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Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview ☆

Alfred H. Schinkel *, Johan W. Jonker

Division of Experimental Therapy, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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ABSTRACT

Active drug efflux transporters of the ATP binding cassette (ABC)-containing family of proteins have a major impact on the pharmacological behavior of most of the drugs in use today. Pharmacological properties affected by ABC transporters include the oral bioavailability, hepatobiliary, direct intestinal, and urinary excretion of drugs and drug-metabolites and -conjugates. Moreover, the penetration of drugs into a range of important pharmacological sanctuaries, such as brain, testis, and fetus, and the penetration into specific cell- and tissue compartments can be extensively limited by ABC transporters. These interactions with ABC transporters determine to a large extent the clinical usefulness, side effects and toxicity risks of drugs. Many other xenotoxins, (pre-)carcinogens and endogenous compounds are also influenced by the ABC transporters, with corresponding consequences for the well-being of the individual. We aim to provide an overview of properties of the mammalian ABC transporters known to mediate significant transport of clinically relevant drugs.

Contents

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* Corresponding author. Tel.: +31 20 5122046; fax: +31 20 5122050.
E-mail address: a.schinkel@nki.nl (A.H. Schinkel).

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1. General properties of drug-transporting ABC transporters

This review concentrates on those mammalian ABC transporters [1] that have been demonstrated to have a well-defined role in the transport of clinically relevant drugs. This means that we limit ourselves to the following proteins: P-glycoprotein (P-gp), the multidrug resistance proteins (MRPs) 1–5, and breast cancer resistance protein (BCRP). The family of mammalian ABC transporters, however, is far more extensive, and functionally highly diverse. With the ever-expanding structural and functional diversity of potential drugs, we expect that in the future many more ABC transporters will fall into this class. Several reviews have recently been written on P-gp and MRPs [2–9], and some aspects of these transporters are described in greater detail elsewhere in this issue. We will therefore primarily discuss general principles of the transporters, referring to these reviews, whereas BCRP is discussed in somewhat more detail. Also, we will focus on consensus insights here, rather than discussing currently controversial issues.

All of the ABC drug efflux transporters discussed here are primarily located in the plasma membrane, where they can extrude a variety of structurally diverse drugs, drug conjugates and metabolites, and other compounds from the cell. Export of these compounds occurs in an active, ATP-dependent manner, and can take place against considerable concentration gradients. ATP hydrolysis provides the energy for this process. Based on their predicted two-dimensional structure in membranes, the drug efflux transporters discussed here can be divided into four classes (Fig. 1). Discovered first, the P-glycoprotein polypeptide consists of two very similar halves, each containing 6 putative transmembrane segments, and an intracellular ATP binding site. The first extracellular loop in P-gp is heavily *N*-glycosylated. The same overall architecture is found in MRP4 and MRP5, two members of the MRP family, but N-linked glycosylation occurs most likely on the fourth extracellular loop here. MRP1, MRP2, and MRP3 have the same basic structure as MRP4 and MRP5, but in addition they have an N-terminal extension consisting of 5 putative transmembrane segments. As a consequence, the N-terminus of these proteins is located extracellularly, and also glycosylated in MRP1. The most recently discovered ABC drug efflux transporter is BCRP. Unlike the discussed transporters, this is a half-transporter, consisting of only a single N-terminal, intracellular ATP binding site, followed by 6 putative transmembrane segments. The last extracellular loop is in all likelihood *N*-glycosylated [10]. Based on analogy with the other mammalian ABC drug transporters and bacterial ABC drug 'half'-transporters such as LmrA and MsbA [11,12], and biochemical data [13–15] it is very likely that BCRP functions as a homodimer. All the transporters possess to a greater or lesser extent extracellular *N*-glycosylation branches (Fig. 1). Based on in vitro studies on P-gp [16] it appears that this *N*-glycosylation is not necessary for the basic transport function of these transporters. However, *N*-glycosylation probably has an important cell-biological role for these proteins, helping in stabilizing membrane insertion and possibly routing to, and stability in the plasma membrane.

Some basic properties of the drug efflux transporters, and their official gene nomenclature according to the Human Genome Nomenclature Committee are given in Table 1. A more extended list of properties of mammalian ABC transporters can be found at the website developed by Dr. M. Müller (http://nutrigene.4t.com/humanabc.htm). In the following sections we provide general descriptions of each of the transporters.

2. MDR1 drug-transporting P-glycoprotein (ABCB1)

2.1. Substrates for MDR1 P-glycoprotein

P-glycoprotein (P-gp) was discovered by Juliano and Ling [17], and is possibly the best studied ABC drug efflux transporter to date (see e.g. [5–8,18–20]). Its most striking property is the diversity in structure of substrates that can be transported, including a vast number of drugs applied for a range of therapeutic applications. The therapeutic diversity of a selection of transported substrates is illustrated in Table 2. (For consistency, in this review we will refer to compounds transported by the efflux transporters as 'substrates', whereas compounds that have only been shown to inhibit transporters will be called 'inhibitors' or 'blockers'.) Many cytotoxic anticancer drugs are transported by P-gp, which was first identified because it was overexpressed in cell lines made resistant to such cytotoxic drugs. Owing to the broad substrate specificity of P-gp, the cells displayed cross-resistance to many different cytotoxic drugs, hence the name multidrug resistance (MDR).

There are few common structural denominators for transported P-gp substrates. They are usually organic molecules ranging in size from less than 200 Da to almost 1900 Da. Many contain aromatic groups, but non-aromatic linear or circular molecules are also transported. Most of the efficiently transported molecules are uncharged or (weakly) basic in nature, but some acidic compounds (e.g., methotrexate, phenytoin) can also be transported, albeit at a low rate. The only common denominator identified so far in all P-gp substrates is their amphipathic nature. This may have to do with the mechanism of drug translocation by P-gp: it has been postulated that intracellular P-gp substrates first have to insert into the inner hemileaflet of the cell membrane, before being 'flipped' to the outer hemileaflet, or perhaps being extruded directly into the extracellular medium by P-gp [21]. Only amphipathic molecules would have the proper membrane insertion properties. Both MDR1 P-gp and the highly related MDR3 P-gp can transport intrinsic (amphipathic) membrane components such as phosphatidylcholine and analogues thereof, which supports the notion that substrates are taken from the inner hemileaflet [22–24].

As most P-gp substrates are quite hydrophobic, in principle they can diffuse passively across biological membranes at a reasonable rate. For cell-biological and pharmacological studies, this means that in the absence of active transport, P-gp substrates will cross membranes and (in vivo) penetrate into tissues and pharmacological compartments. It

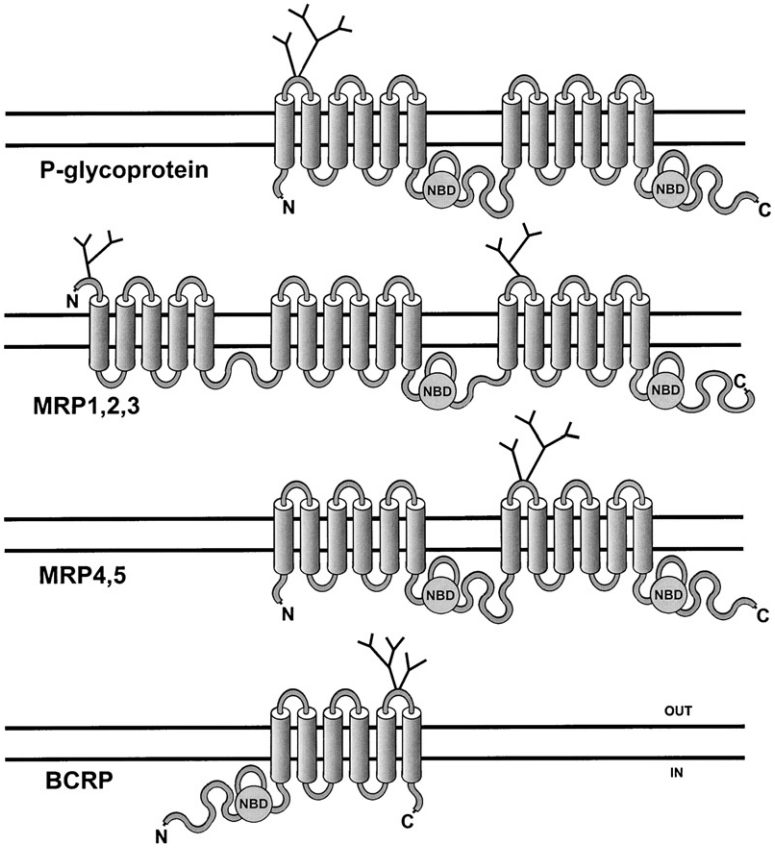

Fig. 1. Predicted secondary structures of drug efflux transporters of the ATP-binding cassette family. Four classes are distinguished here, based on predicted structure and amino acid sequence homology. (1) P-glycoprotein consists of two transmembrane domains, each containing 6 transmembrane segments, and two nucleotide binding domains (NBDs). It is *N*-glycosylated (branches) at the first extracellular loop; (2) MRP1, 2 and 3 have an additional aminoterminal extension containing 5 transmembrane segments and they are *N*-glycosylated near the N-terminus and at the sixth extracellular loop; (3) MRP4 and 5 lack the aminoterminal extension of MRP1–3, and are *N*-glycosylated at the fourth extracellular loop; (4) BCRP is a 'half transporter' consisting of one NBD and 6 transmembrane segments, and it is most likely *N*-glycosylated at the third extracellular loop. Note that, in contrast to the other transporters, the NBD of BCRP is at the aminoterminal end of the polypeptide. BCRP almost certainly functions as a homodimer. N and C denote amino- and carboxy-terminal ends of the proteins, respectively. Cytoplasmic (IN) and extracellular (OUT) orientation indicated for BCRP applies to all transporters drawn here.

also means that a contribution of active (back-)transport by P-gp will only result in noticeable distribution effects if the rate of active transport for a certain compound is substantial relative to the passive diffusion rate. If not, the pump activity will be overwhelmed by the passive diffusion component.

