Efflux Transporters and their Clinical Relevance

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Abstract: It is increasingly recognized that efflux transporters play an important role, not only in chemo protection e.g. multi-drug resistance, but also in the absorption, distribution, and elimination of drugs. The modulation of drug transporters through inhibition or induction can lead to significant drug-drug interactions by affecting intestinal absorption, renal secretion, and biliary excretion, thereby changing the systemic or target tissue exposure of the drug. Few clinically significant drug interactions that affect efficacy and safety are due to a single mechanism and there is considerable overlap of substrates, inhibitors, and inducers of efflux transporters and drug metabolizing enzymes, such as CYP3A. As well, genetic polymorphisms of efflux transporters have been correlated with human disease and variability of drug exposure. Accordingly, this review will discuss drug interactions and suitable probe substrates, as well as, the clinical relevance of the variability and modulation of efflux transporters and the exploitation of substrates as diagnostic tools. An update is given on inhibitors, which clinically reverse drug resistance and minimize the risk of metabolic interactions.

Keywords: Efflux transporters, P-glycoprotein, multi-drug resistance.

INTRODUCTION

ATP-binding cassette (ABC) efflux transporters are important in many biological processes and are present in many organisms and tissues. There is an increasing awareness of their relevance in the disposition of drugs. The importance of ABCB1, (P-glycoprotein, Pgp or MDR1) was first recognized in the multi-drug resistance (MDR) of tumor cells in response to chemotherapy treatment [1]. Substrates for these transporters include, not only pharmaceuticals, but also sugars, amino acids, glycans, cholesterol, phospholipids, peptides, proteins, and toxins [2]. ABCB1 and other efflux transporters are expressed in tumor cells and, as well as, in many normal tissues including the surface of epithelial cells in the intestine, restricting the oral absorption of substrates; in the blood-brain barrier, preventing substrates from entering the brain; in the fetal-maternal barrier of the placenta, protecting the fetus from toxins; in the bile canalicular membrane of hepatocytes; and in lymphocytes (Table **I**). Thus, in addition to the extrusion of endogenous substrates and toxins, these efflux ABC transporters are currently appreciated for their role in modulation of the absorption, distribution, and elimination of drugs.

The modulation of drug transport, through inhibition or induction of transporters, can lead to significant drug-drug interactions and, most importantly, to drug treatment resistance. There is a significant overlap between ABCB1 and CYP3A4 substrates and inhibitors (as well as inducers) [3-5]. Thus, some drug interactions, previously believed to be cytochrome P450 (CYP) mediated, are now considered to be due, at least in part, to the inhibition of transport proteins. It is thus often difficult to distinguish between *in vivo* P450 inhibition and inhibition of transport processes. Furthermore, inhibition of non-drug transporters such as the bile acid transporters can indirectly lead to reduced biliary clearance of a non-substrate drug.

Table I. Efflux Proteins Relevant to Drug Disposition and Tissue Distribution [7, 84-86]

	Previous names	Substrates	Location
ABCB1	MDR1, Pgp	Neutral and cationic compounds, lipids	Intestine, liver, kidney, blood brain barrier, lymphocytes
ABCC1	MRP1	GS-X, organic anions, lipids	Widespread
ABCC2	MRP2, cMOAT	GS-X, organic anions, glutathione disulfide, β-lactam antibiotics	Liver, kidney, intestine
ABCG2	MXR, BCRP, ABCP	Anthracyclines, mitoxantrone	Placenta, intestine, breast, liver, veins and capillaries

EFFLUX TRANSPORTER DRUG-DRUG INTERACTIONS: INHIBITION

Inhibition of ABCB1 or other efflux transporters expressed in the intestine, liver, and kidney can increase systemic and/or target tissue exposure and reduce clearance of substrates by increasing intestinal absorption, decreasing renal secretion and/or biliary excretion. Few clinically

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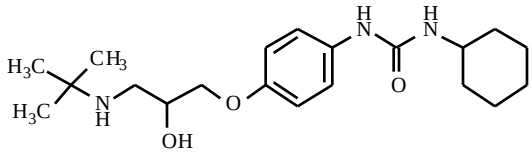
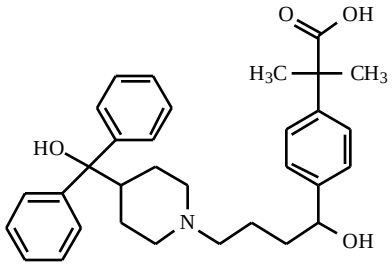
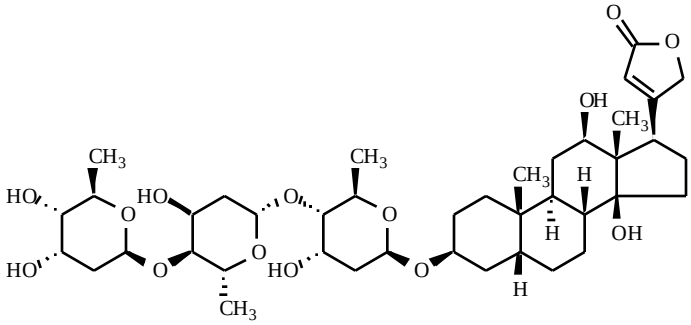
significant drug interactions affecting efficacy or safety have been demonstrated to be based on a single mechanism through transporter inhibition. The overlap of specificity of substrates and inhibitors for ABCB1 (and/or other transporters) with those as drug metabolizing enzymes, such as CYP3A4, makes it difficult to distinguish the underlying mechanism of the observed effects. For inhibitors, the use of IC_{50} ratios for CYP3A4 and ABCB1 inhibition has been proposed as a means to assess the relevance of the various systems (metabolism or transport) to the effect [6, 7]. For substrates, it is essential to establish the relative importance of the efficiencies of substrate efflux by the transporter(s) and metabolism by the P450, as well as the concentrations at the sites of drug transport and metabolism of the substrate *in vivo*. For chemotherapeutic regimes it is important to exploit the inhibition of ABCB1 with little effect on CYP3A4 metabolism to increase efficacy and minimize the risk of CYP3A-mediated metabolic interactions [6]. The use of inhibitors of ABCB1 activity to clinically reverse multi-drug resistance in cancer chemotherapy and distinguish the interaction from interactions with metabolic enzymes and other transporters will be discussed in more detail later in this review.

