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# The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME–Tox)

**Gergely Szakács[1,2], András Váradi[2], Csilla Özvegy-Laczka[1] and Balázs Sarkadi[1]**

[1] National Medical Center, Institute of Haematology and Immunology, Membrane Research Group of the Hungarian Academy of Sciences, H-1113 Budapest, Diószegi u. 64, Hungary
[2] Institute of Enzymology, Hungarian Academy of Sciences, H-1113 Budapest, Karolina u. 29, Hungary

**ATP binding cassette (ABC) drug transporters play an important role in cancer drug resistance, protection against xenobiotics, and in general in the passage of drugs through cellular and tissue barriers. This review explores how human ABC transporters modulate the pharmacological effects of various drugs, and how this predictable ADME–TOX modulation can be used during the process of drug discovery and development. We provide a description of the relevant human ABC drug transporters and review the models and assay systems that can be applied for the analysis of their expected drug interactions. The use of the *in vitro*, *in vivo*, *in silico* models, their combination, and the emerging clinical information are evaluated with respect to their potential application in early drug screening.**

## Introduction—ADME–TOX and ABC transporters in drug discovery

During the process of drug discovery in many cases a new compound or lead molecule is found to act with high affinity and specificity on a desired drug target. However, medical application of this compound will be greatly influenced by its absorption, distribution, metabolism, excretion, and toxicity (ADME–Tox) parameters. Therefore, the fate of the new compound within the body should also be estimated. Moreover, predictive studies should be performed as early as possible in drug development, in order to save a great deal of time, effort, animal (or even human) lives, and money.

In the past years pharmaceutical companies have realized the need of including membrane transporter studies in addition to basic drug metabolism assays in the early ADME–Tox investigations. It has become common knowledge that for crossing tissue barriers, in addition to some basic physical characteristics, including molecular size, charge distribution, and hydrophobicity, drug interactions with membrane transporters are key determinants. Although membranes are rapidly passed by various hydrophobic compounds, and hydrophilic molecules only slowly penetrate these barriers, it is now considered more as a rule, than an exception, that a given

*Corresponding author:* Sarkadi, B. (sarkadi@biomembrane.hu)

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pharmacological agent will interact with a (set of) membrane transporter(s) at some point of its route in our body [1].

Classical drug metabolism was earlier divided into Phase I and Phase II. Usually, Phase I metabolism represents the oxidation (in some cases reduction) of the foreign compounds, minimizing the compounds' direct interactions with intracellular targets, while Phase II reactions involve the conjugation (e.g. glucuronidation, or glutamylation) of the partially detoxified metabolites, to make them harmless, water soluble metabolites. (It is worth noting that in some instances, Phase I reactions can activate (pro-)drugs (e.g. cyclophosphamide), and conjugates can be precursors of mutagenic and carcinogenic radicals.) By now it has been realized that two additional steps, called Phase 0 and Phase III of drug disposition, are just as important as the previously known processes. These phases involve the modulation of the cellular *entry* and *exit*, respectively, of either the unmodified or metabolized compounds. In Phase 0, a large number of more-or-less selective transporters can increase or decrease the cellular entry of various compounds before they would reach the intracellular compartments or cross tissue barriers. In fact, this mechanism results in a significant modulation of pharmacological effects, by not allowing, or greatly augmenting drug interactions with intracellular targets. The term Phase III was introduced for the process of efficient elimination of the already detoxified molecules, also carried out by multiple transporters [2].

Since all tissue barrier functions, including absorption in the gut, extrusion in the liver or kidney, transport through the blood–brain, blood–testis, or the feto-maternal barrier, involve specific, mostly polarized cell layers, Phase 0 and Phase III drug disposition is directly relevant to all ADME–Tox features of the pharmaceutical agents. By learning the details of these processes we hope to be able to make reasonable predictions for the relevant parameters of newly devised compounds.

Numerous recent reviews deal with the key transporters involved in these important steps [3–5], and here we refer to only the two major groups of proteins involved in drug transport. The first group includes the so-called 'uptake transporters', which are multispecific solute carrier (SLC) transporters, facilitating the cellular entry or exit of a wide range of compounds, without the direct involvement of ATP hydrolysis. The current classification of the uptake transporters distinguishes transporters for organic anions and for organic cations. The SLCO gene family encodes for the organic anion transporting polypeptides (OATPs). The SLC22 gene family encodes for the organic cation transporters (OCTs), the organic cation/carnitine transporters (OCTNs), and the organic anion transporters (OATs) [6,7]. Some of these transporters perform obligatory exchange of organic compounds (e.g. OAT3), while in others transport is modulated and/or driven by monovalent ions and the membrane potential (e.g. OCTN, for recent reviews see refs [8–11].

The second major group of transporters involved in cellular drug disposition are the so-called multidrug resistance (MDR) ATP binding cassette (ABC) proteins, comprising the main subject of the current review. They were named multidrug resistance proteins because of their important role, recognized more than 30 years ago, in cancer drug resistance. Many of the anticancer drugs are transported substrates of these proteins, thus MDR–ABC protein overexpression in cancer cells results in multidrug resistance. While their role in cancer is still an important subject of research, by now it has been generally accepted that MDR–ABC transporters are key players in our physiological cellular defense system. By recognizing and removing a large variety of toxic agents from the living cells, they are major players in Phase 0 and Phase III of drug disposition, thus in the processes shaping general ADME–Tox characteristics.

ABC proteins involved in drug transport are large, membrane-bound proteins, built from a combination of membrane-spanning regions and cytoplasmic ATP binding domains. They bind and hydrolyze ATP, and drug transport is directly linked to their ATPase activity [12]. In humans, the major MDR–ABC proteins include the MDR1/*P*-glycoprotein (ABCB1), several members of the MRP (ABCC) family (MRP1-9), and the ABCG2 (MXR/BCRP) transporter. Although most of their recognized substrates are of hydrophobic nature, MDR–ABC pumps are also capable to extrude a variety of amphipathic anions and cations. On the basis of this 'promiscuous' behavior and the overlapping substrate recognition patterns, these pumps form a functional network, capable to extrude a very wide range of foreign (xenobiotic) substrates.

In our recent review we speculated that the network of MDR–ABC transporters forms a 'chemoimmunity' system [13], which dynamically protects our body against the accumulation of foreign chemical agents. As explained above, hydrophobicity or the amphipathic character of a drug may allow a rapid penetration through the membrane lipid layers, but transporters selectively modulate their entry and exit through cellular barriers. Whereas MDR1–Pgp transports unmodified neutral or positively charged hydrophobic compounds, substrates of the ABCC subfamily members (the MRPs) and ABCG2 extend to organic anions and Phase II metabolic products. In fact, this synergism between the efflux systems and the metabolizing/conjugating enzymes provides a very efficient network for drug elimination. There is a considerable overlap in the substrate specificities and regulation of Cytochrome P450s (CYPs) and the MDR–ABC transporters, especially of MDR1–Pgp. CYP3A, the major Phase I drug metabolizing enzyme and MDR1–Pgp play complementary roles in intestinal drug metabolism, where, through repeated extrusion and re-absorption, MDR1–Pgp ensures elongated exposure of the drugs to the metabolizing enzyme [14].

In the following chapters we present the basic structural and functional features of the key MDR–ABC protein players in drug disposition, as well as the relevant *in vitro* and *in vivo* assays for the determination of their expression and function. We emphasize the possible drug/transporter and drug/drug interactions, as well as factors contributing to the prediction of ADME–Tox properties.

## Structure and mechanism of action of ABC drug transporters

### *Building blocks and the mechanism of action of ABC drug transporters*

The evolutionarily conserved family of the ATP binding cassette (ABC) proteins is present in practically all the living organisms from prokaryotes to mammals. The human genome encodes 48 ABC proteins. Sequence alignments revealed that these proteins can be grouped into seven subfamilies, from A to G. According to a general consensus, all functionally active ABC transporters contain a minimum of two ABC units and two TMDs (see Table 1). ABC

