeability Assay to Identify ABC Transporter Substrates: Evert Gut Sac Model

The in vitro everted gut sac model was first introduced in 1954 by Wilson and Wiseman [87]. Since then modifications and improvements have been made to the model to increase the viability of tissue and to maintain intact mucosal epithelium that mimic the in vivo conditions [88, 89]. The everted sac model has been explored to carry out pharmacokinetic investigations of the impact of efflux transport modulators on the absorption of drugs [90–94]. The main advantage of this technique with respect to in vitro assays is that frequently results from the everted intestinal sac model have been in agreement with in vivo findings [92, 95–98]. On the other hand, the cell culture assays conducted in either human cancer lines expressing ABC tansporters or transfected cell lines with high density of transporter molecules, which is required for their function to be predominant over membrane permeation and passive diffusion processes, have high negative predictive value, but often give false positives [99]. Indeed, there are evidences that the absorption rate of test compounds is not always similar in the everted gut sac than in the Caco-2 monolayer [100]. For oral absorption studies, Caco-2 cell line experiments were approved by the Food and Drug Administration, but these cell lines are not always the right substitute for the ex vivo everted gut sac model [101]. For that reason, new drug candidates screened for interactions in silico and in vitro culture assays during early stages of drug development should be tested in a subsequent step by ex vivo assays such as everted gut sac, the isolated intestinal perfusion, or Ussing chamber system [102].

The advantages of this everted gut sac model as an ex vivo tool to study the mechanisms and kinetics of drug absorption are a relatively large surface area available for absorption and the presence of a mucus layer [103]. On the other hand, the tissue viability is one of the main limiting parameters having a maximum of approximately 2 h [88].

Different animals have been chosen for everted gut sac experiments, but the everted rat intestinal sac is the most commonly used [87, 88, 90, 104]. The reproducibility of the rat everted gut sac

suggests that this ex vivo model is a useful tool for studying transport of substrates and modulators of ABC transporters [93].

### 4.1 Localization of ABC Transporters along the Intestinal Tract

One of the keys for the success of the analysis of the participation of ABC transporters in drug absorption by the everted gut sac technique is to select the correct intestinal segment. A cellular and subcellular localization description of the most frequently studied ABC transporters in human and in rats is briefly described below. For a full scan of the intestinal expression of Pgp (MDR1/ABCB1), BCRP, and MRP2 (ABCC2) in male and female rats, consult McLean et al. [105].

The expression of Pgp increases from proximal to distal portions in the apical membrane of the intestinal crypts in human, resulting in the highest expression levels in the colon [106, 107]. In the same way, it shows an increase from proximal to distal regions in the rat [105].

In the rat and human intestine, MRP2/ABCC2 mRNA expression is highest in the apical membrane of the enterocytes in the duodenum and subsequently decreases in direction to the terminal ileum and colon where it is only minimal [103, 108]. Protein levels of MRP2 in rats decrease along the intestinal axis from proximal to distal parts [105].

Transcription of BCRP in the human jejunum is higher than that of MDR1 and comparable with that of MRP2 [109]. BCRP/ABCG2 mRNA is also expressed with apical localization in the epithelium of the small intestine of the rat showing duodenum and jejunum levels similar to those found for MRP2 and then decreasing slightly to the more distal portions [109]. In the large intestine, a continuous decrease of the BCRP mRNA toward the distal portions is observed finding in the rectum a half of the levels found in the small intestine [110]. However, protein levels of BCRP show an arcuate pattern with the highest expression toward the end of the small intestine in the rat [90, 105].

On the other hand, MRP3/ABCC3 localizes to the basolateral membrane of enterocytes in rats. It is expressed in low levels in the duodenum and jejunum but markedly increases in the ileum and colon [108].

There are not sex differences in the mRNA and protein expression levels of ABC transporters in the entire intestine of the rat [105].