The distinction between drug interactions caused by CYP3A4 and ABCB1 inhibition was apparent in clinical studies with the antidiarrheal agent, loperamide. Loperamide, a potent opiate, is not normally associated with central nervous system side effects, such as analgesia and respiratory depression, since ABCB1 efflux presents an effective barrier at the blood-brain barrier [8]. When loperamide was coadministered with ritonavir (a CYP3A4 and ABCB1 inhibitor) [9], there was a change in systemic exposure to loperamide which did not cause adverse central nervous system effects. It was evidenced in this study that the clinical interaction was most likely due to CYP3A4 inhibition and not due to modulation of ABCB1. However, when quinidine (also a CYP3A4 and ABCB1 inhibitor) [8, 10] was given in combination with loperamide, respiratory depression resulted, which could not be explained by the changes in systemic loperamide exposure, demonstrating the potential importance of ABCB1 inhibition in drug-drug interactions.

SUITABILITY OF *IN VIVO* SUBSTRATES TO ASSESS ABCB1 DRUG INTERACTIONS

ABCB1 is the most studied efflux protein in drug disposition. Three of the most commonly used probe substrates for assessing ABCB1 modulation by drugs *in vivo* are talinolol, fexofenadine, and digoxin (Table **II**). These three compounds are actively secreted into the intestine and undergo negligible biotransformation. However, relative low doses must be used *in vivo* to observe significant effects, including effects of allelic polymorphisms on transporter function. It is known that susceptibility to drug interactions involving intestinal efflux (as well as gut metabolism) are observed more for the drugs administered at lower doses. Typically, the role of ABCB1 in disposition of the probe substrates in humans was defined

Table II. Common *In Vivo* ABCB1 Probe Substrates

Chemical name	Structure
talinolol	
fexafenadine	
digoxin	

in vitro, by cellular efflux assays (e.g. the polarized monolayers Caco-2, a human colon carcinoma cell line and the porcine renal tubular cell line, LLC-PK1, expressing human ABCB1) and *in vivo*, by inhibition or induction of ABCB1 and evaluation of changes in pharmacokinetic parameters of the ABCB1 substrate. Clinical studies that have addressed changes in pharmacokinetics of probe substrates with respect to polymorphisms in the *ABCB1* gene have also provided some information about the role of ABCB1. However, the association of particular single nucleotide polymorphisms (SNPs) or haplotypes with ABCB1 function and clinical outcomes remain controversial and will be discussed later.

Talinolol, a selective β_1-adrenoceptor antagonist for the therapy of cardiovascular disease, has been used to examine the roles of efflux transporters in limiting oral bioavailability. Talinolol is thought to be a good marker for evaluating efflux transporters, as it has low intestinal permeability, active intestinal secretion, and a lack of significant metabolism [11, 12]. Talinolol (60% of the dose) is primarily eliminated renally (40% excreted nonrenally in the feces) mainly as unchanged drug [12-14]. ABCB1 has been implicated in the intestinal efflux of talinolol from *in vitro* studies using Caco-2 mono-layers. The ratio of basolateral (b) → apical (a) to a→b transport of talinolol was 9 and 10 for the *R* and *S* enantiomers, respectively (at 0.2 mM). This efflux was saturable and inhibited by the ABCB1 inhibitor, verapamil [15].

Talinolol, as a probe drug for ABCB1 activity in humans, has also been characterized *in vivo* [16-18] using an intestinal steady-state perfusion method. Intravenous talinolol (range of mean intralumenal talinolol concentration 0.8-2 μM) was found to be secreted into the human small intestine, this secretion was reduced with intralumenal perfusion of *R*-verapamil (565 μM) by 29-56% [16]. Further implications of ABCB1 involvement in talinolol disposition are evidenced in a report of interactions with the ABCB1 inducer, rifampin, and will be described later in this review [17]. In a clinical drug interaction study between talinolol (50 mg oral) and *R*-verapamil (120 mg oral) there was a 25% decrease in oral bioavailability of talinolol [19], not the expected increase, as was the case for the ABCB1 substrate, digoxin [20-24] nor as would be expected from the intestinal steady-state perfusion experiment [16]. This decreased oral bioavailability of talinolol was explained, in part, by reduced verapamil concentrations due to CYP3A4 metabolism in the intestine and potential stimulation of ABCB1 ATPase activity at low verapamil concentrations (if the concentrations had reached below 20 μM) [19]. In addition, it is possible that the oral doses of talinolol were not low enough to observe a maximal inhibitory effect. A 50 mg talinolol dose would correspond to approximately 0.5 mM if dissolved in a volume of 250 mL. In the intestinal steady-state perfusion method, the inhibition of talinolol secretion into the intestinal lumen by *R*-verapamil (565 μM) was evidenced with apparently lower concentrations of talinolol (intralumenal concentrations of 0.8-2 μM) [16]. If the ABCB1 is saturated, especially if the ABCB1 protein levels are low, absorption would be dominated by passive diffusion and not active secretion. In fact, saturation of talinolol oral clearance in humans is evidenced at a 100 mg dose, and decreased bioavailability is apparent at lower talinolol doses (50 and more so at 25 mg) [15]. In general, the doses of digoxin are at least 50 to 100-fold lower than that of talinolol, and most likely more sensitive to inhibition. The concentration of the probe substrate for transport by ABCB1 must be at least within the concentration range of linear first order kinetics of transport in order to accurately assess inhibitory properties of other drugs.