2.2. Inhibitors of MDR1 P-glycoprotein

It was soon recognized that transport of cytotoxic and other substrates for P-gp can also be inhibited by certain compounds [25]. These so-called 'reversal agents' or 'P-gp blockers' will be discussed in greater depth elsewhere in this issue, but we will present some general considerations here. Many of the initially identified inhibitors, like the calcium channel blocker verapamil or the immunosuppressive agent cyclosporin A, turned out to be themselves transported substrates of P-gp, suggesting that they act as competitive inhibitors. For other inhibitors no significant transport by P-gp could be demonstrated, indicating that they probably work through other mechanisms. Still, it may be that some of the latter compounds are just diffusing so quickly across membranes that transport by P-gp, although it does occur, is not detectable (see discussion in [20]).

Initial thoughts on clinical application of P-gp inhibitors were focused on reversing MDR in chemotherapy-resistant tumor cells that contain significant amounts of P-gp, but later insights indicated that such inhibitors might also be useful to modulate the general pharmacological behavior of drugs in the body (see below). The P-gp inhibitors that were initially recognized, such as verapamil, are actually relatively poor P-gp inhibitors in vivo, and they frequently have their own pharmacodynamic effects that put severe restrictions on the plasma levels that can be safely achieved in patients. We will therefore briefly discuss five so-called second- or third-generation P-gp inhibitors, that were selected specifically for their high P-gp-inhibiting capacity, and for lack of other pharmacodynamic effects (Table 3).

SDZ PSC 833 (or PSC 833) is a cyclosporin A analogue that does not have the immunosuppressive effect of cyclosporin A, and can be given at quite high dosages to patients [19,26]. PSC 833 is a high-affinity, but slowly transported substrate for MDR1 P-gp, which probably acts as an effective inhibitor because its release from P-gp is very slow [27].

Table 1
Properties of human ABC drug efflux transporters.

Protein	Gene name	Alternative names	Size (aa)	Polarized localization (in MDCK or kidney cells)
MDR1 P-gp	ABCB1	PGY1, GP170	1280	Apical
MRP1	ABCC1	MRP	1531	Basolateral
MRP2	ABCC2	cMOAT, cMRP	1545	Apical
MRP3	ABCC3	MOAT-D, cMOAT-2	1527	Basolateral
MRP4	ABCC4	MOAT-B	1325	Apical
MRP5	ABCC5	MOAT-C, pABC11	1437	Basolateral
BCRP	ABCG2	MXR, ABCP	655	Apical

Additional information can be found on the web site of M. Müller: http://nutrigene.4t.com/humanabc.htm.

Table 2
Some clinically relevant transported substrates of MDR1 P-glycoprotein.

Analgesics
Asimadoline
Morphine (poor substrate)
Anticancer drugs
Vinca alkaloids (vinblastine, vincristine)
Taxanes (paclitaxel, docetaxel)
Anthracyclines (doxorubicin, daunorubicin, epirubicin)
Anthracenes (bisantrene, mitoxantrone)
Epipodophyllotoxins (etoposide, teniposide)
Actinomycin D
Methotrexate (poor substrate)
Topotecan (poor substrate)
HIV protease inhibitors
Saquinavir
Ritonavir
Nelfinavir
Indinavir
Lopinavir
Amprenavir
H_2-receptor antagonists
Cimetidine
Anti-gout agents
Colchicine
Antidiarrheal agents
Loperamide
Antiemetics
Domperidone
Ondansetron
Calcium channel blockers
Verapamil (poor substrate)
Cardiac glycosides
Digoxin
Immunosuppressive agents
Cyclosporin A
FK506
Corticoids
Dexamethasone
Hydrocortisone
Corticosterone
Triamcinolone
Pesticides, anthelmintics, acaricides
Ivermectin
Abamectin
Amebicides
Emetine
Antibiotics
Erythromycin
Gramicidin D
Valinomycin
Diagnostic dyes
Rhodamine 123
Hoechst 33342

Table 3
Some efficacious inhibitors of MDR1 P-glycoprotein.

PSC 833 (Valspodar)
GF120918
LY335979
XR9576
OC144-093

Although PSC 833 is an efficient P-gp inhibitor, it does have the complication that it is also an inhibitor of cytochrome P450 3A4 (CYP3A4), one of the main drug-metabolizing enzymes in the body [28]. Consequently, when administered to patients, next to inhibiting P-gp, it may have additional effects on the clearance of drug substrates that are degraded by CYP3A4. Many cytotoxic anticancer drugs that are P-gp substrates, such as etoposide and doxorubicin, are also extensively degraded by CYP3A4. Therefore, co-administration with PSC 833 can intensify the toxic side effects of these anticancer drugs, necessitating a dose reduction for safe treatment of the patient (reviewed in [19]). The main dose-limiting toxicity of PSC 833 itself in patients is ataxia. PSC 833 is currently tested in a number of Phase III clinical trials for reversal of drug resistance in tumors.

GF120918 (also called GG918) is a highly effective P-gp inhibitor developed specifically for this purpose [29]. It can be given at very high oral dosages to mice and patients without obvious toxic effects [30]. In mice, GF120918-treatment improves the response of implanted P-gp-containing tumors to chemotherapy. GF120918 also does not appear to affect the clearance of intraperitoneally administered doxorubicin in mice, which suggests that it does not have very extensive effects on enzymes metabolizing this drug (like CYP3A). However, GF120918 does interact with at least one other drug efflux transporter, namely BCRP [31], but this is not necessarily a disadvantage (see later). Currently running clinical trials with GF120918 are amongst others testing inhibition of P-gp in the intestine [30].

LY335979 is another specifically developed, highly effective P-gp inhibitor [32]. Like GF120918, it improves chemotherapy response in mice with transplanted P-gp-containing tumors, and it does not clearly affect the plasma clearance of intraperitoneally administered doxorubicin or etoposide. However, this was only tested with intravenously or intraperitoneally administered LY335979. Whether LY335979 could be orally active is as yet unclear. In line with the absence of pronounced plasma pharmacokinetic interactions, LY335979 has much lower affinity for CYP3A than for P-gp [33] LY335979 is currently being tested in clinical trials [33].

Two other compounds, XR9576 and OC144-093 appear to be very promising P-gp inhibitors as well [34,35]. Both have high affinity for P-gp, they can be given both orally and intravenously to improve the chemotherapy response of transplanted P-gp-containing tumors in mice, and they do not affect the plasma pharmacokinetics of intravenously administered paclitaxel. The latter point again suggests that XR9576 and OC144-093 do not have extensive interaction with paclitaxel-metabolizing enzymes.

Obviously, this discussion of P-gp inhibitors cannot be comprehensive, but it is clear that by now there are many P-gp inhibitors with increasingly suitable properties for clinical use. Future studies will tell us whether they can have a marked impact on clinical multidrug resistance.

2.3. Pharmacological functions of P-glycoprotein

MDR1 P-gp is mainly (though not exclusively) present in epithelial cells in the body, where it localizes to the apical membrane [36]. As a consequence, transported P-gp substrates are translocated from the basolateral to the apical side of the epithelium (Fig. 2). This can have dramatic consequences for the pharmacological behavior of substrate drugs. Many of these pharmacological aspects were first directly demonstrated in knockout mice that lack one or both of the two murine homologues of human MDR1 P-gp, i.e. Mdr1a and Mdr1b P-gp. Insights obtained from these mice have been recently reviewed [7,8,18], and many aspects are also discussed in more detail elsewhere in this issue. We will therefore limit ourselves here to a brief listing of the most important locations and pharmacological functions of drug-transporting P-gp, and refer to the reviews for details.