As described above, the localization of ABC transporters not always matches thorough the intestine between rat and human beings. Moreover, differences exist between the mRNA expression and the subsequent translation to protein because of post-transcriptional modifications. Furthermore, it is important to note that expression levels of transporters can be modified under various physiopathological conditions, in the presence of polymorphisms, and by administration of drugs. For example, we

observed overexpression of BCRP in the ileum after chronic oral administration with the antiretroviral efavirenz that reduces its own intestinal penetration [90]. Yumoto et al. demonstrated that in situ intestinal absorption of methotrexate, a substrate of Mrp2, is decreased by oral treatment with ursodeoxycholic acid that causes Mrp2 upregulation [92]. Moreover, the metabolic syndrome generated by enhanced fructose intake in rats decreases the protein expression and activity of ileal Pgp, thus increasing the bioavailability of rhodamine [93]. Changes in expression level, subcellular localization, and functional properties can all be involved in interindividual differences in drug pharmacokinetics. For that reason, the expression levels of the ABC transporter of interest need to be checked in experimental models before carrying out the everted gut sac technique to achieve optimal experimental conditions and to avoid false results.

### 4.2 Everted Gut Sac Preparation

Under anesthesia with urethane (1.2 g/kg body weight), rapidly remove the jejunum or duodenum or ileum of the intestine and wash each segment with an ice-cold oxygenated Krebs solution (pH 6.5) containing 7 g/l sodium chloride, 0.34 g/l potassium chloride, 1.8 g/l glucose, 0.251 g/l disodium hydrogen phosphate, 0.207 g/l sodium dihydrogen phosphate, and 46.8 mg/l magnesium chloride. Gently evert the washed intestine over a glass rod and divide into segments (5–6 cm each). Clamp one end of the everted intestine and tie with a silk braided suture and then place a short intravenous cannula at the other end and tie the intestine around it using a second braided silk suture making sure it does not break to hang it so that this end is available for the administration of fluids. It is very important to prevent the intestinal wall to enter the cannula being damaged because the fluid inside the bag was lost by these sites giving erroneous results. Previously, enter a stainless thin wire in the cannula cap that acts as a hook at the other end so as to be able to hang the everted sac in the container where it will conduct the test. Hang the everted filled sac in the incubation tube containing drug in 5–10 ml oxygenated Krebs solution at 37 °C alone or in the presence of a fixed dose of ABC inhibitors preventing the sac to touch the walls. Fill the sac with 600–800 μl Krebs solution containing the drug to be tested using a 1 ml syringe. Sampling could be performed at different intervals considering that the same amount of medium as the sample is taken must be replaced. Depending on the transport direction to be analyzed, e.g., serosal to mucosal or the opposite, drugs to be tested could be added in the incubation container or into the sac [90, 93, 103].

There are several factors that need to be taken into account due to the fact that they impact on the outcome and conclusion of everted gut sac studies, e.g., animal factors (age, sex, species, diet, disease state, chronic treatment, and toxicity), intestinal segment (ileum, jejunum, duodenum, and colon), and experimental factors

(e.g., pH, aeration, temperature, concentration of substances). Those factors have been extensively revised by Alam et al. [111].

Finally, to achieve sensitivity and specificity, it is important to select the best quantification method for each compound as spectrophotometry [93, 112], liquid chromatography [92, 93, 96], and radioactivity [106] and to perform the analysis in the presence of highly specific pairs of substrate inhibitors [112].

## 5 Final Remarks

Despite the transporter hypothesis of refractory epilepsy has long been studied (with convincing preclinical evidence and encouraging yet limited clinical evidence), such hypothesis is still underexplored in the drug discovery field to assist the search of new treatments for refractory epilepsy. Although a considerable number of computational models related to the early identification of substrates for ABC transporters have indeed been developed, few of them have been applied specifically in the field of AED discovery.

ABC transporter polyspecificity and interlaboratory variability of experimental data on their substrates and non-substrates constitute major challenges to the development of accurate computational models oriented to the prediction of affinity for efflux pumps. We have reviewed some general criteria to circumvent or at least minimize the impact of such limitations when building computational models for that purpose, prominently, the use of classifiers and meta-classifiers. Whenever applying external (i.e., non-in-house models), estimation of the model's coverage/applicability domain is essential.

Regarding experimental substrate assessment, some relevant issues include measuring interaction with not one but a number of ABC transporters with (partially) overlapping substrate specificity (which often act in a concerted manner and can be subjected to compensatory regulation) and testing a wide range of therapeutically relevant concentrations (from nM to μM) in order to discard possible biphasic responses. The use of small concentrations helps in minimizing the influence of passive diffusion on the study outcome; alternatively, the CETA is a sensitive model to measure the affinity for ABC transporters of highly permeable drugs and/or weak substrates. Experimental variables should be carefully study to enhance the reproducibility of the obtained results [113].