Fexofenadine is a highly selective histamine H_1 receptor antagonist for the treatment of allergic rhinitis and chronic idiopathic urticaria. Fexofenadine is also thought to be a marker for efflux transport as it does not undergo significant biotransformation in humans and has low permeability. Urinary recovery of fexofenadine ranges from 7.6-12% of the dose [25, 26] with 80% recovered in the feces [26]. Only ~5% of the dose is metabolized with approximately 0.5% to 1.5% converted by CYP3A4 to an inactive metabolite and 3.5% transformed to a methyl ester metabolite found only in the feces and thought to be formed by gut microflora (Tech Info Allegra(R), 1997). Human ABCB1 was identified as an efflux transporter for fexofenadine using polarized LLC-PK1 cells and the derivative cell line, L-MDR1, stably transfected with human ABCB1 [27] as well as in Caco-2 cell monolayers [28]. The ratio of b→a to a→b transport of fexofenadine was greater than 5 in Caco-2 cells and was linear up to at least 0.3 mM (solubility limits) and was inhibited by verapamil [28].

Involvement of ABCB1 in fexofenadine transport was also implicated *in vivo*, since the inhibitors of ABCB1, ketoconazole (400 mg/day) and erythromycin (500 mg/8 h) increased the plasma AUC of fexofenadine (120 mg b.i.d.) by 159% and 103%, respectively (Tech Info Allegra(R), 1997). As the case for talinolol, the kinetics of fexofenadine can be saturated and lower concentrations are more appropriate to assess drug interactions involving ABCB1. The AUC of fexofenadine is dose proportional up to 120 mg fexofenadine and higher doses (240 mg) resulted in overproportional increases in systemic exposure [25]. Fexofenadine is also known to be transported by organic anion transporting polypeptide (OATP) [27], which may be of importance for fexofenadine disposition. In fact, using a regional perfusion technique, intralumenally perfused ketoconazole (94 μM) was not able to acutely inhibit the intestinal absorption of fexofenadine (93 μM) even when the perfusion was performed after repeated oral ketoconazole administration (200 mg q.d. for 5 days) [29]. It is thought that the inhibitory effect of ketoconazole on OATP (which was found *in vitro*) may potentially offer some explanation as to the interaction with fexofenadine *in vivo* [27].

As mentioned previously, the role of ABCB1 in digoxin disposition has been well recognized. The co-administration of ABCB1 inhibitors, such as verapamil, talinolol, propafenone, cyclosporine (CsA), nifedipine, itraconazole, amiodarone, dipyridamole, valspodar, and quinidine, with digoxin have led to clinically relevant drug-drug interactions [20-24, 30-33, 33-44]. The relatively large number of pharmacokinetic drug interactions with digoxin, as compared to fexofenadine and talinolol, are most likely due to the relatively low clinical doses (~0.25-1 mg q.d.) of digoxin. In addition, digoxin pharmacokinetics are linear with dose at the usual digoxin dose both after a single dose or at steady-state [45]. Digoxin is absorbed mainly from the proximal

part of the small intestine and the main route of elimination is renal excretion involving tubular secretion, reabsorption, and glomerular filtration [45, 46]. In most patients, more than 80% of digoxin is excreted unchanged in the urine, however, in about 12% of individuals, 20-55% can be excreted as metabolites (mostly dihydrodigoxin) [45]. Approximately 25-28% is eliminated by non-renal routes, such as biliary excretion.

The role of ABCB1 in the disposition of digoxin has also been implicated *in vitro* by the primary basolateral to apical transport of the compound in human ABCB1 transfected cells [47, 48]. This transport was also inhibited by vinblastine, quinidine, CsA, or verapamil [47, 48]. The transport of digoxin by LLC-PK1 cells that expressed an excess of human ABCB1 was found to be saturable at 14 μM [49]. Digoxin has been found recently to be a substrate for the liver specific organic anion transporting polypeptide, OATP8, which may, as well, have functional polymorphisms and contribute to digoxin disposition [50]. The clinical relevance of OATP8 in the disposition of digoxin is unknown.

Among the three mentioned substrates for ABCB1, digoxin is apparently the most sensitive probe substrate for assessing ABCB1 inhibitory potential, most likely due to its lower clinical doses. Talinolol is also a good substrate if used in low doses as it offers specificity for ABCB1. The extent of specificity of the transporter substrates will be continually evolving as more transporters are cloned, expressed, and examined for transport activity, as well as, specificity of inhibitors determined. The clinical relevance of other transporters, e.g. OATP, on the pharmacokinetics of present ABCB1 probe substrates, such as digoxin and fexofenadine, will need further investigation.

EFFLUX TRANSPORTER DRUG-DRUG INTERACTIONS: INDUCTION

The role of transporter induction, specifically ABCB1, in drug-drug interactions has been reviewed recently [7, 51]. There are several clinical reports demonstrating decreased plasma AUC or increased oral clearance of the ABCB1 substrates digoxin, talinolol, or fexofenadine after multiple dosing with inducers of ABCB1 (generally also CYP3A), such as rifampin or St. John's wort (SJW) [17, 52-60]. CYP3A and ABCB1 are often upregulated by common inducers [61]. The co-induction of the genes is associated with activation through the pregnane X receptor (PXR) and binding to promoter regions of each gene contain binding sites for PXR. The DR4 motif in the promoter region of *ABCB1* gene mediates the induction of ABCB1. Whereas, for CYP3A4, a proximal promoter region (missing in the *ABCB1* promoter), as well as, nuclear response elements in the distal promoter region, the XREM (xenobiotic response enhancer module) containing a DR3 motif, is involved in the regulation of the gene [62, 63]. As is the case for common inhibitors of ABCB1 and CYP3A4, changes in the pharmacokinetics (reduced AUC) of substrates of both CYP3A and ABCB1 caused by induction would be difficult to distinguish and the interaction could be due to a combination of both. Analysis of changes in metabolic ratios or the half-life of the drug would help distinguish the drug interaction.