**TABLE 1**

**Key ABC transporters involved in drug resistance and drug disposition**

| Protein name and synonyms | Size of the protein | Tissue distribution | Polarized cell localization | Topology |
|---|---|---|---|---|
| **ABCB1/Pgp/MDR1** | 1280 aa | Blood–brain barrier, Liver, Intestine, Kidney, Placenta, Stem Cells | Apical | TMD1-ABC1-TMD2-ABC2 |
| **ABCB11/BSEP/SPGP** | 1321 aa | Liver | Apical | TMD1-ABC1-TMD2-ABC2 |
| **ABCC1/MRP1** | 1531 aa | Lung, Testes, Kidney, Peripheral Blood Mononuclear Cells, Skeletal and Cardiac Muscle, Placenta | Basolateral (but apical in brain endothelial cells) | TMD0-L0-TMD1-ABC1-TMD2-ABC2 |
| **ABCC2/MRP2** | 1545 aa | Blood–brain barrier, Liver, Intestine, Kidney, Placenta, Lung | Apical | TMD0-L0-TMD1-ABC1-TMD2-ABC2 |
| **ABCC3/MRP3** | 1527 aa | Adrenal gland, Intestine, Pancreas, Gallbladder, Placenta, Liver, Kidney, Prostate | Basolateral | TMD0-L0-TMD1-ABC1-TMD2-ABC2 |
| **ABCC4/MRP4** | 1325 aa | Ovary, Testis, Kidney, Lung, Prostate | Apical, basolateral | TMD1-ABC1-TMD2-ABC2 |
| **ABCC5/MRP5** | 1437 aa | Liver, Testis, Skeletal and Cardiac Muscle, Brain | Basolateral, apical | TMD1-ABC1-TMD2-ABC2 |
| **ABCG2** | 655 aa | Blood–brain barrier, Placenta, Liver, Intestine, Breast, Stem Cells | Apical | ABC-TMD, homodimer |

transporters are large, membrane-bound proteins, built from a combination of characteristic domains, including cytoplasmic ATP binding (ABC) and helical membrane-spanning regions (TMDs). The ABC proteins were named after their conserved, specific ATP binding cassette (ABC) domain, a 200–250 amino acid globular protein unit, which can bind and hydrolyze ATP. The transmembrane domains of ABC transporters are composed in most cases of six membrane spanning helices, and the sites interacting with the transported substrates are most probably located within the TMDs. A minimum of 12 membrane-spanning helices seem to be required to ensure the complex reaction with the transported substrates, bound to high affinity 'on' sites, and unloaded at low-affinity 'off' sites. All recent structural studies indicate a relatively large substrate binding pocket within the transmembrane regions of the ABC transporter proteins. The molecular link, transmitting intramolecular signals between the TMDs and the ABCs, that is, the substrate binding area and the catalytic machinery, respectively, is still unidentified. In order to understand the molecular mechanism of transport, detailed three-dimensional structures of the xenobiotic/multidrug ABC transporters would be required. Currently, such a high-resolution structure of a eukaryotic ABC transporter is not available (the purification and crystallization of pleiotropic membrane proteins is exceedingly difficult). In the past years the community of ABC researchers became enthusiastically optimistic that fundamental questions regarding the mechanism of action of ABC transporters

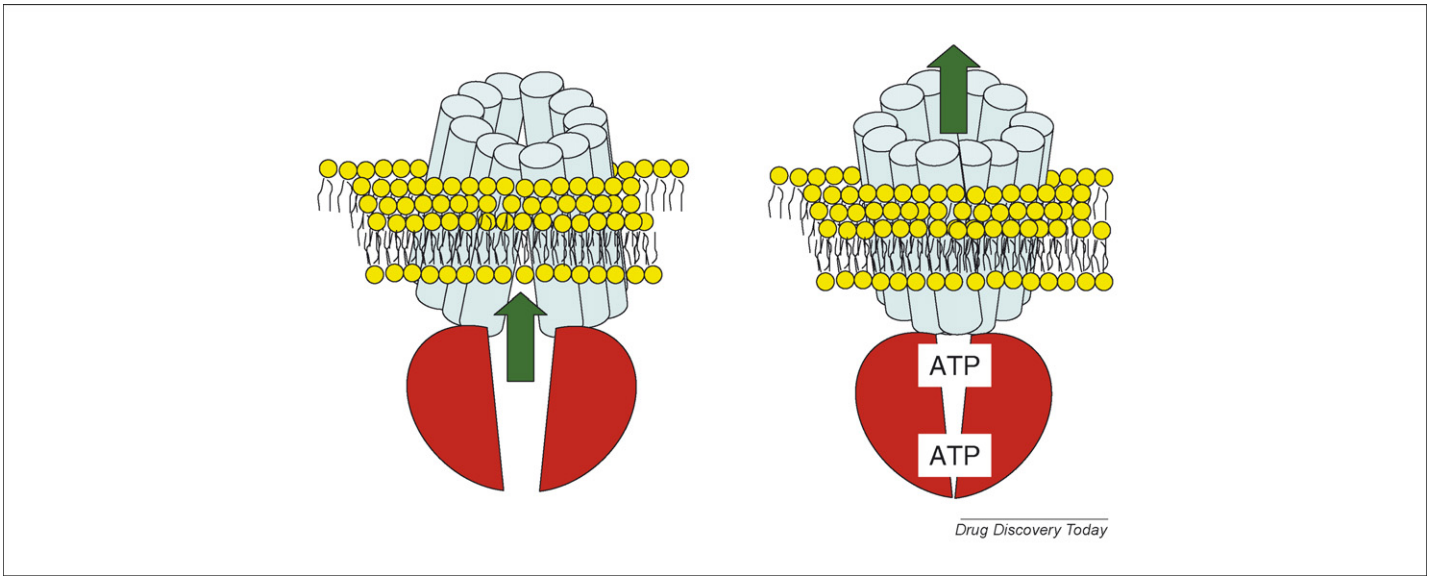


**FIGURE 1**

Schematic representation of the molecular mechanism of ABC transporters [23]. The 12 transmembrane helices (blue) together form a central cavity in the lipid bilayer (yellow). In the left panel, the nucleotide binding domains (NBD, red) are open and nucleotide-free, and the transmembrane helices provide an inward-facing conformation of the transmembrane domains. ATP-driven tight dimerization of the cytoplasmic nucleotide binding domains results in the outward-facing conformation of the TMDs, and ultimately in the dissociation of the transported ligand (see text for details).

**TABLE 2**

**Pharmacologically relevant substances interacting with MDR1, MRP1 and ABCG2**

| | MDR1 | MRP1 | ABCG2 |
|---|---|---|---|
| **Antiarrhythmics** | Amiodarone, Lidocaine, Quinidine | Quinidine | |
| **Antineoplastics** | | | |
| **Anthracyclines** | Doxorubicin, Daunorubicin | Doxorubicin, Daunorubicin, Epirubicin, Idarubicin | Mitoxantrone |
| **Folate based** | | Methotrexate | Methotrexate |
| **Kinase inhibitors** | Imatinib (Gleevec) | Imatinib (Gleevec) | Imatinib (Gleevec), Flavopiridol |
| **Plant alkaloids** | Etoposide, Vincristine, Vinblastine, Topotecan | Etoposide, Vincristine, Vinblastine, Irinotecan, SN-38 | Irinotecan, SN-38, Topotecan |
| **Taxanes** | Docetaxel, Paclitaxel, Ortataxel | Paclitaxel, Ortataxel | Ortataxel |
| **Antidepressants** | Desipramine, Trazadone | | |
| **Antivirals** | Saquinavir, Ritonavir, Indinavir, Nelfinavir | Saquinavir, Ritonavir | Lopinavir, Nelfinavir, Delavirdine |
| **Antibiotics and antifungals** | Cefoperazone, Ceftriazone, Erythromycin, Itraconazole, Ketoconozole, Aureobasidin A | Difloxacin, Grepafloxacin | Ciprofloxacin, Ofloxacin, Norfloxacin |
| **Antimalarials and antiparasites** | Chloroquine, Emetine, Hydroxychloroquine, Quinacrine, Quinine | Chloroquine | |
| **Calcium channel blockers** | Bepridil, Diltiazem, Felodipine, Nifedipine, Nisoldipine, Nitrendipine, Tiapamil, Verapamil | Nifedipine | Nicardipine |
| **Calmodulin antagonist** | Chlorpromazine, Trifluperazine | | |
| **Fluorescent dyes** | BCECF-AM, Calcein-AM, Fluro-2, Fura-2, Rhodamine 123, Hoechst 33342 | BCECF, Calcein, Fluo-3, SNARF | BCECF-AM, Hoechst 33342 |
| **Folates** | | Folic acid, L-leucovorin | Folic acid |
| **Flavonoids** | Quercetin, Kaempferol, Isorhamnetin | Quercetin, Kaempferol, Naringenin | Quercetin, Chrysin |
| **Hormones** | Aldosterone, Cortisol, Deoxycorticosterone, Progesterone Analogs, Testosterone | Sulfate or Glucuronide Conjugates of Estradiol and Estrone | Sulfate or Glucuronide Conjugates of Estradiol and Estrone |
| **Metalloids** | | Sodium arsenite, Sodium arsenate, Potassium antimonite, Potassium, Antimony tartrate | |
| **Immunosuppressants** | Cyclosporin A, Cyclosporin H, Tacrolimus, Sirolimus | Cyclosporin A | Cyclosporin A |
| **Peptides** | Gramicidine D, Valinomycin | GSH, GSSG | |
| **Toxicants** | | Aflatoxin B1, Methoxychlor, Fenitrothion, Chlorpropham | Aflatoxin B1 |