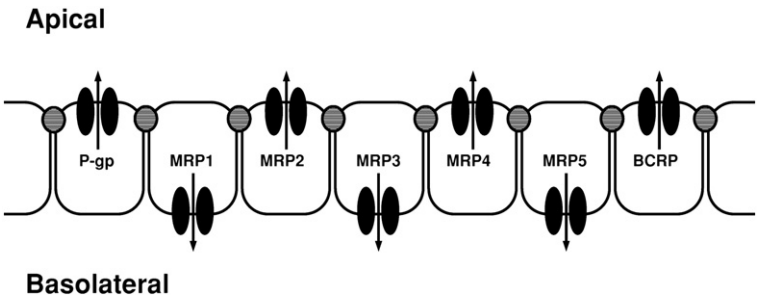

Fig. 2. Subcellular localization of ABC drug efflux transporters in polarized kidney epithelia. P-gp, MRP2, MRP4, and BCRP localize to the apical membranes of kidney epithelial cells. MRP1, 3, and 5 localize to the basolateral membranes of kidney epithelial cells. Note that in prostate acinar cells, MRP4 was detected in the basolateral membrane. The physical barrier between the apical and basolateral plasma membrane is formed by tight junctions (represented by hatched circles).

2.3.1. Blood–brain barrier function

Endothelial cells of the small blood capillaries in the brain are closely linked to each other by tight junctions, and they cover the entire wall of these blood vessels. As a consequence, all compounds that are not small enough to diffuse between the cells have to cross the endothelial cell in order to translocate from the blood compartment into the surrounding brain tissue. Since most MDR1 P-gp substrates are quite hydrophobic, in principle they have the possibility to passively diffuse across the endothelial cell membranes, and thus enter the brain at a reasonable rate. However, owing to a high level of MDR1-type P-gp in the luminal membrane (i.e., facing the blood) of the endothelial cells [37] substrate drugs entering the endothelial cells from the blood are immediately pumped back into the blood. As a consequence, the net penetration of substrate drugs and other substrate compounds from the blood into the brain tissue can be dramatically decreased. In the absence of P-gp in the blood–brain barrier, the brain penetration of P-gp substrate drugs can increase up to 10- to 100-fold, with sometimes dramatic consequences for the clinical applicability and toxicity of compounds (see e.g. [38–40]). The brain is a critical organ, and potentially very sensitive to all kinds of toxic and other pharmacodynamic actions of exogenous compounds. It is quite obvious that P-gp must have evolved in part to protect the brain from damaging effects of xenotoxins that can be taken up with food, or are perhaps generated by pathogenic organisms in the intestine.

2.3.2. Blood–testis and blood–nerve barrier function

These P-gp functions are completely analogous to those found in the blood–brain barrier: part of the capillary vasculature in these tissues has developed similar properties as in the brain, including high content of MDR1-type P-gp in the endothelial cells [37]. Again, it appears that P-gp can help in protecting relatively sensitive and critical tissues from xenotoxic compounds (see e.g. [39–42]).

2.3.3. Fetal–maternal barrier function in the placenta

As first discovered by Lankas et al. [43], MDR1-type P-gp is functional in the placental syncytiotrophoblasts, where it is present in the apical membrane, facing the maternal blood compartment [44]. The syncytiotrophoblasts form the functional barrier between the maternal and fetal blood circulations, and are essential for nutrient and waste product exchange, but also for protection of the fetal circulation. The function of MDR1-type P-gp here appears to be analogous to that in the blood–brain barrier: it protects the highly sensitive developing fetus from substrate xenotoxins and drugs present in the maternal circulation by active back-transport (Fig. 3). This principle has been directly demonstrated using P-gp deficient mutant or knockout mice for at least four different P-gp substrates: an analogue of the pesticide avermectin, the cardiac glycoside digoxin, the HIV protease inhibitor saquinavir, and the anticancer drug paclitaxel [43,45,46]. Quantitative effects could go up to 10–20-fold, and the increased fetal penetration of the avermectin analogue resulted in birth defects. For most therapeutic purposes, low penetration of drugs into the fetus is of course highly desirable, but in the case of HIV treatment it might be advantageous to have reasonable 'drug loading' of the fetus shortly before birth. This might reduce the frequency of vertical transmission of HIV infection, which frequently occurs during birth.

2.3.4. Hepatobiliary and direct intestinal excretion function

MDR1-type P-gp is very abundant in the bile canalicular membrane of hepatocytes, and in the apical (villous) membrane of small and large intestinal epithelium [36]. One predicted function for MDR1-type P-gp in these locations was extrusion of substrate drugs and other compounds from the liver hepatocyte into the bile, and from the intestinal epithelium into the intestinal lumen. As many compounds can readily enter the hepatocytes and intestinal epithelial cells from the blood compartment, this would result in a net excretory function for P-gp. Extruded substrates would ultimately leave the body via the feces, so overall this would result in a plasma clearing and detoxifying function. Indeed, experiments in P-gp knockout animals directly demonstrated that substrate drugs are extruded from the blood into the bile, and

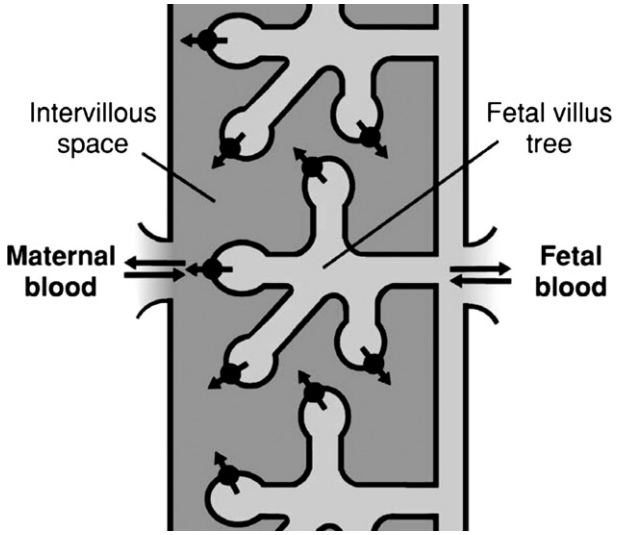

Fig. 3. Highly schematic representation of ABC drug efflux transporter localization and function in the materno–fetal barrier in the placenta. A functional unit of the human placental barrier consists of a fetal villus tree (light grey) containing the fetal blood capillaries (not drawn here). Villus trees are lined with a tight epithelial cell layer, formed by syncytiotrophoblasts, which are in direct contact with the maternal blood freely diffusing throughout the intervillous space (dark grey). Drug efflux transporters, such as P-gp, BCRP, and possibly MRP2 (indicated with ball-and-arrows) are present in the apical membrane of the syncytiotrophoblasts where they prevent drug and toxin entry from the maternal blood into the fetal circulation. The syncytiotrophoblast epithelial layer is presented here by the solid black line lining the villus tree. Maternal arteries and veins regulating blood supply to the intervillous space are schematically drawn here as a single entry–exit gate.

also directly into the intestine, by MDR1-type P-gp [47,48]. For a number of drugs this resulted in a faster clearance of intravenously administered drugs from plasma than in the absence of P-gp, and this has been further corroborated for many other drugs by other groups.

2.3.5. Function in restricting oral bioavailability of drugs and toxins

As MDR1-type P-gp is abundant in the intestinal epithelium, one obvious potential function was that it could restrict the rate at which substrate compounds present in the intestinal lumen enter the bloodstream. Many drugs are P-gp substrates, so this could mean that the bioavailability of many orally administered drugs might be restricted by P-gp. Indeed, in P-gp knockout animals, the oral bioavailability of the anticancer drug paclitaxel was markedly increased [48]. Later experiments by various groups confirmed that the oral bioavailability of many other drugs is limited by P-gp activity as well. For pharmacotherapy purposes, oral bioavailability is a very important factor. Oral drug administration is highly preferred because it is cheap, relatively safe, and patient friendly. However, if a drug has low oral bioavailability, the plasma level of the drug may not attain sufficiently high levels to have therapeutic effect. Also, low drug bioavailability is frequently associated with variable drug uptake, and this can be a problem if the drug has a narrow therapeutic concentration window. Thus, P-gp activity can be a major problem for therapeutic applications of drugs. For this reason, it is of interest for pharmaceutical companies to check at an early stage whether a drug under development is a good P-gp substrate, because this could mean that it is not readily orally available. Depending on the therapeutic category of the drug this could be prohibitive for its ultimate clinical use.

An intriguing hint for a possible natural function of MDR1-type P-gp came from a study by Panwala et al. [49]. When kept in some labs, knockout mice deficient for Mdr1a P-gp developed an inflammation of the large intestine similar to the inflammatory bowel disease sometimes seen in humans. The inflammation was dependent on the presence of intestinal bacteria, and did not seem to result from an abnormal immune response. Although not proven yet, it appears likely that the inflammation results from toxins produced by intestinal bacteria, which are normally kept out of the intestinal wall by Mdr1a P-gp activity. The variable occurrence of this phenotype in different labs probably depends on the presence and abundance of certain toxin-producing bacterial strains in mouse intestinal flora in each lab.

2.3.6. P-gp function in kidney?

Whereas the function of P-gp in the previously discussed tissues has been demonstrated very convincingly, the situation in the kidney is less clear. P-gp is abundant in the apical (luminal) membrane of proximal tubules, so one expects an excretory function here, pumping substrates from blood into urine. However, most pharmacological studies to date using P-gp knockout mouse models have given equivocal results. In some cases the absence of P-gp in the mice resulted in increased renal excretion and even clearance of drugs, which would seem to contradict an excretory function [50]. However, this may in part have to do with a general rerouting of excretory pathways for P-gp substrates in P-gp knockout mice (shifting from primarily hepatobiliary/intestinal/fecal to renal, see e.g. [47]), or with changes in expression of other transporters or enzymes that affect the tested drugs. For instance, it was found that a number of drug-metabolizing enzymes could be upregulated in P-gp deficient animals, but that this was also dependent on the conditions under which the animals were kept [51]. It is therefore as yet uncertain what the excretory role of renal P-gp is, but given the demonstrated functions elsewhere in the body, it seems difficult to imagine that it would not contribute to the renal excretion of at least some P-gp substrate drugs. This question warrants further investigation.