The interaction of rifampin and digoxin has been attributed to induction of intestinal ABCB1. The pharmacokinetics of a single dose (1 mg oral and 1 mg intravenous, i.v.) of digoxin before and after co-administration of oral rifampin (600 mg q.d. for 10 days) was examined in 8 healthy volunteers [52]. The AUC ($AUC_{0\text{-}3h}$ and $AUC_{0\text{-}144h}$) of oral digoxin was significantly reduced by 43% and 30%, respectively during rifampin treatment and was less pronounced after i.v. administration of digoxin ($AUC_{0\text{-}144h}$ was reduced significantly 14.7%). During rifampin treatment, oral bioavailability of digoxin decreased by 30.1% and maximal plasma levels were also significantly reduced. Renal clearance and $t_{1/2}$ of digoxin were not altered by rifampin, which indicated possible selective or more significant up-regulation of ABCB1 in the intestine and not in the liver or kidney. Rifampin increased intestinal ABCB1 protein content in duodenal biopsies (n = 8) 3.5 ± 2.1-fold, compared to levels before rifampin treatment. The increased ABCB1 content correlated with the AUC after oral digoxin but not intravenous digoxin [52]. Rifampin is also a known inducer of P450 enzymes (e.g. CYP3A), however, the lack of change in $t_{1/2}$ of oral or i.v. digoxin argue against the interaction being due to any metabolic clearance, rather it is an effect on the rate and extent of digoxin absorption [52]. In another recent study, an intestinal perfusion catheter technique was used to directly measure intestinal drug transport of digoxin in humans [53]. The impact of up-regulation of intestinal ABCB1 by orally administered rifampin (600 mg q.d.) on the secretion of i.v. administered digoxin was examined [53]. Although not statistically significant, intravenous digoxin was eliminated into isolated segments of the intestine 1.8-fold more during rifampin treatment than in the absence of rifampin.

Rifampin has also been shown to affect the pharmacokinetics of fexofenadine and this has been attributed to induction of ABCB1 in the intestine [54]. In a study with twenty four healthy volunteers, six days of rifampin (600 mg q.d. oral) treatment resulted in a significant increase in oral clearance of fexofenadine (60 mg) (individual increase range 1.3 to 5.3-fold) and a lower C_{max} compared to control values before rifampin treatment. There were no significant effects on fexofenadine $t_{1/2}$, t_{max}, or renal clearance. This suggested that rifampin had an effect on the bioavailability of fexofenadine, rather than the systemic clearance. Further, fexofenadine is not extensively metabolized, and even though rifampin increased the urinary excretion of the dealkylated metabolite, azacyclonol, this metabolite constituted only <0.5% of the oral dose of fexofenadine [54]. As mentioned above, fexofenadine is also known to be transported by OATP [27], however, the contribution of this transporter to fexofenadine distribution is unclear nor is it known whether it is inducible by rifampin.

Talinolol pharmacokinetics are also affected by rifampin treatment in a manner thought to be due to induction of intestinal ABCB1 [17]. Talinolol (30 mg i.v. or long term oral administration, 100 mg for 7 days) kinetics were measured before or after 6 days of oral rifampin treatment (600 mg q.d.). The plasma AUC of intravenous and oral talinolol were significantly lower (21% and 35%, respectively) after rifampin treatment. The metabolic

clearance of talinolol was negligible and was not influenced by rifampin [17].

St John's wort (SJW), *Hypericum perforatum*, is a widely used over-the-counter herbal treatment for depression. SJW is a known inducer of CYP3A4 and ABCB1 [55]. Many side-effects have been reported with SJW, especially with those drugs that are substrates for one or both of these enzymes, such as CsA, digoxin, and indinavir [57, 58, 64-66]. The pharmacokinetics of digoxin were examined in a single-blind, placebo-controlled parallel study after achieving steady-state levels of digoxin, after which subjects were treated orally with 900 mg q.d. SJW extract LI160 (n = 13) or placebo (n = 12) in addition to digoxin (0.25 mg q.d. oral) [58]. Although not statistically significant, there was a trend of an acute interaction with digoxin (increased digoxin C_{max}) observed after a single oral dose of SJW. After 10 days of SJW treatment, there was a 25% decrease of AUC_{0-24h} of orally administered digoxin and a reduction of C_{max} (26%) compared to the control group [58]. In another study, the plasma AUC_{0-7h} after a single oral administration of digoxin (0.5 mg) was reduced 18% following two weeks treatment with SJW extract LI160 (300 mg oral, 3 times a day) [57]. There were also increased levels of duodenal ABCB1 and CYP3A4 protein (1.4- and 1.5-fold, respectively).