Only a few examples are listed. Boxes were left blank where no data are available or no significant drug–protein interactions were found.

would be soon answered on the basis of the high-resolution portraits of a full bacterial MDR–ABC transporter, MsbA [15–17]. These structures accommodated several predictions on the mechanism of ATP binding and/or hydrolysis, and offered a model to explain how the energy of ATP is converted to conformation rearrangements within the transmembrane domains, responsible for the movement of substrates across the membrane. However, in 2006 the publications were withdrawn and the structures were retracted [18]. Fortunately, further high-resolution structures of a bacterial ABC transporter were solved the same year [19]. Although we have only the first snapshots of full bacterial ABC transporters, it is safe to predict that with the availability of further structures and the power of homology model building, more information will emerge to unravel the molecular details of the eukaryote ABC transporters' action. ABC structures reveal similar arrangements of the conserved ATP-hydrolyzing nucleotide binding domains, but unrelated architectures of the transmembrane domains, with the notable exception of a common 'coupling helix' that is essential for transmitting conformational changes. In functional studies, ATP-driven tight dimerization of the cytoplasmic nucleotide binding domains and the concomitant dynamic restructuring of the NBD dimer interface could be linked to conformational changes in the TMDs [20]. Current models suggest a common alternating access and release mechanism, with binding of ATP promoting an outward-facing conformation of the TMDs and dissociation of the hydrolysis products promoting an inward-facing conformation of the 12 transmembrane helices [21]. This basic scheme can, in principle, explain drug extrusion by ABC exporters [22,23] (Figure 1).

### *The role of structure–activity relationship (SAR) in predicting ADMET*

The MDR–ABC transporters are omnivores, transporting a vast array of clinically and toxicologically relevant compounds, including (but certainly not limited to) anticancer drugs, HIV-protease inhibitors, antibiotics, antidepressants, antiepileptics, and analgesics (Table 2).

Bacterial multidrug binding proteins (such as the transcription regulator BmrR from *Bacillus Subtilis*) and their comparison to the best known MDR–ABC transporter, MDR1/Pgp, may provide a clue to explain how these wide-range drug interactions occur. Crystal structures of the bacterial binding proteins indicate that they interact with drugs through van der Waals interactions and hydrophobic stacking [24]. There is no clear consensus regarding the pharmacophore of Pgp substrates except for the finding that they are hydrophobic and tend to have planar aromatic domains and tertiary amino groups [25]. However, there are excellent Pgp substrates that lack planar aromatic rings (e.g. cyclosporin A), or tertiary amino groups (e.g. ivermectin), so these latter two requirements are non-essential.

The analysis of three-dimensional structures revealed that substrates typically contain hydrogen bond acceptor (or electron donor) groups with defined spatial separations. Modeling indicates that the rate-limiting step for the interaction of a substrate with MDR1/Pgp is the partitioning of the compound into the lipid membrane. Conversely, dissociation of the *P*-glycoprotein–substrate complex is determined by the number and strength of the hydrogen bonds formed between the substrate and the transporter. Thus, a compound with a higher potential to form hydrogen bonds with MDR1/Pgp generally acts as an inhibitor [25]. MDR1/Pgp substrates are expected to freely diffuse into the cells, and MDR1/Pgp may recognize them in the context of the plasma membrane [26]. Theoretical models [27] and empirical *in vitro* experiments [28] indicated that MDR1/Pgp may recognize its substrates before they reach the cytoplasm ('preemptive pumping'). Models based on chemical crosslinking studies, photolabeling experiments, homology modeling and pharmacophore patterns [29] suggest that MDR1/Pgp makes different interactions with different drugs, implying the involvement of several, partially overlapping residues. Thus, each substrate appears to define a unique niche in the complex binding pocket through an 'induced-fit' mechanism [30,31].

There are several questions remaining to be answered. Currently it is not known how large substrates (that are far too big to be coerced *in silico* into the presumed translocation pathway) are transported. Therefore, it is not likely that homology models would help predicting ABC transporter–ligand interactions by *in silico* ligand docking. The ligand/substrate binding sites of the multidrug transporters are large, involving several amino acid side chains located in different TM helices. Though we have a long list of these amino acid positions in the case of Pgp and MRP1, the complexity of the interactions makes such predictions basically impossible. This means that screening strategies for transporter-related interactions have to rely on various *in vitro* tests and *in vivo* assays.

## *In vitro* assays and models for exploring the role of ABC transporters in ADME–TOX

Characterization of a compound as a transporter substrate or inhibitor bears significant consequences in drug development, the selection of dosing regimens, the anticipation of toxic effects, and the potential for drug–drug interactions. The pharmacological relevance of ABC transporters has promoted efforts to establish *in vitro* systems for testing drug–transporter or drug–drug interactions. Typically, *in vitro* assays use either cells stably or transiently overexpressing MDR–ABC proteins, or membranes/proteins isolated from these cells. There are assays that can be used in a *direct setup*, where the fate of the investigated substrate is directly followed. However, since the transported drugs/compounds are mostly hydrophobic, their accumulation strongly depends on the availability of intracellular (or intravesicular) binding sites, sequestration, as well as the permeability of the cell membrane. This is the reason why many *indirect methods* (drug-stimulated ATPase activity, fluorescent dye transport, etc.) were developed to characterize drug transport. As we will see, with indirect assays, distinction of substrates and inhibitors is not always straightforward. In the following section we provide a short overview of *in vitro* assay types emphasizing their advantages and limitations. A more detailed description of these techniques is provided in an exhaustive recent review [5].

### *Cell-based assays*

In pharmacological studies, preference is given to genetically engineered cells over cell lines selected in cytotoxic agents to overexpress a particular ABC transporter. Besides mammalian cell lines, bacterial, insect, yeast and Xenopus oocyte cells are used for characterization of ABC proteins [32–37]. These heterologous expression systems produce large amounts of the selected protein and have the ease of genetic selection and manipulation, and/or short generation times of the organism. To study the combined role of influx and efflux transporters, cell lines stably co-expressing ABC transporters and influx transporters are used in more complex ADME–Tox assay systems. Cell-based techniques are suitable for the assessment of kinetic parameters of the transport and can be used in high-throughput mode. A limitation of this setup is that the varying expression levels of the proteins can greatly influence results.

### *Cytotoxicity/chemosensitivity assay/drug resistance*

The cytotoxicity assay is widely used to test MDR–ABC transporter substrates and inhibitors. Stable cell lines overexpressing the studied ABC protein are cultured in increasing concentrations of the tested drug. Surviving cells are counted, and the $IC_{50}$ value (i.e. the concentration of the drug killing half of the cells) is determined. In case the test compound is extruded from cells by an MDR–ABC protein, a difference between the survival of the parental and the MDR–ABC-transporter-expressing cell lines is observed. Inhibitors and reversal agents can also be identified using this method. Inhibitors enhance the toxicity of known toxic substrates in resistant (MDR–ABC-transporter-expressing) cells. Although this assay is quantitative, it is restricted to compounds that show antiproliferative activity. Also, the drug resistance pattern of the cell lines can be very different and highly depends on the expression levels of the MDR–ABC protein.

### *Cellular accumulation*

Transported substrates show lower accumulation in cells expressing the MDR–ABC protein, and inhibitors increase accumulation up to the level observed in parental cells. Cellular contents of compounds with intrinsic fluorescence or a radioactive label can be directly measured, and their steady state accumulation, efflux, or uptake can be determined. Alternatively, compounds can be

followed by sensitive analytical methods, for example, mass-spectrometry. However, cellular sequestration, 'membrane leakage', intracellular binding, and dependence of fluorescence on intracellular milieu of the MDR–ABC substrates complicate quantification. Therefore, indirect assays were developed that use fluorescent compounds meeting the above criteria. In case of Pgp and MRP1, calcein-AM proved to be an ideal reference substrate [38,39]. ABCG2 does not transport calcein-AM. Instead, the accumulation of the Hoechst 33342 dye is followed for detection of ABCG2 function and in drug screening projects [40]. Alternatively, rhodamine 123 and daunomycin (Pgp substrates), BODIPY–prazosin (Pgp and ABCG2 substrate) or pheophorbide A (ABCG2 substrate) are used as fluorescent probes. Unfortunately, only a few test compounds have intrinsic fluorescence or are available in radioactively labeled form, therefore mainly the indirect setup is used. Since both substrates and inhibitors can hinder the accumulation of the fluorescent substrate, the indirect setup does not distinguish between substrates and inhibitors. Also, false results can be obtained if a test compound is itself toxic to the cells.