## Acknowledgments

The authors' work is supported by Agencia de Promoción Científica y Tecnológica (PICT 2010-1774, PICT 2013-3175), CONICET, Universidad Nacional de La Plata, and Argentinean National Institute of Medications (INAME, ANMAT).

## References


1. Brodie MJ, Barry SJ, Bamagous GA et al (2012) Patterns of treatment response in newly diagnosed epilepsy. Neurology 78:1548–1554
2. Sinha S, Siddiqui KA (2011) Definition of intractable epilepsy. Neurosciences (Riyadh) 16:3–9
3. Löscher W, Schmidt D (2011) Modern antiepileptic drug development has failed to deliver: ways out of the current dilemma. Epilepsia 52:657–678
4. Löscher W, Klitgaard H, Twyman RE et al (2013) New avenues for anti-epileptic drug discovery and development. Nat Rev Drug Discov 12:757–776
5. Kwan P, Brodie MJ (2005) Potential role of drug transporters in the pathogenesis of medically intractable epilepsy. Epilepsia 46:224–235
6. Remy S, Beck H (2006) Molecular and cellular mechanisms of pharmacoresistance in epilepsy. Brain 129:18–35
7. Schmidt D, Löscher W (2005) Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. Epilepsia 46:858–877
8. Fang M, Xi ZQ, Wu Y et al (2011) A new hypothesis of drug refractory epilepsy: neural network hypothesis. Med Hypotheses 76:871–876
9. Löscher W, Klotz U, Zimprich F et al (2009) The clinical impact of pharmacogenetics on the treatment of epilepsy. Epilepsia 50:1–23
10. Rogawski MA, Johnson MR (2008) Intrinsic severity as a determinant of antiepileptic drug refractoriness. Epilepsy Curr 8:127–130
11. Schmidt D, Löscher W (2009) New developments in antiepileptic drug resistance: an integrative view. Epilepsy Curr 9:47–52
12. Kobow K, El-Osta A, Blumcke I (2013) The methylation hypothesis of pharmacoresistance in epilepsy. Epilepsia 54(Suppl S2):41–47
13. Talevi A, Bruno-Blanch LE (2012) In: Rocha L, Cavalheiro EA (eds) Pharmacoresistance in epilepsy. From genes and molecules to promising therapies, 1st edn. Springer, New York, NY
14. Rees DC, Johnson E, Lewison O (2009) ABC transporters: the power to change. Nat Rev Mol Cell Biol 10:218–227
15. Talevi A, Bruno-Blanch LE (2012) Efflux transporters at the blood-brain barrier. Therapeutic opportunities. In: Montenegro PA, Juárez SN (eds) The blood-brain barrier: new research, 1st edn. Nova, New York, NY
16. Glavinas H, Krajscsi P, Cserepes J et al (2004) The role of ABC transporters in drug resistance, metabolism and toxicity. Curr Drug Deliv 1:27–42
17. Abuznait AH, Kaddoumi A (2012) Role of ABC transporters in the pathogenesis of Alzheimer's disease. ACS Chem Neurosci 3:820–831
18. Potschka H, Fedrowitz M, Löscher W (2001) P-glycoprotein and multidrug resistance-associated protein are involved in the regulation of extracellular levels of the major antiepileptic drug carbamazepine in the brain. NeuroReport 12:3557–3560
19. Potschka H, Fedrowitz M, Löscher W (2003) Multidrug resistance protein MRP2 contributes to blood-brain barrier function and restricts antiepileptic drug activity. J Pharmacol Exp Ther 306:124–131
20. Clinckers R, Smolders I, Meurs A et al (2005) Quantitative in vivo microdialysis study on the influence of multidrug transporters on the blood-brain barrier passage of oxcarbazepine: concomitant use of hippocampal monoamines as pharmacodynamics markers for the anticonvulsant activity. J Pharmacol Exp Ther 314:725–731
21. van Vliet EA, van Schaik R, Edelbroek PM et al (2006) Inhibition of the multidrug transporter P-glycoprotein improves seizure control in phenytoin-treated chronic epileptic rats. Epilepsia 47:672–680
22. Brandt C, Bethmann K, Gastens AM et al (2006) The multidrug transporter hypothesis of drug resistance in epilepsy: proof-of-principle in a rat model of temporal lobe epilepsy. Neurobiol Dis 24:202–211
23. Höcht C, Lazarowski A, González NN et al (2007) Nimodipine restores the altered hippocampal phenytoin pharmacokinetics in a refractory epileptic model. Neurosci Lett 413:168–172
24. Jambroszyk M, Tipold A, Potschka H (2011) Add-on treatment with verapamil in pharmacoresistant canine epilepsy. Epilepsia 52:284–291
25. Tishler DM, Weinberg KI, Hinton DR et al (1995) MDR1 gene expression in brain of patients with medically intractable epilepsy. Epilepsia 36:1–6
26. Dombrowski SW, Desai SY, Marroni M et al (2001) Overexpression of multidrug resistance genes in endothelial cells from patients with refractory epilepsy. Epilepsia 42:1501–1506
27. Sisodiya SM, Lin WR, Harding BN et al (2002) Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. Brain 125:22–31
28. Aronica E, Gorter JA, Jansen GH et al (2003) Expression and cellular distribution