Another ABCB1 substrate, fexofenadine, has been found to be affected by SJW [59, 60]. In one study, fexofenadine (60 mg) was administered orally before treatment with a single dose of SJW (900 mg) and after two weeks of SJW treatment (300 mg, 3 times a day) [59]. The single-dose administration of SJW resulted in a 45% increase in the C_{max} of fexofenadine and 20% decrease in oral clearance with no change in $t_{1/2}$, t_{max}, and renal clearance. This was indicative of acute inhibition of ABCB1 in the intestinal wall and, further, the excretion of azacyclonol (the minor CYP3A-catalyzed fexofenadine metabolite) was not affected by acute SJW administration. This acute inhibition of ABCB1 by SJW was shown previously with digoxin [58]. Long term administration of SJW, on the other hand, did not cause a significant decrease in C_{max} or AUC of fexofenadine or in $t_{1/2}$, t_{max}, and oral clearance. In contrast, a recent study found that SJW extract LI160 did increase the oral clearance of fexofenadine by almost 2-fold (94% increase) with a decrease in C_{max} of 39%. There were no changes in fexofenadine $t_{1/2}$ or t_{max} [60]. The differences in the results between the two studies were attributed to the time of fexofenadine administration or possible differences in SJW preparations. In the former study, fexofenadine was administered 1 hour after a single dose of SJW, which resulted in the apparent acute inhibition of ABCB1 (decreased oral clearance of fexofenadine). Long term SJW administration resulted in no change in fexofenadine kinetics, possibly due to no net effect of the acute inhibition and induction by SJW [59]. In the latter study, by contrast, fexofenadine was administered 10 hours after the SJW dose and the induction of ABCB1 was apparent (increased oral clearance of fexofenadine) after long term SJW dosing [60].

POLYMORPHISMS IN THE *ABCB1* GENE

Polymorphisms of the efflux transporters have been associated with changes in the disposition of drugs as well as linked to some inherited diseases. A number of SNPs have been identified in the *ABCB1* gene [67-71]. The polymorphism in exon 26 of the *ABCB1* gene (C3435T) is a silent mutation (no associated amino acid change) that has been focused upon due to its association with altered ABCB1 transporter function *in vivo* and ABCB1 expression in human duodenum [71] and in peripheral blood cells ($CD56^+$ natural killer cells) [72]. The 3435C/C genotype was found to occur with a frequency of 21% in Caucasians and 35% in Japanese [68, 71]. The 3435C/T genotype was found to occur with a frequency in Caucasians of 48-50% and in Japanese, 53% [68, 71, 73]. The frequency of the 3435 T/T genotype was 24-28.6% in Caucasians and in Japanese, 12% [68, 71, 73, 74]. Hoffmeyer et al. found a significant correlation of the C3435T genotype in Caucasians with decreased expression of ABCB1 in the duodenum (2-fold lower for homozygous T allele (T/T) compared to C/C individuals) and ABCB1 function (increased steady-state plasma concentrations of digoxin) [71]. This mutation is also associated with altered ABCB1 function in peripheral $CD56^+$ blood cells [72]. Individuals of the 3435C/C genotype had significantly higher ABCB1 activity measured by rhodamine efflux of the blood cells than those of T/T genotype, this was associated with lowered ABCB1 expression in the cells of the T/T individuals [72]. Several other groups have also found an increase in digoxin bioavailability and/or area under the curve (AUC) and C_{max} in Caucasian and Japanese individuals with the T/T genotype [75, 75-77].

Contradictory to the previously mentioned reports, however, one group did not find the same effects of the C3435T genotype on digoxin pharmacokinetics or duodenal ABCB1 gene expression in Japanese individuals, in fact, the opposite effect was found [73, 74, 78]. In individuals with the 3435T/T genotype there was a suppression of duodenal absorption, decreased plasma levels of digoxin, and increased gene expression of ABCB1 in the duodenum compared to the C/C genotype [73, 74, 78, 79]. In line with these results, Kim et al. found that the 3435T/T genotype was associated with a lowered fexofenadine (180 mg) AUC in Caucasians [70]. In contrast, other reports indicated no significant changes in fexofenadine (180 mg), digoxin (1 mg), or talinolol (100 mg) pharmacokinetics in Caucasian individuals in association with the 3435C/C and T/T allelic groups [80-82].

It is unclear as to the reasons for the discrepancies, but it may indicate that further investigations addressing haplotypes and not only SNPs is necessary. It is increasingly apparent that it is difficult to account for correlations of genotype and phenotype by one SNP (e.g. C3435T) and even two (C3435T and G2677T/A). The C3435T polymorphism is highly linked with the SNP at exon 21, position 2677 (G2677T/A), which results in amino acid changes Ala893Thr and Ala893Ser, respectively, and with C1236T in exon 12 [70, 83]. This linkage of the 3 alleles was found to occur in 62% of European Americans and 13% of African Americans [70]. The C3435T SNP and the G2677T/A SNP in Japanese were also closely linked (94%) [83]. The functional change of the G2677T mutation was examined and enhanced efflux of digoxin was found by the cells expressing the mutant allele compared to the wildtype [70]. This data is in-line with the results of

decreased plasma levels of digoxin in individuals with the 3435T/T genotype, since this allele is highly linked with the G2677A/T allele [70, 73, 74, 79]. However, this does not explain the results of other studies which found increased plasma digoxin concentrations in Caucasian and Japanese individuals with mutations in these alleles even when the haplotypes of the two SNPs were evaluated [75, 76]. Further correlation of ABCB1 haplotypes with its expression and function may be essential to establish the relationship between genotype and phenotype and explain the contradictory results.