### *Transcellular ('vectorial') transport*

*In vivo*, drugs have to cross the pharmacological barriers in order to get absorbed (intestinal epithelial cells), distributed (blood–brain barrier endothelial cells), or excreted (hepatocytes, proximal tubule epithelial cells). This transcellular movement is modeled by cellular monolayer efflux ('vectorial transport') assays. In cellular monolayer efflux measurements the direct transport activity of the MDR–ABC transporter and the permeability of the test compound through a polarized cell monolayer are evaluated. Cells are seeded on a membrane surface (e.g. in a transwell), and at 100% confluency, the test compound is added into the apical or the basolateral solution. At desired time points the concentration of the given compound is determined in both the apical and the basolateral compartments. The apical to basolateral (A–B) and basolateral to apical (B–A) permeability is then determined, and a ratio of the two transport rates, reflecting the function of the MDR–ABC transporter is calculated. In case of apically localized efflux transporters such as Pgp or ABCG2, the basolateral to apical flux will dominate (B–A > A–B) [41,42]. A net flux ratio >2 for the investigational drug indicates transport, often mediated by Pgp. This ratio is reversed in case of a functioning MRP1 that is localized basolaterally [43]. When used in indirect setup, the B–A and A–B rates of transport of known substrates are determined in the presence and absence of the tested compound. If the test compound interacts with the MDR–ABC transporter, the difference between transport rates will decrease. These experiments require the use of known MDR–ABC transporter substrates and (if possible, specific) inhibitors (Figure 2).

Cell lines applied in this assay have to meet certain criteria: they have to polarize and express the given MDR–ABC protein in the appropriate membrane compartment. Cell lines frequently

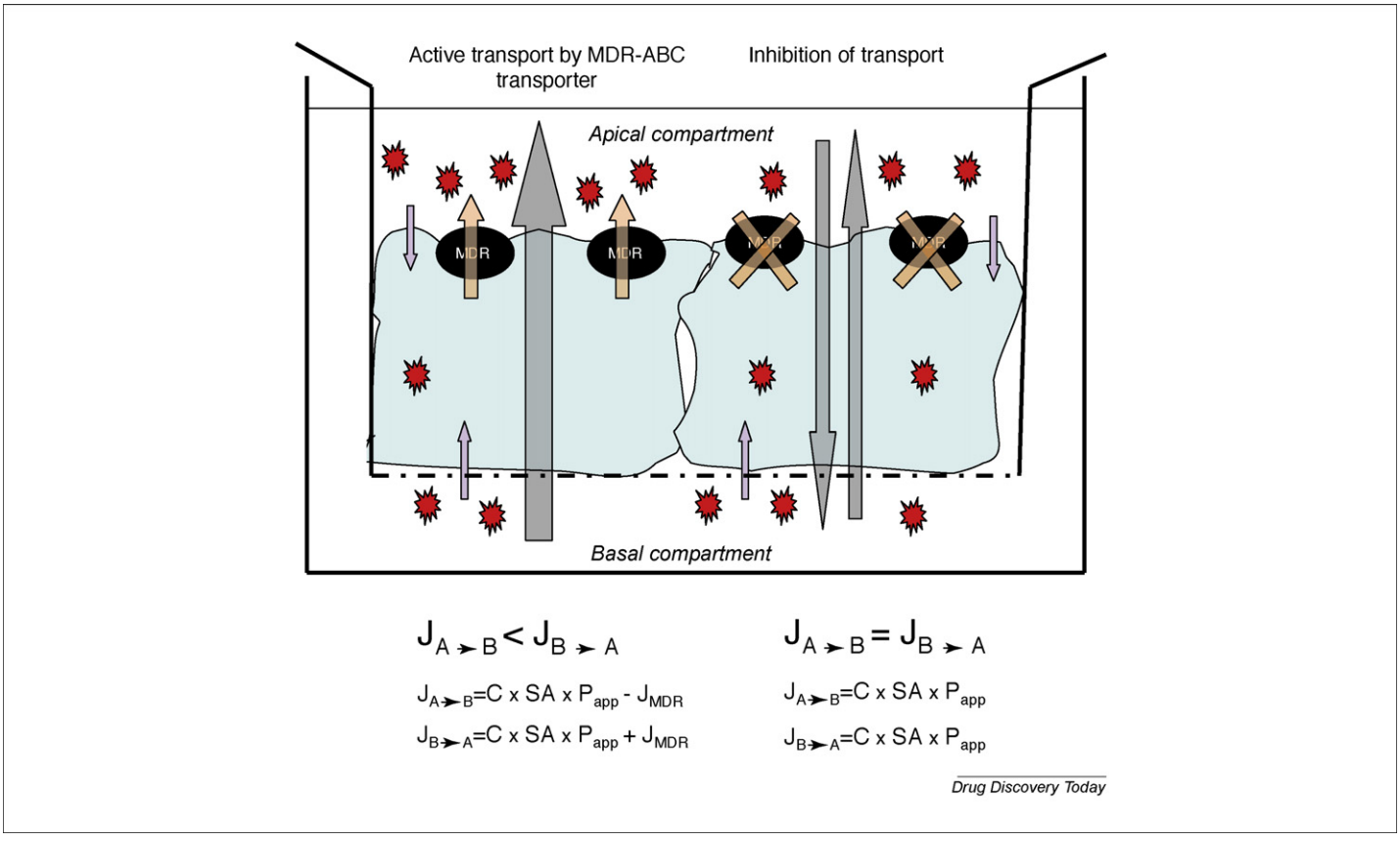


**FIGURE 2**

Model of transcellular transport by an apically localized MDR–ABC transporter (adapted from reference [45]). In a tight monolayer culture of polarized cells, the test compound has to cross the cellular membranes in order to penetrate through the cells. In case of an apically localized MDR–ABC transporter, the basal to apical ($J_{B-A}$) flux of the transported substrate (represented by red stars) will dominate (left panel). When the function of the transporter is inhibited, passive diffusion will determine the distribution of the substrate, and the basal to apical and the apical to basal ($J_{B-A} = J_{A-B}$) fluxes will be equal (right panel). C: concentration of the drug in the donor compartment, SA: surface area, $P_{app}$: passive permeability and $J_{MDR}$: transport due to MDR–ABC transporter activity.

used in pharmacological screens include the human colonic adenocarcinoma (Caco-2), Madin-Darby canine kidney (MDCK) and porcine kidney epithelial (LLC-PK1) cells. Caco-2 cells have characteristics that resemble intestinal epithelial cells such as the formation of a polarized monolayer, with well-defined brush border on the apical surface, and intercellular junctions. Measuring the rate of transport across the Caco-2 monolayer is thought to model absorption across the gut wall. To establish specific roles of MDR–ABC transporters, Caco-2 cells are used in conjunction with specific pump inhibitors. Alternatively, transfected versions of the canine kidney cell lines MDCKI, MDCKII, or the porcine kidney epithelial cells LLC-PK1 are used. More advanced models allow the sequential and concerted action of multiple transporters [44].

To model distribution, primary cultured cells, such as brain endothelial cells, conjunctiva and alveolar epithelial cells can also be used [5]. An important constraint is that the test compounds should not be toxic to the cells (within the time frame of the experiment) and should have a moderate passive diffusion (permeation) rate. If a drug has a high passive transport, rapid re-distribution will diminish the effect of the MDR–ABC transporter on the net transport. Despite technical challenges (long cell culturing time, expression levels depend on culture conditions and passage number), the transcellular transport assay is considered the best *in vitro* model of the *in vivo* interaction, that is, the absorption in the intestine, distribution through blood–brain barrier, and excretion in the liver or kidney.

### *Membrane/protein-based assays*

Membranes isolated from cells described above contain high amounts of the overexpressed MDR–ABC transporter, which enables the characterization of a specific transporter (in cells, the background activity of endogenous proteins often confounds the interpretation of the results). In membrane-based assays, functional characterization of ABC transporter–drug interactions may follow the (i) catalytic activity of the transporter, (ii) the binding of the compounds to the transporter, or (iii) the actual substrate transport. These assays are relatively straightforward and amenable to high throughput. In addition, membranes can be easily maintained after preparation.

## *ATPase activity measurement*

Translocation of compounds by ABC transporters through membranes is energized by ATP hydrolysis. In case of MDR–ABC transporters, substrates usually increase the rate of ATP hydrolysis ('substrate stimulated ATPase activity'). ATPase activity can be estimated by quantifying ATP consumption, ADP release or the liberation of inorganic phosphate [45]. Membrane vesicles prepared from MDR–ABC transporter overexpressing cells (mammalian cells with no/low ecto-ATPase activity or insect cells) or isolated and reconstituted proteins are suitable for measurement of ATP hydrolysis. The profile of the drug-stimulated ATPase reflects the nature of interaction: compounds may be substrates, inhibitors, or may have no effect on the transporter ATPase activity. In the presence of transported substrates, the ATPase activity of the transporter usually increases (activation protocol). Inhibitors, or compounds transported at a lower rate inhibit the ATPase activity of the stimulated transporter (inhibition protocol). In general, most of the transported compounds stimulate the ATPase activity.