of multidrug transporter proteins in two major causes of medically intractable epilepsy: focal cortical dysplasia and glioneuronal tumors. Neuroscience 118:417–429

29. Aronica E, Gorter JA, Redeker S et al (2005) Localization of breast cancer resistance protein (BCRP) in microvessel endothelium of human control and epileptic brain. Epilepsia 46:849–857
30. Lazawoski A, Massaro M, Schteinschnaider A et al (2004) Neuronal MDR-1 gene expression and persistent low levels of anticonvulsants in a child with refractory epilepsy. Ther Drug Monit 26:44–46
31. Kubota H, Ishihara H, Langmann T et al (2006) Distribution and functional activity of P-glycoprotein and multidrug resistance-associated proteins in human brain microvascular endothelial cells in hippocampal sclerosis. Epilepsy Res 68:213–228
32. Ak H, Ay B, Tanriverdi T et al (2007) Expression and cellular distribution of multidrug resistance-related proteins in patients with focal cortical dysplasia. Seizure 16:493–503
33. Feldmann M, Koepp M (2012) P-glycoprotein imaging in temporal lobe epilepsy: in vivo PET experiments with the Pgp substrate [11C]-verapamil. Epilepsia 53(Suppl 6):60–63
34. Feldmann M, Asselin MC, Liu J et al (2013) P-glycoprotein expression and function in patients with temporal lobe epilepsy: a case-control study. Lancet Neurol 12:777–785
35. Summers MA, Moore JL, McAuley JW (2004) Use of verapamil as a potential P-glycoprotein inhibitor in a patient with refractory epilepsy. Ann Pharmacother 38:1631–1634
36. Ianetti P, Spalice A, Parisi P (2005) Calcium-channel blocker verapamil administration in prolonged and refractory status epilepticus. Epilepsia 46:967–969
37. Schmitt FC, Dehnicke C, Merschhemke M et al (2010) Verapamil attenuates the malignant treatment course in recurrent status epilepticus. Epilepsy Behav 17:565–568
38. Pirker S, Baumgartner C (2011) Termination of refractory focal status epilepticus by the P-glycoprotein inhibitor verapamil. Eur J Neurol 18:e151
39. Nicita F, Spalice A, Papetti L et al (2014) Efficacy of verapamil as an adjunctive treatment in children with drug-resistant epilepsy: a pilot study. Seizure 23:36–40
40. Borlot F, Wither RG, Ali A (2014) A pilot double-blind trial using verapamil as adjuvant therapy for refractory seizures. Epilepsy Res 108:1642–1651
41. Haerian BS, Lim KS, Tan CT et al (2011) Association of ABCB1 gene polymorphisms and their haplotypes with response to antiepileptic drugs: a systematic review and meta-analysis. Pharmacogenomics 12:713–725
42. Yu L, Liao WP, Yi YH et al (2015) ABCB1 G2677T/A polymorphism is associated with the risk of drug-resistant epilepsy in Asians. Epilepsy Res 115:100–108
43. Lv WP, Han RF, Shu ZR (2014) Associations between the C3435T polymorphism of the ABCB1 gene and drug resistance in epilepsy: a meta-analysis. Int J Clin Exp Med 7:3924–3932
44. Li SX, Liu YY, Wang QB (2015) ABCB1 gene C3435T polymorphism and drug resistance in epilepsy: evidence based on 8,604 subjects. Med Sci Monit 21:861–868
45. Zhang C, Kwan P, Zuo Z et al (2012) The transport of antiepileptic drugs by P-gp. Adv Drug Deliv Rev 64:930–942
46. Stépien KM, Tomaszewski M, Tomaszewska J et al (2012) The multidrug transporter P-glycoprotein in pharmacoresistance to antiepileptic drugs. Pharmacol Rep 64:1011–1019
47. Luna-Tortós C, Fedrowitz M, Löscher W (2008) Several major antiepileptic drugs are substrates for human P-glycoprotein. Neuropharmacology 55:1364–1375
48. Löscher W, Luna-Tortós C, Römermann K et al (2011) Do ATP-binding cassette transporters cause pharmacoresistance in epilepsy? Problems and approaches in determining which antiepileptic drugs are affected. Curr Pharm Des 17:2808–2828
49. Cerveny L, Pavek P, Malakova J et al (2006) Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. Epilepsia 47:461–468
50. Römermann K, Helmer R, Löscher W (2015) The antiepileptic drug lamotrigine is a substrate of mouse and human breast cancer resistance protein (ABCG2). Neuropharmacology 93:7–14
51. Nakanishi H, Yonezawa A, Matsubara K et al (2013) Impact of P-glycoprotein and breast cancer resistance protein on the brain distribution of antiepileptic drugs in knockout mouse models. Eur J Pharmacol 710:20–28
52. Uchida Y, Ohtsuki S, Katsukura Y et al (2011) Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. J Neurochem 117:333–345
53. Potschka H, Luna-Munguia H (2014) CNS transporters and drug delivery in epilepsy. Curr Pharm Des 20:1534–1542