ABCC1 (MRP1), ABCC2 (MRP2), AND ABCG2 (BCRP) EFFLUX TRANSPORTERS

ABCC1 (MRP1), ABCC2 (MRP2), and ABCG2, the breast cancer resistance protein (BCRP) are also implicated in the disposition of drugs in humans. ABCC1 is located in a wide range of tissue types, but has low expression in the liver. ABCC2 is located on the bile canalicular membrane and is involved in the biliary excretion of glucuronide, glutathione, and sulfate conjugates, as well as, non-conjugated anionic compounds. ABCC2 is also expressed in the intestine on the apical membrane of enterocytes and renal proximal tubule epithelia [84]. There is a considerable overlap with substrates of ABCC2 and ABCC1 and a list of substrates of ABCC1 and ABCC2 has been described previously and in Table **I** [84, 85]. ABCG2 is present in the placenta, intestine, breast, liver, veins, and capillaries [86]. Substrates of ABCG2 include mitoxantrone, anthracyclines, bisantrene and topotecan, and there is some overlap with those substrates of ABCB1 [87].

The clinical relevance of ABCC1, ABCC2, and ABCG2 in cancer chemotherapy and HIV-1 nucleoside reverse transcriptase inhibitor resistance is recognized, however how these efflux transporters contribute also to drug-drug interactions in general and pharmacokinetic variability in humans is less directly defined [84, 88, 89]. There are identified polymorphisms in ABCC1, however, the functional importance, gene expression, or clinical consequence of the polymorphisms in drug disposition has not been shown thus far [67, 90-92]. The clinical relevance of ABCC2 has been more defined in humans, as the Dubin-Johnson syndrome is a result of a deficiency of ABCC2, leading to deficient transport of conjugated bilirubin and anionic conjugates into the bile [85]. In addition to mutations associated with Dubin-Johnson syndrome, other polymorphisms of the *ABCC2* gene have been identified, however, have not shown clinical functional significance [67, 84, 91, 93]. Similar to ABCB1, ABCC2 protein expression is inducible by PXR ligands [89, 94].

It is implicit that modulation by induction or inhibition of these efflux transporters would have an impact on drug disposition, however, due to overlap in substrates, inhibitors and inducers of these efflux transporters as well as drug metabolizing enzymes, an interaction involving a combination of many enzymes is likely. For example, clinical drug interactions involving the potent anticancer agent, irinotecan (CPT-11), can be explained inpart by several mechanisms involving metabolism and active transport. Irinotecan is eliminated mainly by fecal elimination, likely through biliary elimination of the parent drug and its metabolites [95]. Irinotecan is converted to its active metabolite, SN-38, by carboxyesterases and then it is glucuronidated by UDP-glucuronosyl transferase (UGT) 1A1/7. Irinotecan is also metabolized by CYP3A4 to form two metabolites, APC and NPC [36]. Irinotecan is a substrate for ABCB1 and ABCC2 [95-97]. Irinotecan and SN-38 are also substrates for ABCG2 and SN-38 and the SN-38-glucuronide are transported by ABCC2 [95, 96]. In a clinical study, the effect of enzyme-inducing anticonvulsants (EIAs, e.g. phenytoin, phenobarbital, and carbamazepine) on the disposition of irinotecan and metabolites was examined in pediatric patients [98]. These types of inducers are known to increase expression of ABCB1, ABCC2, CYP3A4, and UGT enzymes, all enzymes involved in the clearance of irinotecan. There was a 1.5-fold increase in the median clearance of irinotecan lactone of those patients receiving EIAs and a 2-fold decrease in the median systemic exposure to SN-38 lactone (the lactone is the pharmacologically active form) [98]. There was, however, no observed statistically significant increases in the systemic exposure to APC or NPC, suggesting that the induction of CYP3A4 could not explain the differences in the kinetics [98]. There was an increased glucuronidation ratio of SN-38G/SN-38 in those patients treated with EIAs, which suggested induction of UGT enzymes, but the reduced systemic exposure could not be completely explained by UGT induction alone [98]. As well, induced levels of transporters, such as ABCB1 and ABCC2, by the EIAs also could lead to the increased clearance of irinotecan. Similarly, in another clinical study of patients with malignant glioma receiving irinotecan and phenytoin, there was a reduction in the AUC of irinotecan, SN-38, and SN-38G by 40, 25, and 25%, respectively, compared to patients with metastatic colorectal cancer not receiving antiepileptics [99]. Due to the overlap in the inducers and substrate specificity of efflux transporters and metabolizing enzymes, the interaction of irinotecan and EIAs is likely due to modulation of several elimination pathways.

EFFLUX TRANSPORTER AND DISEASE

Many inherited human diseases have been associated with defects in ABC transporters [2]. A defective ABCA1 cholesterol transporter leads to Tangier disease [100, 101] and a mutation in ABCC2 (MRP2; cMOAT) leads to hyperbilirubinemia, the Dubin-Johnson syndrome (as mentioned above), and jaundice in animal models and in patients [102, 103]. ABCB1 has been shown to transport lipids such as ceramide analogs [104] and platelet activating factor [105, 106] from the cytosol to the extra cellular environment. The C3435T allele frequency was found to vary significantly in different populations ranging from less than 20% in African populations to about 50% in Caucasians [107]. Low frequency of the *ABCB1 3435T/T* genotype in Africans compared to Caucasians with a higher frequency [107] correlated with the incidence of inflammatory bowel disease, i.e. ulcerative colitis, which is much less prominent in populations of African origin as compared to a Caucasian population [86, 108, 109]. A causal relationship between ABCB1 expression and ulcerative colitis is further supported by a study of Schwab et al. [109], which reported a 2-fold increase in the development of ulcerative colitis in patients with the *ABCB1 3435T/T* genotype and attributed it to an impaired barrier