In the case of Pgp, exceptions were noted, and some substrates were shown to stimulate activity at lower, and inhibit the ATPase at higher concentrations. ABC proteins typically exhibit a basal ATPase activity, which is probably related to an endogenous stimulation and/or a partial uncoupling [13]. Furthermore, the ATPase activity may also be affected by the lipid environment and the experimental conditions. Both problems had to be overcome before an insect cell based screening assay for the study of ABCG2 was established. When expressed in mammalian cells, ABCG2 demonstrates an ATPase activity that can be further stimulated by its substrates [46]. However, in Sf9 insect cells, wild-type ABCG2 (R482) exhibited a very high basal ATPase activity that could not be (or only very poorly) stimulated by known ABCG2 substrates [47,48]. Recently, we and others have found that in cholesterol-loaded Sf9 membranes significant stimulation of the ATPase (and vesicular transport) activity of wild-type ABCG2 could be reached [46,49].

The application of the vanadate-sensitive membrane ATPase assay for drug interaction studies circumvents the problems of measuring the transport of hydrophobic substrate compounds, if indeed MDR–ABC transporter ATPase is closely coupled to transport activity. The ATPase assay is suitable for high-throughput testing of drugs, investigating drug–drug interactions and determining the kinetic parameters of MDR–ABC transporter drug interaction. A major disadvantage is that the interpretation of the results is not always straightforward, as substrates transported at a lower rate may not affect or even inhibit the basal ATPase activity. Therefore, this indirect assay does not distinguish well between substrates and inhibitors.

An additional way, related to the ATPase cycle, to measure drug interactions with an MDR–ABC transporter is to examine the formation of the catalytic intermediate ('trapped adenine nucleotide') by using labeled ATP or ADP. However, this technique is more suitable for the detailed examination of a specific drug interaction with the transporter and is not routinely used for drug screening purposes (see ref [13]).

### *Drug binding/photoaffinity labeling*

Photolabeling is used to detect substrate or modulator binding to MDR–ABC tranporters. Photoaffinity-labeled compounds are incubated with membranes enriched in ABC transporter and UV-irradiated to permanently attach the label to the proteins. The radioactively labeled ABC transporter is then detected by size fractionation followed by autoradiography. Frequently used photolabeling agents are iodoarylazidoprazosin (IAAP) and [(3)H]azidopine for Pgp and ABCG2 [50,51]. [$^{3}$H]LTC$_4$, *N*-(hydrocinchonidin-8′-yl)-4-azido-2-hydroxybenzamide (IACI) and a tricyclic isoxazole (LY475776) were used for screening drug–drug interactions with MRP1 [52], azidophenyl agosterol for MRP1 and Pgp, and [$^{125}$I]iodoaryl azido-rhodamine123 (IAARh123) for Pgp, MRP1, and ABCG2, for review see ref [5]. This technique can be employed for high-throughput drug–drug interaction screening; however, it does not distinguish between substrates and inhibitors and it is time consuming.

### *Vesicular transport*

A more direct measurement of substrate translocation and its modulation can be achieved by the quantitation of the intravesicularly trapped substrates in vesicular transport assays. In this assay the accumulation of the test compound into inside-out membrane vesicles prepared from cells, or reconstituted transporter-membrane vesicles is measured. Given the orientation of ABC transporters in cells (where the NBDs are in the intracellular compartment), in inside-out vesicles, the NBDs face the incubation media (accessible to ATP and other chemicals), and substrates are actively transported into the vesicles. Rapid filtration, using glass fiber filters or nitrocellulose membranes, is used to separate the vesicles from the incubation solution. The quantity of the transported unlabeled molecules can be determined by high resolution, high sensitivity analytical methods. Alternatively, the compounds are radiolabeled or a fluorescent tag is attached, and the radioactivity or fluorescence retained on the filter is quantified. In case of MRP1, leukotriene $C_4$ ($LTC_4$), [$^3$H]17β-estradiol-17 β-D-glucuronide, estrone 3-sulfate, [$^3$H]vincristine [53], methotrexate, and NEM–GS [54], while in case of ABCG2 methotrexate, [$^3$H]17β-estradiol-17 β-D-glucuronide, estrone 3-sulfate, [$^3$H]dehydroepiandrosterone sulfate (DHEAS) [55,56] can be used as test substrates. The limitations of this assay are that labeled versions are not available for every compound. Another drawback of this method is that hydrophobic compounds may non-specifically bind to lipid membranes and filters, and also, may rapidly leak out from the vesicles. For example, in case of Pgp that transports mainly lipophilic compounds, no such transport assay could be established for its most relevant substrates.

## *In vivo* assays and model systems

Determining the drug transport capacity of the transporters and extrapolating the results to assess the pharmacological impact of a given drug–transporter interaction has remained a difficult task. Besides technical problems, a major problem is the relevance of *in vitro* experiments to the *in vivo* role of MDR–ABC transporters. The *in vitro* models described above clearly demonstrate the ability of ABC transporters to restrict the cellular uptake and the transcellular passage of drugs. On the basis of the analysis of drug–transporter interactions – at least theoretically – it should be possible to predict the penetration of compounds through pharmacological barriers. When considering the *in vivo* role of ABC transporters in drug disposition, the question is whether drugs can freely cross the pharmacological barriers or their passage is restricted by ABC transporters, and whether drugs can influence the passage of other compounds through the inhibition of ABC transporters. Generation of a series of ABC transporter KO mice has provided valuable tools for answering these questions.

### *Pgp–KO animals—absence of Pgp has a profound effect on the tissue distribution of substrate compounds*

Since mice have two mdr1 genes (matching together the human mdr1 gene), Pgp's contribution to drug disposition *in vivo* could be fully appreciated only in the *mdr*1a/1b double-knockout mouse [57]. Surprisingly, the knockouts were viable and fertile. Thus, the first conclusion was that Pgp does not play an essential role in normal homeostasis—at least in the well-controlled environment of the laboratory. Apart from multidrug-resistant cancer cells, Pgp is expressed in normal tissues (the tissue distribution of the mouse mdr gene products closely matches that of the human Pgp). Pgp is found in the apical (lumenal) membranes of epithelial cells, lining organs regulating drug distribution. This expression pattern in excretory tissues and barriers suggested a physiological role in the protection of the organism against orally ingested natural toxins.

Indeed, when the animals were challenged with various toxic agents, the knockouts proved to be hypersensitive, as a result of a profound difference in the tissue distribution of Pgp–substrate compounds. Numerous studies have demonstrated that *P*-glycoprotein limits the brain penetration of drug substrates. The first indication of Pgp's role in the blood–brain barrier came from an accidental discovery. Ivermectin, an antihelminthic drug proved toxic exclusively to the mdr1a (−/−) mice, at a dose routinely used by the caretakers of the animal facility. The 50–100-fold increased sensitivity to orally administered ivermectin was due to an increased accumulation of ivermectin in the brain [57]. Since ivermectin is a *bona fide* Pgp substrate, it was concluded that transport by Pgp at the BBB protected wild-type mice from its neurotoxic activity. This observation has opened the way to a series of investigations assessing the role of Pgp in the BBB. Increased CNS exposure, accompanied by enhanced central activity of peripherally administered drugs, such as loperamide, morphine [58], and amprenavir [59] was observed in the knockout animals (for a comprehensive list, see ref [60]. Combined, these results demonstrated that Pgp could significantly reduce the central nervous system (CNS) access of compounds, thus reducing their brain disposition and changing their overall pharmacological or toxicological effects.

The gastrointestinal tract, which represents the first line of defense against orally ingested toxins and drugs, is ligned by cells that express high levels of Pgp. In the KO mice, the AUC of orally administered taxol was found to be significantly higher, suggesting that intestinal Pgp may be a major determinant of the reduced uptake of some orally administered drug substrates [61]. Increased oral bioavailability of several HIV protease inhibitors [59], tacrolimus [62], ivermectin, loperamide [58] was observed in the knockout animals (for a comprehensive list, see ref [60]). It has to be noted that since the AUC ratio of KO vs. wild-type animals reflects all Pgp-mediated mechanisms (such as metabolism, drug disposition, and clearance) it may not be used as a direct measure of the effect of Pgp on intestinal absorption. Still, these data suggest that chemical blockade of Pgp will result in the altered oral bioavailability of certain compounds. Studies demonstrating the direct involvement of Pgp in biliary or renal excretion of substrate compounds are scarce. Since both processes involve an extensive array of ABC transporters (see Figure 3), the contribution of Pgp by itself may be modest.