54. Potschka H (2012) Role of CNS efflux drug transporters in antiepileptic drug delivery: overcoming CNS efflux drug transport. Adv Drug Deliv Rev 64:943–952
55. Rosillo-de la Torre A, Luna-Bárcenas G, Orozco-Suárez S et al (2014) Pharmacoresistant epilepsy and nanotechnology. Front Biosci (Elite Ed) 6:329–340
56. Bennewitz MF, Saltzman WM (2009) Nanotechnology for delivery of drugs to the brain for epilepsy. Nanotherapeutics 6: 323–336
57. Ecker GF, Stockner T, Chiba P (2008) Computational models for prediction of interactions with ABC-transporters. Drug Discov Today 13:311–317
58. Chen L, Li Y, Yu H et al (2012) Computational models for predicting substrates or inhibitors of P-glycoprotein. Drug Discov Today 17:343–351
59. Pinto M, Digles D, Ecker GF (2014) Computational models for predicting the interaction with ABC transporters. Drug Discov Today Technol 12:e69–e77
60. Levatić J, Ćurak J, Karlj M et al (2013) Accurate models for P-gp drug recognition induced from a cancer cell line cytotoxicity screen. J Med Chem 56:5691–5708
61. Bikadi Z, Hazai I, Malik D et al (2011) Predicting P-glycoprotein-mediated drug transport based on support vector machine and three-dimensional crystal structure of P-glycoprotein. PLoS One 6:e25815
62. Triballeau N, Acher F, Brabet I et al (2005) Virtual screening workflow development guided by the "Receiver Operating Characteristic" curve approach. Application to high-throughput docking on metabotropic glutamate receptor subtype 4. J Chem Inf Model 48:2534–2547
63. Truchon JF, Bayly CI (2007) Evaluating virtual screening methods: good and bad metrics for the "early recognition" problem. J Chem Inf Model 47:488–508
64. Mysinger MM, Carchia M, Irwin JJ et al (2012) Directory of useful decoys, enhanced (DUD-E): better ligands and decoys for better benchmarking. J Med Chem 55:6582–6594
65. Zhang L, Balimane P, Johnson S et al (2007) Development of an in silico model for predicting efflux substrates in Caco-2 cells. Int J Pharm 343:98–105
66. Huang J, Ma G, Muhammad I et al (2007) Identifying P-glycoprotein substrates using a support vector machine optimized by a particle swarm. J Chem Inf Model 47:1638–1647
67. Talevi A, Bellera C, Di Ianni M et al (2012) An Integrated drug development approach applying topological descriptors. Curr Comput Aided Drug Des 8:172–181
68. Polanski J, Bak A, Gieleciak R et al (2006) Modeling robust QSAR. J Chem Inf Model 46:2310–2318
69. Penzotti JE, Lamb ML, Evensen E et al (2002) A computational ensemble pharmacophore model for identifying substrates of P-glycoprotein. J Med Chem 45:1737–1740
70. Li WX, Li L, Eksterowicz J et al (2007) Significance analysis and multiple pharmacophore models for differentiating P-glycoprotein substrates. J Chem Inf Model 47:2429–2438
71. Svetnik V, Wang T, Tong C et al (2005) Boosting: an ensemble learning tool for compound classification and QSAR modeling. J Chem Inf Model 45:786–799
72. Cao DS, Huang JH, Yan J et al (2012) Kernel k-nearest neighbor algorithm as a flexible SAR modeling tool. Chemom Intell Lab Systems 114:19–23
73. Gantner ME, Di Ianni ME, Ruiz ME et al (2013) Development of conformation independent computational models for the early recognition of breast cancer resistance protein substrates. Biomed Res Int 2013:863592
74. Di Ianni ME, Talevi A, Castro EA et al (2011) Development of a highly specific ensemble of topological models for early identification of P-glycoprotein substrates. J Chemometr 25:313–322
75. Leong MK, Chen HB, Shih YH (2012) Prediction of promiscuous P-Glycoprotein inhibition using a novel machine learning scheme. PLoS One 7:e33829
76. Zhu H, Tropsha A, Foruches D et al (2008) Combinatorial QSAR modeling of chemical toxicants tested against *Tetrahymena pyriformis*. J Chem Inf Model 48:766–784
77. Oltra-Noguera D, Magas-Sanjuan V, Centelles-Sangüesa A et al (2015) Variability of permeability estimation from different protocols of subculture and transport experiments in cell monolayers. J Pharmacol Toxicol Methods 71:21–32
78. Rodriguez YI, Abalos IS, Lozano V et al (2011) Standardization of Caco-2 cell culture as in vitro model for intestinal permeability. Lat Am J Pharm 30:33–38
79. Birske-Anderson MJ, Finley JW, Newman SM (1997) The Influence of culture time and passage number on the morphological and physiological development of Caco-2 cells. Exp Biol Med (Maywood) 214:248–257