function. While these data would indicate that active ABCB1 protects against the onset of the disease by preventing toxins from entering the cells, active ABCB1 expression in peripheral blood lymphocytes and intestinal epithelial cells also have been implicated in glucocorticoid treatment resistance [110]. The ABCB1 transporter system may also be involved in the trafficking of molecules involved in normal human lymphocyte function, reported by Coon *et al*. [111]. Subsequently, it was found that ABCB1 mediated the transport of cytokines such as interleukins 2 and 4, as well as, interferon γ in normal peripheral T lymphocytes [112]. Furthermore, a physiological role for ABCB1 during the mobilization of human dendritic cells and for their migration from the periphery to lymph nodes was identified [113]. Such lymphocyte homing is triggered by the immune modulator FTY720 [114] which targets the sphingosine 1-phosphate receptor [115]. Efflux transporters could play a major role in FTY720-mediated lymphocyte homing as studies in mice indicated that FTY720 enhances T-cell ABCB1 and ABCC1 (MRP1) efflux activity and promoted the accumulation of 5-lipoxygenase-dependent metabolites in culture. This, in turn, enhanced T cell migration and VLA-4α- and CD44-dependent lymph node homing [116]. While these mechanistic studies in human *in vitro* and in animals would suggest a major role of efflux transporters in immune modulation, there are insufficient clinical data to demonstrate clinical relevance.

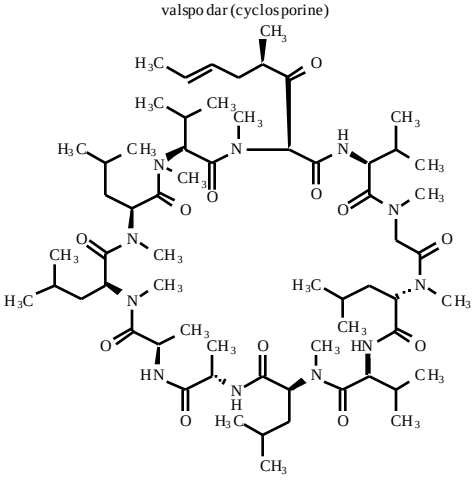
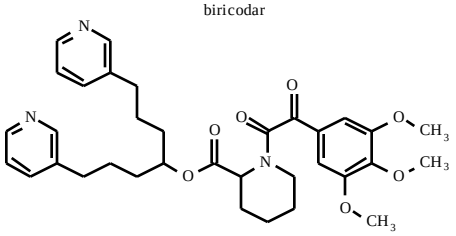
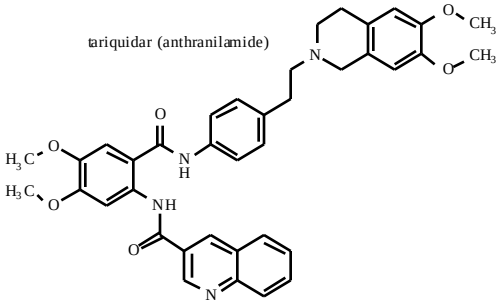
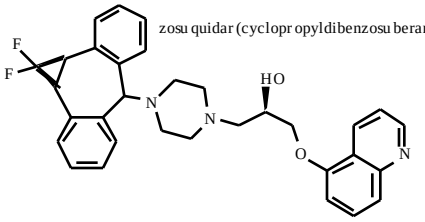
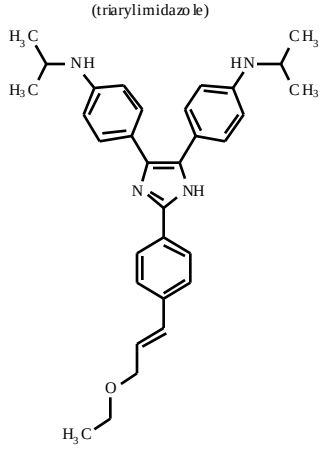
OVERCOMING RESISTANCE TO CHEMOTHERAPEUTIC AGENTS

Up to eight ABC proteins are thought to be involved in drug resistance [2], however the multi-drug resistance proteins, ABCB1, ABCC1, ABCC2, and ABCG2 are most recognized. These transporters can be present initially in a tumor or emerge by clonal expansion, which eventually can lead to drug resistance due to transporter-mediated drug efflux from the cell. ABCB1 transports organic cations [117] and can lead to resistance of anthracyclines, vinca alkaloids, colchicines, epipodophyllotoxins, and paclitaxel, while ABCC2 transports organic anions and can lead to resistance against methotrexate [118]. Non-ionic compounds such as vincristine and etoposide may be transported as glutathione, glucuronide, or sulfate conjugates or may be cotransported with glutathione. ABCG2 expression can lead to resistance towards mitoxantrone, topotecan, and irinotecan. Inhibition of the transport function, therefore, appears to be a sensible strategy to overcome drug resistance.