### *Abcg2–KO mice—a unique phenotype associated with the increased bioavailability of substrate compounds*

The substrate specificity as well as the tissue distribution pattern of ABCG2 has several features reminiscent of Pgp. Like Pgp, ABCG2 is expressed in sites that influence the fate of administered drugs: in the canalicular membrane of the liver, in the epithelia of small intestine, colon, kidney, as well as the capillary endothelial cells of the blood–brain barrier [63–65].

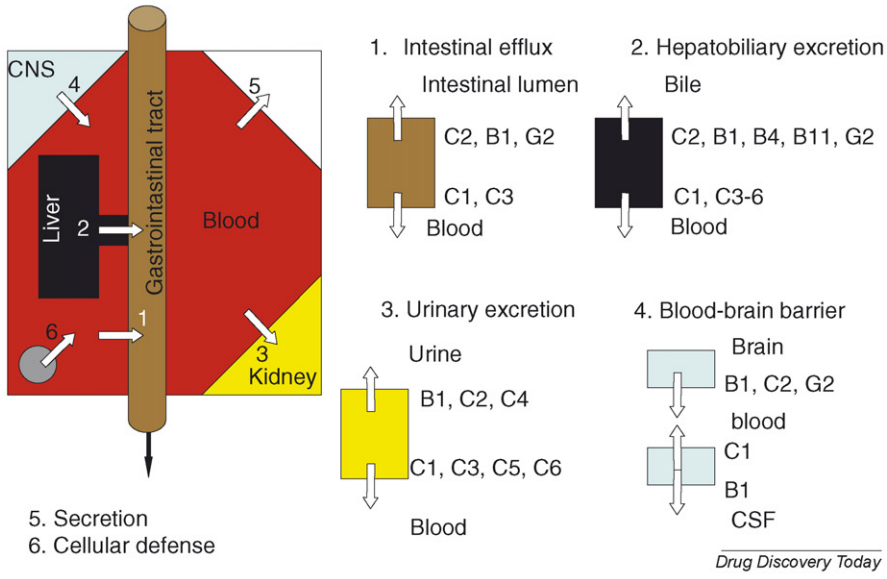


**FIGURE 3**

ABC transporters determine oral bioavailability, tissue penetration and cellular accumulation, and excretion. Several ABC transporters are expressed in the basolateral or apical side of epithelial cells ligning pharmacological barriers. Together, they play a significant role in determining basic pharmacokinetic parameters reflected in the plasma concentration time profile (AUC). Apical ABC transporters in the intestinal epithelium extrude compounds back to the gastrointestinal lumen (1). Drugs that escape this 'first line of defense' reach the liver via the portal system (blood), where they are subject to further metabolism and biliary excretion (2). Excretion of the drugs into the bile may be considered a 'second line of defense', provided by the same set of transporters (ABCB1, ABCC2, ABCG2), passing the drugs back to the gastrointestinal lumen (enterohepatic circulation). Drugs reaching the systemic circulation after escaping first pass extraction by the liver (first pass effect) have to cope with the kidneys, which are equipped with numerous ABC transporters promoting the active extrusion of compounds (3). Drug targets are often shielded by organ–blood barriers (such as the blood–brain and the blood–CSF barrier (4)) and ultimately the cellular membrane (e.g. the membranes of CD4+ lymphocytes in the case of anti-HIV therapy) (6). Drug secretion into specific compartments (such as milk (5)) is also regulated by ABC transporters.

On the basis of the lessons learned with the Pgp-knockouts, a phenotype of the Abcg2-deficient animals was only expected outside of the controlled environment of an animal facility. This was indeed the case: Abcg2 proved to be non-essential in mice, as the knockouts were almost indistinguishable from wild-type littermates. However, under various environmental or pharmacological challenges, several alterations were demonstrated [66,67]. Abcg2 knockouts kept on an alphalpha diet developed UV-light-induced skin lesions [67]. The pathomechanism of the lesions could be explained by the increased plasma levels of a chlorophyll degradation product, pheophorbide A, found in alphalpha. Normally, the transport activity of Abcg2 limits the gastrointestinal uptake of this compound, whereas the knockouts demonstrated an increased intestinal absorption and decreased biliary secretion of this toxin. Further compounds that exhibited elevated plasma levels, and/or decreased intestinal, fecal, and hepatobiliary excretion in the Abcg2-deficient animals include the food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [68]; glucuronide and sulfate conjugates of drugs and hormones [69]; and nitrofurantoin [70].

Besides the major influence on the systemic exposure and the oral bioavailability of natural substances and drugs, ABCG2 was found responsible for the protection of two exquisitely sensitive and important sanctuaries, the fetus and the stem cells. The placenta is a very effective pharmacological barrier, protecting the fetus from drugs and environmental toxins. In mice, genetic disruption or the chemical blockade of Abcg2 leads to an increased fetal exposure of topotecan, mitoxantrone, and dietary toxins [71]. ABCG2 is believed to enhance the survival of stem cells in the characteristically oxygen-poor environment of the stem cell niche by reducing the accumulation of toxic heme metabolites. This 'metabolic protection' is not essential for normal physiology, as lack of Abcg2 expression did not directly influence stem cell function and differentiation in the KO mice [65]. Although to a lesser extent than Pgp, ABCG2 should also be considered as a limiting factor to the CNS penetration of its substrates. Expressed in the luminal surface of brain microvessels [63], ABCG2 was shown to reduce the brain penetration of intravenously administered Imatinib [72]. Finally, ABCG2 was shown to be responsible for the active secretion of clinically and toxicologically important substrates into milk during lactation [73].

### *MRP1-3–KO animals*

KO models for MRP1-3 gave further support to the role of ABC transporters in xenobiotic defense. Like its human ortholog, the murine mrp1 is expressed in most tissues, at relatively high levels. MRP1 is expressed in the basolateral surface, which usually results in pumping substrates into the blood (in contrast to Pgp, MRP2, and ABCG2 that typically keep their substrates away from the blood). Mrp1–KO mice were found to be viable, healthy, and fertile, exhibiting only a mild phenotype of delayed inflammatory response [74,75].

MRP2, expressed in the apical surface of epithelial cells, has a tissue distribution pattern limited to sites important for xenobiotic

protection, such as hepatocytes and enterocytes. Thus, MRP2 has a pivotal role in the export of organic anions and xenobiotics into the bile and also contributes to protection against orally ingested drugs. The characterization of an Mrp2–KO model was possible before the cloning of the gene by way of analyzing TR rats. The phenotype of this strain corresponds to the human condition called Dubin-Johnson syndrome, which is a form of congenital conjugated hyperbilirubinemia. Symptoms are due to the lack of hepatobiliar export of conjugated anions, such as bilirubin. Given its substrate specificity, MRP2 in the liver is well positioned to play a central role in detoxification by secreting drugs and their metabolites into bile. In addition, MRP2 was also found to limit the gastrointestinal absorption of compounds, such as the dietary carcinogen PhiP [76].

MRP3 is mainly expressed in the kidney, liver, and gut, suggesting a role in the enterohepatic circulation of bile salts. However, recent analysis of Mrp3-deficient mice has not revealed any abnormalities in bile acid homeostasis, indicating that Mrp3 does not have a major role in bile salt physiology [77]. Interestingly, hepatic mrp3 overexpression occurs in obstructive cholestasis and in mrp2–KO animals, suggesting that Mrp2 and Mrp3, expressed in the apical and the basolateral side of liver cells, respectively, provide alternative routes for the excretion of glucuronidated substrates from the liver *in vivo* [78]. This has also been recently observed for a key morphine metabolite, morphine-3-glucuronide (M3G), which is transported both by MRP2 and MRP3. In Mrp2–KO mice the presence of Mrp3 results in an increased sinusoidal transport, while the combined loss of Mrp2 and Mrp3 leads to a substantial accumulation of M3G in the liver, resulting in the prolonged presence of M3G in the plasma [78].

### *Extension and evaluation of the KO models*

Taken together, these results indicate that ABC transporters influence drug distribution by (i) limiting drug absorption in the gastrointestinal tract; (ii) promoting drug elimination in the liver and kidney; and (iii) regulating drug uptake into cells, tissues, or pharmacological compartments.

The expression pattern of ABC transporters suggests a role in the defense against orally ingested xenobiotics (Figure 3). In the era of pharmacotherapy, the evolutionary role of 'xenobiotic protection' translates into 'modulation of pharmacokinetic parameters'. Studies with the ABC–KO animals have highlighted the significance of ABC transporter function in Phase 0–III metabolism and provided valuable information contributing to our understanding of the function of the ABC transporter network. In addition, ever since their first description, the KO models have served as tools for testing and evaluating the pharmacological impact of ABC transporters. As we have seen, the definition of a 'transporter substrate' may depend on the particular assay type. From a pharmacological point of view, a transported compound could be defined as a meaningful substrate if the PK properties (such as the enhancement of brain penetration, improved oral systemic exposure, reduced biliary excretion, etc.) are altered in the KO animals or in wild-type animals co-treated with ABC transporter inhibitors ('chemical knockouts').