2.4. Pharmacological inhibition of P-gp to optimize pharmacotherapy

Given the profound impact of MDR1-type P-gp on a variety of important pharmacological and toxicological processes, it was of great interest to consider whether the available efficient P-gp inhibitors could be used to optimize various aspects of pharmacotherapy. One could also consider whether unplanned P-gp inhibition, owing to co-administration of several drugs, might not sometimes result in unwanted toxicity effects.

Efforts to increase the penetration of anticancer drugs into multidrug resistant tumors containing P-gp were discussed already in the section describing various P-gp inhibitors. A partly related application would concern the chemotherapy treatment of tumors (e.g., gliomas) that might be positioned behind the blood–brain barrier, and therefore poorly accessible to most anticancer drugs. For other diseases of the central nervous system (CNS) it might likewise be desirable to improve the brain parenchyme penetration of drugs. As a proof of principle to show that it is possible to extensively inhibit P-gp in the blood–brain barrier, a number of studies were performed. Indeed, it turned out to be feasible to largely block P-gp in the blood–brain barrier in mouse models, but only when highly efficacious P-gp inhibitors were used [41,52,53]. PSC 833 and GF120918 were shown to be active for this purpose even when administered orally [52,54], and we expect this will be true for several more efficacious P-gp inhibitors with reasonable oral bioavailability.

As pointed out above, oral bioavailability is an important parameter for the practical use of many drugs, and directed inhibition of P-gp in the intestine might improve oral bioavailability of P-gp substrate drugs [55]. The first directed attempts to improve oral bioavailability of the P-gp substrate paclitaxel indeed showed that in both mice and humans, P-gp inhibitors PSC 833, GF120918, and even cyclosporin A could dramatically increase oral availability [30,56–58]. Currently, clinical trials are running in our institute to determine whether such treatment protocols have clear therapeutic benefit in cancer treatment.

In some cases it may be desirable to increase the penetration of drugs into the fetus, although in general one would prefer to minimize fetal drug penetration to prevent toxic effects. For efficacious anti-HIV drugs, it may be useful to increase fetal drug penetration in the period shortly before delivery to minimize the chance that there will be mother-to-child transmission of the virus during birth. Indeed, we have demonstrated in a mouse model that even oral administration of the P-gp inhibitors PSC 833 and GF120918 can substantially increase the fetal penetration of the HIV protease inhibitor saquinavir, to levels that suggest that placental P-gp was completely inhibited [45]. Whether it is sufficiently safe to try this procedure in humans should be carefully investigated, given the risk of unexpected toxicities for the unborn child.

3. Multidrug resistance(-associated) protein 1 (MRP1, ABCC1)

3.1. General properties of MRP1

MRP1 was first identified in a cell line made highly resistant to a cytotoxic drug (doxorubicin), and subsequent analysis showed that it conferred MDR against a range of anticancer drugs [59,60]. Substrate anticancer drugs (Table 4) include *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, mitoxantrone and methotrexate (see e.g. [2,3,62]). Unlike P-gp, MRP1 does not confer high levels of resistance to paclitaxel or bisantrene in cells [61,63–65]. In further contrast to P-gp, MRP1 functions mainly as a (co-)transporter of amphipathic organic anions. It can transport hydrophobic drugs or other compounds (e.g. the inflammatory mediator leukotriene C4 (LTC_4)) that are conjugated or complexed to the anionic tripeptide glutathione (GSH), to glucuronic acid, or to sulfate [66–69]. In fact, efficient export of several non-anionic anticancer drugs by MRP1 is dependent on a normal cellular supply of GSH (see e.g. [70,71], and

Table 4
Some cytotoxic (anticancer drug) substrates of MRP1.

Epipodophyllotoxins	Etoposide
	Teniposide
Vinca alkaloids	Vincristine
	Vinblastine
Anthracyclines	Doxorubicin
	Daunorubicin
	Epirubicin
	Idarubicin
Camptothecins	Topotecan
	Irinotecan/SN-38
Anthracenedione	Mitoxantrone
Heavy metal oxyanions	Arsenite
	Trivalent antimony

Data are taken from studies in cells transfected with human MRP1 or murine Mrp1, and in murine cells lacking Mrp1. Note that murine Mrp1 has a much lower capacity to transport anthracyclines than human MRP1 [82].

it is likely that MRP1 exports drugs such as vincristine and etoposide by co-transport with reduced GSH [72,73]. Non-organic heavy metal oxyanions like arsenite and trivalent antimony are also transported by MRP1, in all likelihood complexed to GSH [71].

In contrast to P-gp, MRP1 localizes to the basolateral membrane of epithelial cell layers [68], and its substrates are therefore transported towards the basolateral side of epithelia. Knockout mice lacking Mrp1 are viable and fertile, but they do show deficiencies in LTC_4-mediated inflammatory reactions, suggesting that secretion of LTC_4 is an important physiological function of MRP1 [74–76].

3.2. Pharmacological functions of MRP1

Even though MRP1 localizes predominantly to the basolateral membrane of epithelial cells, it still has important pharmacological and toxicological functions. Wijnholds et al. [74,77] showed that Mrp1 knockout mice are more sensitive to the toxicity of intravenously administered etoposide in the oropharyngeal mucosal layer and testicular tubules. This can be explained by the fact that these cells are shielded from blood-borne toxins by epithelia that have the basolateral membrane facing the blood circulation. Moreover, Mrp1 in the basolateral membrane of choroid plexus epithelial cells can mediate a substantial clearance of etoposide from the cerebrospinal fluid, indicating that this compartment is also protected by Mrp1 [78]. Other studies demonstrated that a combined deficiency of Mdr1a and Mdr1b P-gps and Mrp1 in knockout mice resulted in a dramatically increased sensitivity to intraperitoneally administered vincristine (up to 128-fold), but also to etoposide (3.5-fold), whereas a P-gp deficiency alone resulted in a 16- and 1.75-fold increased sensitivity to this drug, respectively [79]. In this case, greatly increased vincristine toxicity was observed in bone marrow and the gastrointestinal mucosa, suggesting that these compartments are normally extensively protected by the P-gp and/or Mrp1 transporters.

At the cellular level, the endogenous expression of MRP1 (and P-gp) can already contribute substantially to the basal resistance of cell lines to a range of cytotoxic anticancer drugs, as was demonstrated in vitro with murine fibroblast and embryonic stem cell lines deficient for murine Mrp1 and/or Mdr1a and Mdr1b [80,81]. A marked increase in sensitivity to epipodophyllotoxins, *Vinca* alkaloids, anthracyclines, topotecan and SN-38, and arsenite was found in these lines due to Mrp1 deficiency. Such contributions can well explain the markedly increased drug sensitivity of bone marrow and intestinal epithelial cells deficient for Mrp1, and especially when drug-transporting P-gp is also absent. For the extrapolation of pharmacological studies in (knockout) mice and possibly rats to humans, it is important to be aware that the murine Mrp1 is much less efficient in transporting anthracyclines than human MRP1 [82].

3.3. Inhibitors of MRP1

So far, it has been much more difficult to find good small molecule inhibitors for MRP1 than for P-gp, especially ones that work in intact cells. This probably has to do with the preference of MRP1 for anionic compounds as substrates and inhibitors: most anionic compounds enter cells poorly, so it may be difficult to obtain sufficient intracellular concentrations of the inhibitor for efficacious inhibition. A variety of inhibitors of MRP1 has been described. Some examples are the LTC_4 analogue MK571, LTC_4 itself, S-decylglutathione, sulfinpyrazone, benzbromarone and probenecid (see e.g. [62,68,83–85]). P-gp inhibitors like cyclosporin A and PSC 833, with reasonable cellular penetration, do inhibit MRP1, but only with low affinity and (obviously) poor specificity [66]. For specific in vivo inhibition of MRP1 the general organic anion transporter inhibitors sulfinpyrazone, benzbromarone and probenecid are also not very suitable, as they extensively affect organic anion *uptake* systems as well. Moreover, these compounds need to be used at relatively high concentrations. For the aim of specifically inhibiting MRP1 activity in vivo, for instance to improve anticancer chemotherapy, better MRP1 inhibitors will have to be developed, with reasonable specificity and cellular penetration properties, and low cytotoxicity.