80. Garberg P, Ball M, Borg N et al (2005) In vitro models for the blood-brain barrier. Toxicol In Vitro 19:299–334
81. Wang Q, Rager JD, Weinstein K et al (2005) Evaluation of the MDR-MDCK cell line as a permeability screen for the blood–brain barrier. Int J Pharm 288:349–359
82. Tavelin S, Graski J, Taipalensuu J et al (2002) Applications of epithelial cell culture in studies of drug transport. Methods Mol Biol 188:233–272
83. Navarro C, González-Álvarez I, González-Álvarez M et al (2011) Influence of polyunsaturated fatty acids on cortisol transport through MDCK and MDCK–MDR1 cells as blood–brain barrier in vitro model. Eur J Pharm Sci 42:290–299
84. Varma MVS, Sateesh K, Panchagnula R (2005) Functional role of P-glycoprotein in limiting intestinal absorption of drugs: contribution of passive permeability to P-glycoprotein mediated efflux transport. Mol Pharm 2:12–21
85. Kakumoto M, Takara K, Sakaeda T et al (2002) MDR1-mediated interaction of digoxin with antiarrhythmic or antianginal drugs. Biol Pharm Bull 25:1604–1607
86. Karwatsky J, Lincoln MC, Georges EA (2003) A mechanism for P-glycoprotein-mediated apoptosis as revealed by verapamil hypersensitivity. Biochemistry 42:12163–12173
87. Wilson TH, Wiseman G (1954) The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J Physiol 123:116–125
88. Barthe L, Woodley JF, Kenworthy S et al (1998) An improved everted gut sac as a simple and accurate technique to measure paracellular transport across the small intestine. Eur J Drug Metab Pharmacokinet 23:313–323
89. Ugolev AM, Bagiian AA, Ekkert LG (1980) New method of studying membrane hydrolysis and transport as well as metabolic processes in the small intestine in vitro (everted small intestinal sac with bilateral oxygenation). Fiziol Zhurnal SSSR Im I M Sechenova 66:1674–1677
90. Peroni RN, Di Gennaro SS, Hocht C et al (2011) Efavirenz is a substrate and in turn modulates the expression of the efflux transporter ABCG2/BCRP in the gastrointestinal tract of the rat. Biochem Pharmacol 82:1227–1233
91. Rong Z, Xu Y, Zhang C et al (2013) Evaluation of intestinal absorption of amtolmetin guacyl in rats: breast cancer resistant protein as a primary barrier of oral bioavailability. Life Sci 92:245–251
92. Yumoto R, Hamada S, Okada K et al (2009) Effect of ursodeoxycholic acid treatment on the expression and function of multidrug resistance-associated protein 2 in rat intestine. J Pharm Sci 98:2822–2831
93. Novak A, Godoy YC, Martinez SA et al (2015) Fructose-induced metabolic syndrome decreases protein expression and activity of intestinal p-glycoprotein. Nutrition 31:871–876
94. Cao F, Zhang H, Guo J et al (2008) Mrp2-related efflux of scutellarin in the intestinal absorption in rats. Pharmazie 63:75–80
95. Patel JP, Korashy HM, El-Kadi AOS et al (2010) Effect of bile and lipids on the stereoselective metabolism of halofantrine by rat everted-intestinal sacs. Chirality 22:275–283
96. Ni L, Yu X, Yu Q et al (2008) Effects of cyclosporine A and itraconazole on permeability, biliary excretion and pharmacokinetics of amlodipine. Drug Metab Lett 2:163–168
97. Machavaram KK, Gundu J, Yamsani MR (2006) Effect of ketoconazole and rifampicin on the pharmacokinetics of ranitidine in healthy human volunteers: a possible role of P-glycoprotein. Drug Metabol Drug Interact 22:47–65
98. Kashimura J, Nagai Y (2007) Inhibitory effect of palatinose on glucose absorption in everted rat gut. J Nutr Sci Vitaminol (Tokyo) 53:87–89
99. del Amo EM, Heikkinen AT, Mönkkönen J (2009) In vitro-in vivo correlation in P-glycoprotein mediated transport in intestinal absorption. Eur J Pharm Sci 36:200–211
100. Park MY, Kwon HJ, Sung MK (2009) Intestinal absorption of aloin, aloe-emodin, and aloesin; A comparative study using two in vitro absorption models. Nutr Res Pract 3:9–14
101. Lafforgue G, Arellano C, Vachoux C et al (2008) Oral absorption of ampicillin: role of paracellular route vs. PepT1 transporter. Fundam Clin Pharmacol 22:189–201
102. van de Kerkhof EG, de Graaf IAM, Groothuis GMM (2007) In vitro methods to study intestinal drug metabolism. Curr Drug Metab 8:658–675
103. Dietrich CG, Geier A, Oude Elferink RPJ (2003) ABC of oral bioavailability: transporters as gatekeepers in the gut. Gut 52:1788–1795
104. Barthe L, Woodley J, Houin G (1999) Gastrointestinal absorption of drugs: methods and studies. Fundam Clin Pharmacol 13:154–168
105. MacLean C, Moenning U, Reichel A et al (2008) Closing the gaps: a full scan of the intestinal expression of p-glycoprotein, breast