As oral bioavailability is an important parameter in the development of drug candidates, the role of ABC transporters, notably the transport activity of Pgp in the gastrointestinal tract should bear significant consequences in the design and formulation of pharmacological agents. Yet studies with the Pgp–KO mice show that the extent of Pgp involvement in oral absorption and hepatobiliary or renal excretion of xenobiotics is somewhat variable [79]. Most orally administered compounds are absorbed through the gut wall by way of passive transcellular diffusion. Given its localization in the apical surface of intestinal cells, Pgp can limit the cellular uptake and thus the transcellular passage of its substrates. *Ex vivo* models, using isolated and perfused intestine clearly demonstrate the involvement of Pgp in the intestinal absorption of its substrates [80]. *In vivo*, the extent of this pharmacokinetic effect is dependent on a complex relationship of transport kinetics, membrane permeability as well as the involvement of other uptake and efflux transporters. Intestinal concentrations of orally administered drugs can reach high levels that saturate the transport activity of Pgp. Since higher concentrations also result in an increased rate of passive influx, substrates can evade the transporter, limiting the contribution of intestinal Pgp to overall drug absorption.

KO studies have shown that Pgp is unlikely to be quantitatively important in the oral bioavailability of a compound unless a very small oral dose is given, or the gastrointestinal dissolution and diffusion rates of the drug are very slow [79]. Accordingly, a group of Pgp substrates such as indinavir, ritonavir, and verapamil show dose-independent absorption kinetics [81]. On the contrary, Pgp substrates including digoxin, paclitaxel, talinolol, and saquinavir

**BOX 1**

**Factors limiting *in vitro-in vivo*-clinical extrapolation of the impact of ABC transporters on ADMET**

**HTP *in vitro* systems**

- Not all assays in use provide direct information on whether a compound is transported by an ABC transporter
- Confusion in interpretation of inhibitory or stimulatory properties of compounds, especially in assays that do not directly measure compound transport.

**Animal models**

- Species differences in transport activity, substrate specificity ([83,60])
- KO mice develop compensatory mechanisms involving various elements of Phase 0–III drug disposition
- WT or KO animals do not model the 'ABC transporter status' of a particular disease

**Human data**

- Inter- and intra-individual variability (polymorphisms, pathophysiological conditions, drug–drug interactions in patients treated with many drugs)
- Ethnic-specific polymorphisms
- Human data are derived from inhibition studies. However, inhibitors are not specific and may affect various elements of Phase 0–III drug disposition
- Lack of standardized methodology to assess the functional expression of ABC transporters

show improved bioavailability when coadministered with Pgp inhibitors [82]. The Pgp–KO model proved very valuable for the testing and prediction of the brain penetration of CNS-targeted compounds through the BBB. As compared with wild-type mice, Pgp substrates (such as vinblastine) showed one to two orders of magnitude higher accumulation in the brain of the knockout animals [61]. On the basis of the evaluation of the brain-to-plasma ratio of further compounds in mdr1a/mdr1b (−/−) and wild-type mice, the ability of the drug to penetrate the human central nervous system can be reliably estimated.

In summary, the KO models and wild-type animals are valuable tools used to evaluate the role of ABC transporters in the pharmacological fate of compounds. However, these models do not take account of the specific changes in the expression pattern and function of ABC transporters in disease conditions (Box 1). Furthermore, development of pharmacoresistance, involving the compensatory upregulation of Phase 0–III enzymes should also be considered whenever drugs are administered chronically. A further constraint is that *in vivo* studies are not compatible with high-throughput screening of drugs, and the knockout mouse system may provide misleading information, since significant species differences exist [83]. Despite the high level of sequence identity between the human and murine ABC transporters, there are remarkable differences in the substrate specificity of the orthologs that bear obvious implications in pharmacological testing. However, for the most part, studies have shown reasonable *in vitro–in vivo* correlations, and comparable human-mouse *in vivo* effects on systemic drug disposition.

## Relevance of MDR–ABC transporters in human pharmacology

The activity of a drug ultimately depends on the ability of the compound to reach its target. MDR–ABC transporters constitute an effective pharmacological barrier by restricting the passage of drugs through membranes. Although several ABC transporters have dedicated functions involving the transport of specific substrates, it is becoming increasingly evident that the complex physiological network of ABC transporters plays a significant role in clinical pharmacology. This role is revealed by the tissue distribution of ABC transporters, found highly expressed in important pharmacological barriers (e.g. the epithelium contributing to the blood–brain barrier (BBB)), excretory sites (the biliary canalicular membrane of hepatocytes, the lumenal membrane in proximal tubules of the kidney), and absorption barriers (such as the brush border membrane of intestinal cells) (Figure 3).

### *Drug–drug and drug–food interactions: competition for transport*

Given the wide substrate specificity of MDR–ABC transporters, drug interaction in the membrane penetration (Phase 0) should be significant. Indeed, unexpected drug toxicity is often the result of drug–drug competition during absorption or secretion. Interaction of two Pgp substrates at the level of the transporter provided an explanation for the reduced digoxin clearance in case of coadministration with verapamil or quinidine [84]. Increased absorption of digoxin in the presence of quinidine was directly demonstrated in healthy human subjects by using a multilumen perfusion catheter. The area under the plasma concentration time curve and the maximum plasma concentration of digoxin was significantly higher when luminal quinidine was coadministered, proving that Pgp inhibition in enterocytes increases systemic exposure of orally administered Pgp substrate drugs [85]. Pgp may be partially responsible for the ketoconazole–fexofenadine and erythromycin–fexofenadine interactions [86]. Similarly, competitive interactions between benzimidazoles and MTX for ABCG2-mediated transport result in reduced MTX clearance. Theoretically, this effect may be used advantageously, and pantoprazole may be used to reduce inter-patient variations in systemic MTX exposure by increasing its bioavailability [72]. Drug interaction can also occur through the induction of gene expression. This seems to be the case, for example, during the application of a widely used herbal extract from Saint John's wort (SJW), a herbal medicine to accelerate wound healing, treat nerve pain or depression. Chronic exposure by SJW reduces the bioavailability for a number of drugs because of the induction of both CYP3 and Pgp expression and activity [87]. Through the induction of Pgp, Rifampin was also shown to decrease the intestinal absorption and thus the AUC of coadministered drugs [88].

When drugs compete for MDR–ABC transporters, various food constituents should also be considered as players. This is especially true in the case of natural breakdown products as well as toxic agents obtained during fermentation or preservation of food products.

Without detailing these aspects, it has to be mentioned that flavonoids, which provide much of the flavor and color of food products (see the 'French paradox', probably caused by flavonoids in red wine), are important modifiers of several physiological processes and are also exported by ABCG2. Thus flavonoid consumption may significantly alter the pharmacokinetics, increase the toxicity or the antitumor action of ABCG2 substrate compounds [89].

### *Lessons learned with Pgp inhibitors*

Cancer cells readily co-opt the ancient mechanism providing chemoimmunity as a shield against chemotherapy. Since most of the routinely used anticancer agents of the current repertoire are Pgp substrates, cancer cells with higher levels of Pgp have a selective advantage during adaptation to the treatment. As a result of drug–drug interaction at the level of Pgp expressed in cancer cells, coadministration of Pgp inhibitors with cytotoxic agents could reverse MDR and improve treatment outcome. After all, Pgp–KO mice are viable and fertile, suggesting that pharmacological modulation of human Pgp represents a safe and effective strategy to thwart MDR cancers. In addition, there seems to be no lack of compounds that have the capacity to inhibit Pgp. *In vitro*, these inhibitors can dramatically sensitize drug-resistant cell lines to known Pgp substrates. Still, despite promising *in vitro* results, successful modulation of clinical MDR through the chemical blockade of drug efflux from cancer cells remains elusive.

Over the years, several generations of Pgp modulators have raised hopes only to fail in clinical trials [90]. The negative results may be explained by several factors, such as the intrinsic toxicity of the modulators and the inadequate design of the trials [90]. More importantly, drug–drug interactions occurring at physiological sites expressing Pgp resulted in the altered distribution of the simultaneously administered cytotoxic compounds. Emblematic

of the failures of second-generation inhibitors, PSC-833 induced pharmacokinetic (PK) interactions that limited drug clearance and metabolism of chemotherapy, thereby elevating plasma concentrations beyond acceptable toxicity. To preserve patient safety, empirical chemotherapy dose reductions were necessary; yet, because PK interactions were generally unpredictable, some patients were probably underdosed while others were overdosed. Although most trials using first- and second-generation inhibitors give reason to doubt the benefit of Pgp modulation, the verdict is still out. Since selective modulation of Pgp in cancer cells is difficult to achieve, attempts to circumvent MDR will have to face the profound effects on the distribution of concomitantly administered drugs. Clearly, the inhibitors used today are much improved from those used in the past, with greater target specificity, lower toxicity, and improved PK profiles. Results from Phase III trials using third-generation inhibitors (such as Tariquidar [91]) will be pivotal in determining whether inhibition of Pgp, or other ABC transporters, can result in improved patient survival.