4. Multidrug resistance protein 2 (MRP2, ABCC2)

4.1. General properties of MRP2

The in vivo function of MRP2 was well studied in advance of identification of the MRP2 gene, as the MRP2 gene is effectively fully deficient in two mutant rat strains (TR^-/GY and EHBR), and in patients that suffer from the Dubin–Johnson syndrome (for reviews see [4,87]). Affected individuals suffer from a recessively inherited conjugated hyperbilirubinemia, which can result in observable jaundice, but overall the clinical phenotype of this disease is relatively mild. The cause of the defect is the absence of MRP2 from the hepatocyte canalicular membrane, where it normally mediates the hepatobiliary excretion of (amongst others) mono- and bis-glucuronidated bilirubin. The MRP2 gene was identified based on its similarity to MRP1, and absence of its expression in homozygously MRP2-deficient rats and humans [88–90].

Identification of substrates transported by MRP2 was based on many different approaches: analysis of compounds transported into bile of normal rats, but not of Mrp2 mutant rats; differential uptake of compounds into inside-out bile canalicular membrane vesicles isolated from normal and Mrp2 mutant rats; transfection or transduction of human MRP2 or rat or rabbit Mrp2 cDNA into cell lines followed by analysis of drug resistance, accumulation of compounds into cells or isolated inside-out membrane vesicles, and transepithelial transport of compounds (see e.g. reviews [2,4]). In one study an MRP2 cDNA antisense construct was used to suppress endogenous MRP2 levels in the HEPG2 hepatocyte cell line [91]. It turns out there are many similarities between the spectrum of compounds transported by MRP2 and MRP1, but there is not a complete overlap. Anticancer drugs transported by MRP2 include methotrexate, anthracyclines (doxorubicin, epirubicin), mitoxantrone, vincristine, vinblastine, CPT-11/irinotecan and SN-38, and possibly cisplatin and etoposide [2,62,91–93]. Like MRP1, MRP2 is primarily an organic anion transporter, so it seems very likely that weakly basic drugs are co-transported with GSH by MRP2. This was strongly suggested by a study of vinblastine transport in MRP2-transduced polarized cells, which occurs stoichiometrically with GSH transport [86]. Moreover, depletion of cellular GSH by treatment with L-buthionine sulfoximine (L-BSO) resulted in decreased substrate transport and drug resistance in MRP2-overexpressing cells [93,94]. Mrp2 is involved in the hepatobiliary excretion of GSH conjugates of inorganic arsenic (As), after administration of arsenite (H_3AsO_3) to rats [95]. MRP2 (and probably MRP1) may therefore also be involved in cellular and organismal protection against arsenic trioxide (As_2O_3), a compound

recently introduced for the treatment of relapsed acute promyelocytic leukemia [96], by extrusion of As-GSH conjugates. The observation that cellular GSH levels correlate inversely with the sensitivity to arsenic trioxide is in line with this possibility, although direct detoxification of As by GSH complexation may be as relevant [97].

Other well-defined substrates of MRP2 include many amphipathic anionic drugs and endogenous compounds, encompassing GSH-, glucuronide-, and sulfate conjugates. Some examples are reduced and oxidized GSH, LTC_4, LTD_4, LTE_4, estradiol-17β(β-D-glucuronide) ($E_2$17G), *p*-aminohippurate, *S*-glutathionyl 2,4-dinitrobenzene (GS-DNP), (*S*-glutathionyl-)sulfobromophthalein, glucuronidated SN-38, pravastatin, and the organic anion transport inhibitor sulfinpyrazone (see e.g. [4,86]). Moreover, some food-derived (pre-)carcinogens and glucuronide conjugates thereof are also transported by Mrp2 [94,98].

4.2. Pharmacological and toxicological functions of MRP2

Unlike MRP1, but similar to MDR1 P-gp, MRP2 localizes to the apical membrane of polarized cell lines in which it is expressed [92]. Moreover, in vivo MRP2 is found in a range of tissues important for the pharmacokinetics of substrate drugs, namely, next to the bile canalicular membrane of hepatocytes (human, rat), in the luminal membrane of the small intestinal epithelium (human, rat, rabbit), and in the luminal membrane of the proximal tubules of the kidney (rat, human) [99–102]. In human jejunum, MRP2 mRNA levels as measured by RT-PCR were amongst the highest of all tested ABC transporters [103].

The role of MRP2 in hepatobiliary excretion has been amply demonstrated (see e.g. [104]), but MRP2 in intestine and kidney could also contribute to direct intestinal and active renal excretion of substrate compounds, and intestinal MRP2 could limit oral uptake of compounds. Indeed, using TR^- rats, Dietrich et al. [94,98] demonstrated that the MRP2 substrate PhIP, a food-derived carcinogen, is limited in its oral bioavailability by Mrp2, and that Mrp2 mediates direct intestinal and hepatobiliary excretion of PhIP. Extrapolating to the large number of possible MRP2 substrates, it may well be that these are also affected by intestinal MRP2 activity in oral bioavailability and/or direct intestinal excretion.

Recently, Mrp2 was also found to be localized to the luminal membrane of endothelial cells of the small blood capillaries in rat brain [105], and in humans MRP2 has been detected in the apical syncytiotrophoblast membrane of term placenta [106]. In analogy with MDR1 P-gp present at these locations, MRP2/Mrp2 might limit the brain and fetal penetration of a range of substrate compounds present in (maternal) plasma. Overall, there is fairly extensive overlap between MRP2 and MDR1 P-gp tissue distribution, so it is likely that these two proteins have considerable overlap in pharmacological and toxicological protective functions, albeit with different (but partially overlapping) sets of substrates.

4.3. Inhibitors of MRP2

As with MRP1, the selection of currently available small molecule inhibitors of MRP2 that can be used in intact cells is quite limited [2,4]. Obviously, many of the anionic transported substrates of MRP2 will readily serve as competitive inhibitors when applied in in vitro systems where MRP2 is present in an inside-out (vesicle) orientation. Some examples are LTC_4, MK571, phenolphtalein glucuronide and fluorescein methotrexate [107–109]. However, such compounds frequently do not penetrate most normal cells to a sufficient extent to obtain useful levels of inhibition. It should be noted, though, that normal hepatocytes have a multitude of carrier uptake systems for organic anions, so that in vivo drug–drug interactions mediated by such anionic MRP2 inhibitors in the liver could be highly relevant. For MRP2 substrates that are co-transported with GSH, GSH depletion with L-buthionine sulfoximine (L-BSO) can be used to inhibit transport (see e.g. [94,110]).

MRP2 inhibitors that have been demonstrated to work to a greater or lesser extent in intact cells include cyclosporin A, sulfinpyrazone, benzbromarone, probenecid, PSC 833, PAK-104P and MK571 (see e.g. [62,86,110,111]). Note that with some of these inhibitors, also dependent on the transported substrate analyzed, paradoxical increases in MRP2-mediated transport were observed. The background of this phenomenon will be discussed later. It is further clear that compounds that *specifically* inhibit MRP2 are hard to come by, as all the compounds identified so far have considerable activity against MRP1, and sometimes also against MDR1 P-gp.

5. Stimulative co-transport of substrates by MDR1 P-gp, MRP1, and MRP2: potential for drug–drug interactions?

There is increasing evidence that a number of ABC transporters can transport multiple different substrates at the same time, and, perhaps more importantly, that this co-transport can result in a marked increase in the efficiency of transport of one or both of the substrates. The first example concerns the need for reduced GSH to obtain efficient transport of vincristine and etoposide by MRP1 [72,73]. In both of these studies it was shown that GSH stimulates MRP1-dependent transport of etoposide or vincristine, and, conversely, that etoposide or vincristine stimulates MRP1-dependent transport of GSH. There are no indications that covalent conjugates are formed between GSH and the drugs. The simplest explanation for these data is that both compounds are transported together, and that in the absence of one of the compounds, the other compound is transported poorly, if at all.

For MDR1 P-gp a similar situation was described: Shapiro and Ling [112] found evidence for two distinct, positively cooperating drug binding and drug transport sites. One site had a preference for the model substrate Hoechst 33342, the other for rhodamine 123. Each compound stimulated P-gp-mediated transport of the other compound. A number of other transported drug substrates for P-gp (though not all) could be classified as belonging to the Hoechst ('H') or rhodamine ('R') classes, depending on their ability to stimulate rhodamine transport and inhibit Hoechst transport, or the inverse, respectively. An attractive explanation for these results is the presence of two distinct drug binding/transport sites, each with preference (but not necessarily an absolute preference) for a distinct set of drugs or other compounds. Efficient translocation of the bound drugs/compounds will only occur when both of these sites are occupied. The marked stimulation of MDR1 P-gp-mediated transport of one set of drug substrates, coinciding with inhibition of transport of another set of drug substrates by a series of synthetic small molecules found by Kondratov et al. [113] might well be explained by the same mechanism.

In an elaborate series of experiments, Evers et al. [86] and Bakos et al. [109] found indications that MRP2 also has the capacity for cross-stimulation of transport of different classes of compounds. Sulfinpyrazone and vinblastine, both transported MRP2 substrates, stimulated GSH transport, and vinblastine transport occurred stoichiometrically with GSH transport. In vitro, sulfinpyrazone, indomethacin, and penicillin G could markedly stimulate transport of *N*-ethylmaleimide-GS by MRP2. Sulfinpyrazone stimulation of $E_2$17G transport by MRP2 was also observed by Ito et al. [114].