cancer resistance protein, and multidrug resistance-associated protein 2 in male and female rats. Drug Metab Dispos 36:1249–1254. doi:10.1124/dmd.108.020859

106. Fricker G, Drewe J, Huwyler J et al (1996) Relevance of p-glycoprotein for the enteral absorption of cyclosporin A: in vitro-in vivo correlation. Br J Pharmacol 118:1841–1847
107. Suzuki H, Sugiyama Y (2000) Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. Eur J Pharm Sci 12:3–12
108. Rost D, Mahner S, Sugiyama Y et al (2002) Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine. Am J Physiol Gastrointest Liver Physiol 282:G720–G726
109. Taipalensuu J, Törnblom H, Lindberg G et al (2001) Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. J Pharmacol Exp Ther 299:164–170
110. Gutmann H, Hruz P, Zimmermann C et al (2005) Distribution of breast cancer resistance protein (BCRP/ABCG2) mRNA expression along the human GI tract. Biochem Pharmacol 70:695–699
111. Alam MA, Al-Jenoobi FI, Al-Mohizea AM (2012) Everted gut sac model as a tool in pharmaceutical research: limitations and applications. J Pharm Pharmacol 64:326–333
112. Perrone MG, Inglese C, Berardi F et al (2013) Comparative evaluation of two dye probes in the rat everted gut sac model for unambiguous classification of P-gp substrate and inhibitor. J Pharmacol Toxicol Methods 67:5–8
113. Löscher W, Langer O (2010) Imaging of P-glycoprotein function and expression to elucidate mechanisms of pharmacoresistance in epilepsy. Curr Top Med Chem 10:1785–1791