## Clinical pharmacology implications of MDR–ABC transporters

### *Improving oral bioavailability and CNS penetration*

The hope behind ABC transporter targeted anticancer chemotherapy was that ABC modulation in cancer cells could be achieved without significant consequences in the general pharmacokinetic parameters of the concomitantly administered drugs. Although results of clinical trials have been disappointing, the failures opened an alternative way for the development of the inhibitors, to improve the oral bioavailability and CNS penetration of drugs. There is a clear pharmacological need to overcome barriers maintained by ABC transporters in tissue–blood interfaces. Improving the oral bioavailability of anticancer drugs would be a major improvement in the treatment of tumors requiring a prolonged chemotherapy.

Promising results of preclinical and clinical Phase I and II studies indicate that paclitaxel, docetaxel, and topotecan can indeed be administered orally in combination with inhibitors of drug transport [92]. The oral administration of GF120918, a dual inhibitor of ABCG2/Pgp, significantly increased the oral absorption and systemic bioavailability of topotecan [93]. This study also provided a clinical proof of a concept that was based on *in vitro* experiments (topotecan is an ABCG2 substrate [94]) and *in vivo* studies [71].

Pgp presents a barrier to hydrophobic compounds that would otherwise penetrate the blood–brain barrier by passive diffusion. Pgp may thereby reduce the efficacy of agents targeted to the CNS to treat epilepsy, central infections (such as HIV), or brain tumors [95]. While the pathogenesis underlying pharmacoresistance in epilepsy is unclear, upregulation of efflux transporters is viewed as a probable cause of drug resistance. This hypothesis is supported by the findings of elevated expression of efflux transporters in epileptic foci in patients with drug-resistant epilepsy, and experimental evidence that some commonly used antiepileptics are substrates [95]. Treatment of brain tumors is especially limited because of low central penetration of anticancer agents. Ample evidence proves that in mice, CNS penetration of anticancer drugs can be improved by the concomitant use of Pgp inhibitors. However, convincing human pharmacology data offering a clinical proof of the concept are missing [96].

Still, there is an indirect evidence to suggest that clinical application of Pgp blockers will improve the BBB permeability of drugs that currently display insufficient brain penetration for effective therapy. Although the antidiarrheal loperamide is a potent opiate, it does not produce opioid central nervous system effects at usual doses in patients. However, when loperamide was coadministered with the Pgp-inhibitor quinidine, central opioid effects, such as respiratory depression became prominent [97].

Positron emission topography (PET) enables *in situ*, real-time measurements of pharmacological compounds in various pharmacological compartments. For the assessment of pharmacological distribution, including detailed pharmacokinetic parameters, drugs are labeled with a positron-emitting isotope. Using this setup, PSC 833, a second-generation Pgp-inhibitor was shown to increase the brain penetration of the Pgp substrate [11C]verapamil in the living brain of nonhuman primates [98]. The use of such non-invasive technologies should revolutionize how we think about the role of ABC transporters in ADMET. By developing non-toxic derivatives of transporter substrates, it will become possible to assess drug distribution, accumulation, or elimination directly in the patients. By monitoring drug levels, side effects can be correlated to real-time pharmacokinetic data, and 'surrogate' assays can be developed to evaluate the efficacy of the treatment. For example, to ensure abrogation of the MDR phenotype, surrogate assays can be performed to assess the effect of the inhibitor in each patient. This may either be done *ex vivo*, by using flow cytometry to measure Pgp function in CD56+ cells taken from patients treated with inhibitors, or '*in vivo*' using 99mTc-sestamibi or other imaging modalities to directly image accumulation of Pgp substrates within tumors [99].

### *Individualized medicine—role of MDR–ABC transporter polymorphisms*

According to recent studies, most MDR–ABC transporters have several polymorphic variants, which may significantly influence their activity, substrate recognition, and regulation. These interindividual differences are translated to possible metabolic differences, and responses to drug and toxin exposure. The exact determination of these differences in the future may greatly help the predictive efforts during the application of drugs with significant possible side effects.

In MDR1–Pgp, as of today, more than 50 single nucleotide polymorphisms (SNPs) have been reported (see: www.ncbi.nlm.nih.gov/SNP/GeneGt.cgi?geneID=5243). There are several detailed investigations related to the possible function of these polymorphic variants, with little clear-cut evidence for their role in substrate recognition and transport activity. One notorious example is the 'silent' polymorphism in exon 26 (C3435T), which was initially identified as a potential factor in modulating Pgp activity [100]. However, many subsequent studies questioned such an alteration, suggesting that C3435T has either no such effect, or that possible Pgp modulation is caused by the linkage disequilibrium, forming a common haplotype of this SNP with other nonsynonymous polymorphisms, for example, G2677T. Intriguingly, a recent study found that although both RNA and protein levels, as well as turnover rates were similar in the C3435T and wild-type Pgp, the altered protein conformation in the polymorphic variant may cause a functional difference [101].

As to the polymorphic variants of ABCG2, analysis of the sequences in the human population identified several single nucleotide polymorphisms (up to 330 SNPs), and their effects on protein expression and function has been the subject of numerous recent studies. A sequence variant Q126X, leading to premature termination of protein synthesis, was consistently observed in certain Japanese cohorts, while absent in different Caucasian and African American groups. Two ABCG2 protein variants, V12M, and Q141K were found in relatively high frequencies, with significant differences in allele frequencies in different areas of the world, and these may indeed have an effect on ADME–Tox parameters [102,13].

In order to clarify the possible physiological or pathological relevance of ABCG2 polymorphisms, several studies attempting the functional characterization of the variants were performed. Interestingly, the results of the different research groups, regarding expression levels, localization and functionality, are still contradictory. As a short summary, these variants may not substantially alter the substrate specificity of ABCG2, while a reduced protein expression of Q141K may indeed affect drug disposition.

De Jong *et al.* investigated patients with solid malignant tumors, receiving irinotecan treatment [103]. According to this study, the pharmacokinetic parameters of irinotecan and SN-38 were not significantly different between patients carrying the wild-type ABCG2 or at least one polymorphic allele. However, one of the two homozygous individuals showed increased accumulation of SN-38 and SN-38 glucuronide, indicating that the K141 homodimer may have an impaired function. According to a recent study, in five patients, carrying one K141Q allele, after oral administration of diflomotecan, no difference was found in serum levels as compared with individuals with the wild-type genotype [104]. However, after intravenous administration of the drug, the plasma concentration in the heterozygous patients was markedly increased as compared with the individuals with the wild-type genotype.

## Prospects for drug discovery—save money by spending on transporter assays

As described in detail in the above sections, MDR–ABC transporters have a key role in regulating ADME–Tox. Yet this role is less appreciated in the process of drug discovery and development. One crucial and generally recognized problem in pharmacology is oral availability of a new compound. As of now, simple empirical rules and models, based on molecular descriptors and physicochemical properties are used to predict absorption or permeability properties (oral bioavailability). Lipinski's 'rule of five' is based on the cut-off values of four parameters: Log *P* (where *P* is the octanol–water partition coefficient), H-bond donors, H-bond acceptors, and the molecular weight [105]. However, there are many compounds that do not comply with the Rule of Five and most exceptions are found among those recognized by members of the xenobiotic transporter network [106].

There are several possible ways to assess susceptibility to transport and the relevance of the transporter-based effects. A straightforward solution is to set up *in vitro* screening assays. The pharmacological industry is currently establishing medium-throughput transporter assays; the drawbacks, pitfalls and the potential of this approach are presented in the previous sections. It is important to understand the limitations of such screening systems since false estimations of drug distribution can lead to erroneous clinical trials and ultimately to study failures (Box 1).

One of the challenges is to choose the appropriate and most convenient assay, another is to implement and validate the results. According to the current guidelines of the Food and Drug Administration, such investigations should be performed in the early stage of drug development, using 'suitable *in vitro* probes'. For predicting *in vivo* drug–transporter interactions, the FDA recommends cell-based transport assays, such as Caco-2 and Pgp-transfected forms of the MDCK or LLC-PK1 cells (www.fda.gov).

All these current developments strongly suggest that, given the variety of drugs affected by ABC transporter-mediated interactions, it should be of tremendous value to know the susceptibility of drug candidates for transport in the early stages of drug development. Technologies are available in this regard, and a huge amount of money, work and animal lives may be saved by understanding and applying this complex methodology.

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