It should be noted that interactions between cross-stimulatory or -inhibitory compounds sometimes appear very complex (see e.g. [20,115,116]), so the molecular interactions may be more involved than can be explained by the presence of just two distinct but coupled drug binding/transport sites. For instance, in P-gp the presence of a third drug binding site has been proposed, which allows stimulation or inhibition of transport of drugs bound elsewhere, but which is not itself a transport site [116].

The degree of transport stimulation observed with the various transporters is sometimes considerable (4-fold or more). This could mean that upon co-administration of such stimulatory compounds, the increase in transporter function in various pharmacological barriers might substantially alter the pharmacological handling of substrate drugs. Especially in the intestinal epithelium, the bile canalicular membrane, the blood–brain and the maternal–fetal barrier the effects could be considerable. It will therefore be interesting to study whether these cross-stimulation phenomena also occur in animals and humans. If so, this will add yet another element to the increasing number of potential drug–drug interaction mechanisms.

6. Multidrug resistance protein 3 (MRP3, ABCC3)

6.1. General properties of MRP3

MRP3 has not been studied as extensively yet as MRP1 and MRP2. Most work to date was done on human MRP3 and rat Mrp3. Based on its amino acid sequence, MRP3 is the MRP family member most closely related to MRP1 (58% identity), and like MRP1, it is present in the basolateral membrane of polarized cells [117–119]. As expected, MRP3 is a quite broad specificity organic anion transporter, with considerable, but not complete overlap in drug substrates with MRP1 and MRP2. Using isolated inside-out vesicles, rat Mrp3 was demonstrated to transport $E_2$17G, methotrexate, and a range of bile salts (taurocholate, glycocholate, taurochenodeoxycholate-3-sulfate, taurolithocholate-3-sulfate). GS-DNP and LTC_4 did not appear to be good substrates, suggesting that rat Mrp3 is not an efficient GS-conjugate transporter [120,121]. Human MRP3 can confer resistance to the anticancer drugs etoposide, teniposide, and methotrexate, and increase the efflux of GS-DNP from cells, and its uptake into inside-out membrane vesicles [118,122]. Zeng et al. [122,123] also reported low vincristine and methotrexate resistance in MRP3-overexpressing cells, and MRP3-dependent vesicular uptake of methotrexate and glycocholate, but not of taurocholate. Human MRP3 mediates efficient $E_2$17G transport, and moderate GS-DNP and LTC_4 transport [123,124]. Zelcer et al. [124], who studied MRP3 transduced into cell lines with very low basal drug resistance [81], found only resistance to etoposide and teniposide, but not to vincristine. Interestingly, the resistance was not diminished by L-BSO treatment of the cells, suggesting that export was not GSH-dependent. Transport of GSH itself could also not be demonstrated in vesicles or polarized cells with high MRP3 levels [118,124]. Etoposide-glucuronide was found to be an excellent substrate for MRP3, but etoposide-resistant MRP3-overexpressing cells demonstrated only efflux of free etoposide, indicating that etoposide itself is transported by MRP3 [124].

A number of classical organic anion transport inhibitors such as benzbromarone, indomethacin, probenecid, and sulfinpyazone, used at non-cytotoxic concentrations, could reverse the MRP3-mediated decrease in etoposide accumulation [124].

6.2. Pharmacological and physiological functions of MRP3

MRP3 is expressed in liver, small and large intestine, adrenal gland, and to a lower extent in pancreas and kidney [117,125,126]. In normal liver, MRP3 is primarily found at low levels in the basolateral membrane of hepatocytes, and in the intra-hepatic bile duct epithelial cells [119,126]. The basolateral membrane of the gallbladder epithelium also contains MRP3 [127]. In MRP2/Mrp2-deficient patients and rats, and with some pharmacological treatments resulting in cholestasis, a strong upregulation of MRP3/Mrp3 is observed in the liver [119,126,128]. In kidney MRP3 is localized to the distal convoluted tubules and the ascending loops of Henle [126].

Based on its substrate specificity and tissue distribution as identified so far, several putative functions for MRP3 can be considered. An attractive possibility is that MRP3 could play a role in the cholehepatic and enterohepatic circulation of bile salts: bile salts entering the epithelium of the bile ducts from the bile, or the epithelium of the small and large intestine from the intestinal lumen, could be actively transported towards the bloodstream by MRP3 in the basolateral membrane, and thus become available for recycling through the liver [119,121]. In analogy with MRP1, MRP3 may also contribute to a toxicological defense function, by eliminating a range of toxic organic anions, notably glucuronide conjugates, from various epithelial cell types. In view of its ability to confer resistance to several anticancer drugs it might contribute to intrinsic or perhaps acquired chemotherapy resistance in some tumors. In adrenal gland there could be a role in elimination of steroid conjugates like $E_2$17G or similar compounds. However, at this point all these functions are speculative. The generation and analysis of Mrp3-deficient knockout mice may help to elucidate these matters.

7. Multidrug resistance protein 4 (MRP4, ABCC4)

A full-length MRP4 cDNA was first reported by Lee et al. [129]. As discussed above, MRP4, like MRP5, lacks the N-terminal domain of 5 putative transmembrane segments present in MRP1–3. Two different RNA expression studies yielded somewhat different tissue distribution results, indicating that MRP4 is expressed in lung, kidney, bladder, gallbladder, and tonsil [125], or very highly in prostate, next to moderate expression in lung, skeletal muscle, pancreas, spleen, thymus, testis, ovary, and small intestine [129]. Work by Schuetz et al. [130] suggested that MRP4 can transport the antiviral agent and AMP analogue 9-(2-phosphonylmethoxyethyl)adenine (PMEA), as a cell line made highly resistant to PMEA had amplified and overexpressed the MRP4 gene. These cells were further cross-resistant to the GMP analogue PMEG, and to the antiviral drugs azidothymidine (AZT), and 2′,3′-dideoxy-3′-thiacytidine (3TC). PMEA and AZT-monophosphate were much more rapidly effluxed in the PMEA-resistant line than in the parental line. Importantly, the antiviral efficacy of PMEA, AZT, and 3TC against human immunodeficiency virus (HIV) was also decreased substantially in the PMEA-selected cell line.

Using MRP4-transfected NIH3T3 cells, Lee et al. [131] could confirm low-level resistance to PMEA, and resistance to short-term methotrexate exposure, but not to AZT or 3TC. This apparent discrepancy with the results by Schuetz et al. might be explained by relatively low MRP4 expression in the transfected NIH3T3 cells, and possibly selection for additional changes in the PMEA-selected cell lines that favor efficacy of MRP4 in extruding nucleoside monophosphates, like reduced adenylate kinase activity [130]. Chen et al. [132] further reported MRP4-mediated resistance to the anticancer drugs 6-mercaptopurine (6-MP) and thioguanine (TG). Also, using high-level expression in a baculovirus expression system, they demonstrated that MRP4 can mediate ATP-dependent accumulation of cyclic GMP, cyclic AMP, and $E_2$17G into inside-out membrane vesicles.

An immunohistochemical study by Lee et al. [131] localized human MRP4 to the basolateral membrane of acinar cells in the prostate. In contrast, Van Aubel et al. [133] found human and rat MRP4 primarily in the brush border (apical) membrane of proximal tubular cells in the kidney. It may be that MRP4 is present at different subcellular locations in different epithelial tissues, but further studies are warranted before drawing definitive conclusions on the subcellular localization of MRP4, as there is always a risk of cross-reactivity with other MRP family members.

At this point in time, we do not consider it very useful to speculate on possible physiological and pharmacological roles of MRP4, since there are as yet too many uncertainties with respect to exact substrate specificity and tissue distribution. A possible contribution to chemotherapy resistance to the anticancer drugs 6-MP and TG, or to the antiviral drugs AZT and 3TC should be considered. As with the other MRP family members, it may be useful to generate and analyze Mrp4 knockout mice for further characterization of the in vivo function(s) of this protein.

8. Multidrug resistance protein 5 (MRP5, ABCC5)

As for MRP4, the analysis of MRP5 is still in its infancy. Human MRP5 cDNA was cloned, expressed, and studied in various cell lines by several groups [134–136]. McAleer et al. [134] found that an ectopically expressed MRP5 fused to the C-terminus of Enhanced Green Fluorescent Protein (EGFP) localized primarily to the plasma membrane of human HEK293 cells. These cells further displayed low-level resistance to cadmium chloride and potassium antimonyl tartrate, but not to a range of anticancer drugs and other heavy metal oxyanions. The accumulation of several anionic dyes (FDA, CMFDA, BCECF) was markedly decreased in the EGFP-MRP5 containing cells. Further support for MRP5 as an organic anion transporter came from work of Wijnholds et al. [135]: MRP5, when expressed in polarized MDCK-II cells, localized primarily to the basolateral membrane, where it could mediate the efflux of GS-DNP, but also GSH. The GS-DNP efflux could be fully inhibited with the classical organic anion transport inhibitor sulfinpyrazone. HEK293 cells overexpressing MRP5 further acquired moderate resistance to 6-MP, TG, and PMEA. In these cells, no resistance to cadmium chloride or potassium antimonyl tartrate could be demonstrated, contrasting with the results of McAleer et al. [134]. Wijnholds et al. [135] could further show that PMEA itself, and the nucleoside monophosphate derivative of 6-MP, thio-IMP, were extruded from the cells by MRP5. Jedlitschky et al. [136] used vesicles derived from a hamster cell line transfected with MRP5 to demonstrate that 3′,5′-cyclic GMP and 3′,5′-cyclic AMP can be transported by MRP5. This transport could be inhibited with probenecid, but more effectively with the phosphodiesterase inhibitors trequensin and sildenafil (also known as Viagra). It is presently unknown whether the phosphodiesterase inhibitor activity of these latter compounds is related to their ability to interfere with MRP5. Given the wide variety in pharmacodynamic activities of compounds known to also inhibit ABC transporters, we think it more likely that this combination of properties is coincidental.

Precise immunohistochemical localization of MRP5 is still lacking in the absence of suitable antibodies, but MRP5 RNA is widely expressed throughout most tissues tested [125]. Highest expression was found in brain and skeletal muscle [134], and MRP5 is also present in erythrocyte membranes [136]. In what (sub-)cellular location and in what subpopulations of cells in various tissues MRP5 is expressed will be important information for establishing its possible physiological and pharmacological roles. Knockout mice lacking Mrp5 have already been generated, but these apparently do not display immediately obvious abnormalities [135]. A more detailed characterization of such mice may help in the future analysis of MRP5 function.

9. Breast cancer resistance protein (BCRP, ABCG2)

9.1. BCRP structure and function

Although BCRP was discovered last of the ABC drug efflux transporters discussed in this chapter, there is already ample evidence that, similar to MDR1 P-gp and MRP2, it could have a substantial impact on the general pharmacology of a range of substrate drugs. Moreover, its activity can be effectively modulated in vivo with inhibitors. We will therefore discuss this protein in somewhat more detail.

BCRP was first cloned based on its overexpression in a highly doxorubicin-resistant MCF7 breast cancer cell line (MCF-7/AdrVp) [137–139]. Transfection of *BCRP* cDNA demonstrated that BCRP itself could confer resistance to mitoxantrone, doxorubicin and daunorubicin, and that it acted by energy-dependent (most likely through ATP hydrolysis) extrusion of its drug substrates [13,137]. Because the gene was isolated from a breast cancer cell line, it was called the breast cancer resistance protein (BCRP) gene. It should be stressed, though, that there is no indication that its expression is specific for breast cancer cells, or that BCRP should play a significant role in chemotherapy-resistance in breast cancer. *BCRP* cDNA sequences were also cloned by Miyake et al. [140] and Allikmets et al. [141], who called the gene *MXR* (for mitoxantrone resistance) and *ABCP* (for placental ABC protein), respectively. Human *BCRP* encodes a 655 amino acid ABC protein, containing a single N-terminal ATP binding cassette, followed by 6 putative transmembrane segments (TMSs) (see Fig. 1). Based on structural and sequence homology, *BCRP* belongs to the *ABCG* gene family, containing amongst others the Drosophila *white*, *brown*, and *scarlet* protein genes, the human *white* homologue *ABCG1*, and the more recently identified genes *ABCG5* and *8* [142,143]. *BCRP* was therefore renamed *ABCG2*. The murine homologue, *Bcrp1*, was found to be highly overexpressed in mouse fibroblasts selected for resistance to doxorubicin, mitoxantrone, or topotecan [144]. *Bcrp1* encodes a 657 amino acid protein with 81% identity (87% similarity) to BCRP. Like human BCRP, murine Bcrp1 has been shown to mediate drug resistance through energy-dependent efflux of drug substrates [144]. In drug-selected cell lines highly overexpressing *BCRP* or *Bcrp1*, the gene locus was frequently amplified, but not always, indicating that overexpression can result both from in situ gene activation and gene amplification [140,144,145].

Based on analogy with other ABC (drug efflux) transporters analyzed to date (see above and [11,12,146]), it seemed very likely that the half-transporter BCRP should function as a homo- or heterodimer. Overexpression of only *BCRP* cDNA in mammalian or insect cells already yields functional drug transport and drug-dependent ATP hydrolysis [13,14], suggesting functioning as a homodimer. BCRP homodimer formation was recently demonstrated directly using co-immunoprecipitation. Moreover, the observation of a partial dominant-negative effect of a BCRP mutant on drug resistance mediated by unmutated BCRP further supported the notion that the protein functions as a homodimer [15]. Whether BCRP can also function as a heterodimer with other half-transporters of the ABCG class, or other ABC classes, is not known.

When overexpressed in drug-selected or transfected cell lines, BCRP primarily localizes to the plasma membrane, in accordance with its capacity to efficiently extrude drug substrates from the cell [10,147,148]. Murine *Bcrp1* expressed in polarized canine or porcine epithelial cell lines mediated apically directed transport of its drug substrates, indicating that Bcrp1 localizes to the apical membrane in polarized cells [149]. Subsequent immunohistochemical studies of human BCRP and pharmacological studies in mice (see below) confirmed this notion.

9.2. Drug resistance mediated by BCRP/Bcrp1

There is considerable, but varying overlap in anticancer drug substrate specificity between BCRP on the one hand and P-gp, MRP1, and MRP2 on the other hand (see above and [150]). Cell lines selected for resistance to mitoxantrone, topotecan, doxorubicin, SN-38 (the active metabolite of the camptothecin analogue irinotecan/CPT-11), flavopiridol, or the experimental indolocarbazole topoisomerase I inhibitors NB-506 and J-107088 all overexpressed *BCRP* or *Bcrp1*, strongly suggesting that these drugs are transported substrates for BCRP and Bcrp1 [144,151–155]. Cytological dyes such as rhodamine 123, Lysotracker Green, and BBR3390, as well as the fluorescent conjugate BODIPY-prazosin, also demonstrated decreased accumulation in *BCRP*-overexpressing cells [137,150,156–158]. Little or no resistance was found to the anticancer drugs vincristine, paclitaxel or cisplatin, indicating that these are not transported substrates for BCRP/Bcrp1.

Drug-selected *BCRP*- or *Bcrp1*-overexpressing cell lines variously displayed high cross-resistance to daunorubicin, epirubicin, 9-aminocamptothecin, bisantrene, and in some cases etoposide and teniposide [137,144,150,152,156,159,160]. However, these cross-resistance data obtained from heavily drug-selected cells should be interpreted cautiously. Other drug transporters with partially overlapping substrate specificity can be overexpressed (see e.g. [156,161]), alterations in the cytotoxic targets of the selecting drug can contribute to

resistance, and mutations in the selected drug transporter may alter the substrate specificity (see e.g. [162]).

In some selected cell lines, BCRP appeared to confer high resistance to anthracyclines, whereas in other cell lines there was hardly any resistance to anthracyclines compared to mitoxantrone resistance, which was consistently high. Moreover, topotecan resistance was generally quite high, but low in some *BCRP*-overexpressing lines [137,152,157,159,163]. Robey et al. [158] and Honjo et al. [14] recognized that two *BCRP*-overexpressing lines (MCF-7/AdVp3000 and S1-M1-80, each with high anthracycline resistance) could efflux rhodamine 123 efficiently, whereas all other *BCRP*-overexpressing lines tested could not. Sequence analysis revealed that the rhodamine-extruding lines contained a mutant BCRP, deviating from the 'wild-type' BCRP at arginine 482, which was replaced with either threonine (in the MCF-7/AdVp3000 line) or glycine (in the S1-M1-80 line). The MCF-7/AdVp3000 line had been the source of the first cloned *BCRP* cDNA [137], which therefore represented a mutant BCRP. Further experiments confirmed that the wild-type (R482) BCRP could efficiently extrude mitoxantrone, but not rhodamine 123 or doxorubicin. In contrast, both R482T and R482G mutants efficiently extruded all three compounds [14]. In the parental cell lines, only the wild-type BCRP sequence was found, indicating that in some lines the extended drug selection must have resulted in selection of mutant BCRPs that were more efficient in conferring resistance to the selecting drug (doxorubicin or mitoxantrone).

Depletion of cellular GSH by treatment with BSO of various *BCRP*-overexpressing cell lines did not affect the resistance to, or efflux of anthracyclines or topotecan [10,138,152]. This suggests that GSH is not needed for transport of these drugs by BCRP.

9.3. Inhibitors of BCRP/Bcrp1

GF120918, a highly efficient P-gp inhibitor that can be used in animals and patients [29,30], is also an effective BCRP and Bcrp1 inhibitor [31,144]. Obviously, GF120918 is not a specific BCRP inhibitor, but for some clinical applications this may be an advantage, as two multidrug transporters, BCRP and P-gp, each with their own potentially adverse activity, can be blocked at the same time.

In a systematic search for drug resistance-reversing compounds, fumitremorgin C (FTC), a tremorgenic mycotoxin produced by the fungus *Aspergillus fumigatus*, was found to effectively reverse drug resistance and increase cellular drug accumulation in BCRP-expressing cells [157,160]. FTC inhibited BCRP in vitro at concentrations well below those toxic to cultured cells, and had little effect on P-gp- or MRP1-mediated drug resistance. This relative specificity makes FTC very useful for cell pharmacological studies of BCRP, but its neurotoxic effects preclude application in animals or patients. A number of FTC analogues (mostly pentacyclic diketopiperazines) was generated by the same group [164]. However, none of the compounds tested had substantially better BCRP inhibiting activity than native FTC, and they were frequently more cytotoxic. Whether they can be used in animals or patients is as yet unclear.

Van Loevezijn et al. [165] tested 42 mainly tetracyclic indolyl diketopiperazine analogues of FTC as inhibitors of murine Bcrp1 and human BCRP. The most potent analogues (Ko132 and Ko134) have comparable or greater activity than FTC [164,165]. We recently found that Ko134 has low cytotoxicity in vitro and that it can be given at high oral dosages to mice, which allows modulation of Bcrp1 activity in vivo (Allen et al., personal communication). FTC analogues of this type may thus be useful leads for development of clinical BCRP inhibitors.

By chance it was discovered that an experimental HER tyrosine kinase inhibitor, CI1033, also inhibits BCRP, albeit with moderate affinity [166]. CI1033 also appears to be a transported substrate for BCRP. We now have seen several additional examples of inhibitors designed for other systems that also block BCRP, and it is likely there will be more.

9.4. The tissue distribution of BCRP and Bcrp1

Initial studies on the RNA levels of *BCRP* and *Bcrp1* in tissues yielded rather variable outcomes [137,141,144], and they are therefore best considered together with later immunohistochemical and functional studies [10,149]. Very high *BCRP* RNA expression was found in human placenta, but in murine placenta the expression of Bcrp1 was quite moderate. In contrast, mice displayed highest expression of *Bcrp1* RNA in kidney, where humans appear to have low *BCRP* expression [137,141,149]. The immunohistochemical studies of Maliepaard et al. [10] used two independent monoclonal antibodies, BXP-21 and BXP-34, recognizing human BCRP. BCRP was detected in the placental syncytiotrophoblast plasma membrane facing the maternal bloodstream, in the bile canalicular membrane of the liver hepatocytes and in the luminal membrane of villous epithelial cells in the small and large intestine, locations shared with MDR1 P-gp. BCRP was further found in the apical side of part of the ducts and lobules in the breast, and in the venous and capillary endothelial cells of practically all tissues analyzed, but not in arterial endothelium. BCRP was not detected in human erythrocytes, leukocytes, or platelets [10], but Zhou et al. [167] found that *Bcrp1* mRNA as detected by RT-PCR was present at high levels in primitive murine hematopoietic stem cells. Little or no expression of *Bcrp1* was seen in more differentiated hematopoietic lineages, with the exception of erythroid precursor cells and natural killer lymphocytes that did express some *Bcrp1* [167]. A recent study by Scharenberg et al. [168] demonstrated a very similar distribution of BCRP expression in the human hematopoietic compartment, with high expression in putative hematopoietic stem cells, and much decreased expression in committed progenitor cells, which only comes up again in natural killer cells and erythroblasts, but not in other differentiated lineages.

9.5. Pharmacological and physiological functions of BCRP and Bcrp1

The tissue distribution of BCRP shows extensive overlap with that of P-gp, suggesting that BCRP/Bcrp1 might have a similar role as P-gp in the pharmacological handling of substrate drugs. To address this question, we used the BCRP/Bcrp1 and P-gp inhibitor GF120918 in pharmacological experiments with Mdr1a/1b knockout mice, which lack drug-transporting P-gp. In this way, pharmacological effects of GF120918 observed in these mice would most likely result from Bcrp1 inhibition. Oral co-administration of GF120918 and topotecan resulted in a 6-fold increase in the plasma availability of topotecan [149]. When topotecan was administered intravenously, the plasma clearance and hepatobiliary excretion of intravenously administered topotecan were both about two-fold decreased by oral GF120918, consistent with an excretory role for bile canalicular Bcrp1. The amount of topotecan present in the intestinal contents was reduced three-fold 1 h after topotecan administration in the GF120918-treated mice. Together, the data indicate that the increased topotecan plasma availability after oral GF120918 treatment resulted from a combination of decreased hepatobiliary excretion and more efficient (re-)uptake of topotecan from the intestinal lumen [149]. Intestinal Bcrp1 therefore appears to play a role in limiting (re-)uptake of substrate drugs from the intestine, and hence also their oral bioavailability. Given this functional role, reminiscent of that of MDR1 P-gp, it is noteworthy that in human jejunum, the mRNA level of *BCRP* (like that of *MRP2*) is considerably higher than that of *MDR1* [103]. Whether the respective protein levels reflect these RNA levels is not certain, however.

When [^{14}C]topotecan was administered intravenously to pregnant *Mdr1a/1b* knockout mice, the relative fetal penetration of radioactivity was increased 2-fold owing to oral GF120918 pretreatment [149]. This suggests that Bcrp1 in the placental trophoblast counteracts

the entry of topotecan into the fetus, indicating that Bcrp1 is a protective element of the maternal-fetal barrier.

Taken together, the available data indicate a pharmacological and toxicological protective role for Bcrp1, similar to that of MDR1 P-gp. Protection from naturally occurring xenobiotic toxins is therefore probably (one of) the main normal biological function(s) of BCRP/Bcrp1.

Another tentative physiological role for BCRP/Bcrp1 was suggested by the work of Zhou et al. [167] and Scharenberg et al. [168], which showed that murine Bcrp1 and human BCRP expression is high in a so-called 'side population' (SP) of bone marrow cells, which is defined by its low accumulation of the dye Hoechst 33342. Human BCRP can efflux Hoechst 33342, so it is very likely that the high level of BCRP/Bcrp1 expression in the SP cells is responsible for the low dye accumulation. Importantly, the SP fraction is highly enriched for undifferentiated stem cells, and BCRP/Bcrp1 expression was found to be low or absent in most of the more differentiated hematopoietic lineages. Transplantation of BCRP-transduced bone marrow cells into recipient mice resulted in a lower repopulation capacity than seen with mock-transduced bone marrow cells. Moreover, in a competitive repopulation assay, BCRP-transduced bone marrow cells were less effective than mock-transduced cells in contributing to peripheral red blood cells, thymus, and bone marrow [167]. Also in view of the high Bcrp1 expression in undifferentiated bone marrow cells, it was speculated that the observed effects might be due to a differentiation-inhibiting role of Bcrp1 (possibly by extrusion of a differentiation-inducing substrate). However, any moderately adverse effect of high BCRP overexpression on cell growth and proliferation could also explain the results. Whatever the biological role of Bcrp1 expression in bone marrow stem cells, there can be little doubt that it is responsible for the appearance of the SP-phenotype. As a similar SP phenotype was observed in skeletal muscle stem cells and embryonic stem cells, and again associated with high Bcrp1 expression [167], it could be that high Bcrp1 expression is a general characteristic of various stem cell populations. Several groups are currently working on the generation and characterization of Bcrp1 knockout mice. This will probably allow for elucidation of the various functions of BCRP/Bcrp1.

9.6. Pharmacological inhibition of BCRP to optimize pharmacotherapy?

From our current insights, BCRP function may be relevant in two areas of pharmacotherapy: it could be substantially expressed in some tumors and other malignancies treated with anticancer drugs that are BCRP substrates, and therefore render these cancer cells resistant to chemotherapy; and it may be an important factor in the general pharmacology of substrate drugs, affecting their oral bioavailability, plasma clearance, and hepatobiliary and intestinal elimination. During pregnancy, BCRP in the placenta may also be relevant for pharmacotherapy by protecting the fetus from drug accumulation. Effective, in vivo applicable BCRP inhibitors such as GF120918 are already available, and they can be applied for various purposes, in analogy with the P-gp inhibitors. One possibility is the option to improve penetration of anticancer drugs into tumor cells that express BCRP. Perhaps of more general pharmacotherapeutic relevance is the capability to improve the oral bioavailability of substrate drugs. In fact, the use of BCRP inhibitors to improve the oral bioavailability of BCRP substrate drugs such as topotecan is now the subject of ongoing clinical studies in the group of J.H.M. Schellens in this institute, and initial results look promising [169].

10. Perspective

Over the past 15 years, tremendous progress has been made in understanding the pharmacological and toxicological impact of ABC drug efflux transporters. Once thought to be perhaps just of relevance in making cancer cells resistant to anticancer drugs, it is now clear that they can have a pronounced role in the oral bioavailability and hepatobiliary, direct intestinal, and most likely renal excretion of an extensive range of drugs and toxins. In addition, they contribute to important pharmacological sanctuary sites such as brain, cerebrospinal fluid, testis and fetus, and they can protect individual cells from drug and toxin penetration. The capability to interfere in vivo with the activity of at least some of these transporters may therefore have important pharmacotherapeutic benefits. Moreover, many drug–drug interactions may also be explained by interactions at the level of ABC drug transporters. The challenge for the future will lie in arriving at a complete understanding of the relative impact of all these transporters and their cross-interactions, and their potential interactions with other drug-processing systems such as uptake carriers and drug-metabolizing enzymes. At some point, in the hopefully not-too-distant future, we shall then be able to make reasonably reliable predictions on the behavior of drugs in individual patients.

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