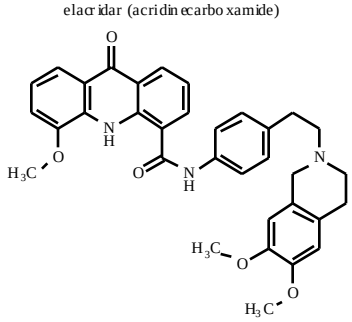
Many of the inhibitors tested clinically (Table **III**), in particular the early ones, such as verapamil, CsA, and valspodar, are not specific and they inhibit multiple transporters and cytochrome P450. For example, valspodar inhibits both ABCB1 and CYP3A4 [119]. Such an inhibition of multiple transporters and enzymes that are involved in the disposition of endogenous compounds, as well as, the clearance of other drugs, including the anticancer agents, can lead to drug-drug interactions resulting in dose adjustments and side effects, due to increased variability in plasma concentrations and inhibition of endogenous functions of these transporters. For example, an empirical 40% dose reduction for etoposide and mitoxantrone resulted in similar plasma concentrations [120] when coadministered with CsA in children with acute leukemia. The increase in etoposide plasma concentrations may be a result of a combination of inhibition of active secretion by ABCB1 and inhibition of metabolism [121]. Whereas paclitaxel, an ABCB1 substrate, is predominantly eliminated via metabolism. ABC transporter inhibition in excretory organs should not significantly influence paclitaxel clearance [119, 122]. Reduction in clearance and increase in pharmacokinetic variability of paclitaxel when coadministered with valspodar are therefore most likely due to inhibition of metabolism [123]. The anthracycline, doxorubicin, is metabolized mainly by reduction and excretion of unchanged doxorubicin into the bile [124]. Thus the increase in plasma concentrations, when coadministered with CsA or valspodar, could be attributed to the inhibition of ABCB1 transport [125]. Additionally, compounds such as CsA and valspodar also inhibit the canalicular bile salt export pump (BSEP), ABCB11, which might reduce bile flow and slow elimination of other agents [126]. Inhibition of transporters may increase tissue concentrations in normal non-tumorigenic tissues as well, such as the brain, which is protected by ABCB1 in the blood brain barrier. This can result in increased severity of side effects typically not observed with the drug alone [127]. Nevertheless, the clinical outcome for reduction of multi-drug resistance in cancer patients is encouraging. For example, the resistance in women with ovarian cancer to paclitaxel was shown to be overcome in approximately 20% of cases with the addition of valspodar, a highly effective ABCB1 modulator [128]. Furthermore, coadministration of valspodar with mitoxantrone, etoposide, and cytarabine was well tolerated during a phase I trial and led to a complete remission in 38% of the patients with ABCB1 expression and function [129].

To determine the optimal dose of the MDR modifier and to better distinguish the pharmacokinetic effects towards the anticancer agent from the desired pharmacological endpoint of transport inhibition in tumor cells, surrogate endpoints have been developed in recent years. Rhodamine accumulation in $CD56^+$ T cells from patients receiving ABCB1 modulators can be determined *ex vivo* to assess ABCB1 inhibition [130-132]. In this assay, efflux of rhodamine from $CD56^+$ cells is measured before treatment with the ABCB1 inhibitor and again after treatment. The results were encouraging in that, at clinically achievable concentrations, *in vivo* inhibition of ABCB1 efflux was achieved and, importantly, this inhibition was monitored *ex vivo* in clinical trials. For non-blood born tumors such as liver, renal, and breast tumors, radiopharmaceutical substrates have been successfully employed for *in vivo* tumor detection, imaging, and functional transport activity. Technecium-99m Sestamibi and Technetium-99m Tetrofosmin are both substrates for ABCB1 and to a lesser extent ABCC1, but not for ABCG2 [133]. Through scintigraphy with these radiopharmaceutical transporter substrates, tumor visualization is enhanced and the methodology is sensitive to distinguish between different strength of inhibitors e.g. vinblastine *vs.* valspodar [134, 135]. When biricodar, an inhibitor of ABCB1 and of ABCC1 [136, 137], was given with Technetium-99m Sestamibi, the increase in Technetium-99m Sestamibi concentrations was, however, greater in the liver than in the

Table III. Specificity of Multidrug Resistance Modulators for Inhibition of Transport and Metabolism

Compound	Generic name (Structural class)	ABCB1	ABCC1/2	ABCG2	CYP3A	References
PSC833	valspodar (cyclosporine) 	Inhibitor K_i 2.8-300 nM[a]	Inhibitor K_i 29 μM[b]	no	Inhibitor K_i 1 μM	[119, 126]
VX-710	biricodar 	Inhibitor[c]	Inhibitor[c]	no[d]	?	[137, 142]
XR-9576	tariquidar (anthranilamide) 	Inhibitor EC_{50} 25.4-487 nM[e]	?	?	Inhibitor IC_{50} >70μM	[146, 163, 164]
LY335979	zosuquidar (cyclopropyldibenzosuberane) 	Inhibitor EC_{50} <100 nM[e]	?	?	Inhibitor K_i 3.8 μM	[158]
ONT093/ OC1440935	(triarylimidazole) 	Inhibitor EC_{50} 9-90 nM[e]	no	?	Inhibitor K_i 39.8 μM	[151]

(Table 3). contd.....

Compound	Generic name (Structural class)	ABCB1	ABCC1/2	ABCG2	CYP3A	References
GF-120918	elacridar (acridinecarboxamide) 	Inhibitor EC_{50} 20-512 nM[e]	no	Inhibitor	Inhibitor K_i 10.9 μM	[146, 147, 150]

[a]determined in rat canalicular membrane vesicles

[b]K_i value determined by inhibition of leukotriene C_4 transport into membrane vesicles from cells specifically expressing human ABCC2 [169]

[c]determine in humans *in vivo* and reversal of drug resistance in human cells expressing various levels of ABCB1 and/or ABCC2

[d]no modulation of topotecan (an ABCG2 substrate) sensitivity in cells resistant to doxorubicin [137, 170]

[e]EC_{50} is the concentration of inhibitor that produced half-maximal reversal of anti-cancer agent resistance in ABCB1 expressing cultured cells

tumor, an indication of differences in drug distribution or in transporter expression between liver and tumor [138]. Nevertheless, rapid tumor clearance of Technetium-99m Sestamibi could predict the lack of tumor response to drugs affected by MDR [139]. Slow tracer clearance did not predict a positive response through chemotherapy, indicating ABCB1 independent mechanisms for chemotherapy resistance.

In spite of the fact that most substrates/inhibitors for ABCB1 are also substrates/inhibitors for CYP3A, the inhibitory potency for ABCB1 appears to dissociate from inhibition of CYP3A [6]. Newer inhibitors of ABC transporters (Table **III**) in development for chemotherapy resistance were intended to be more specific and to have little effect on cytochrome P450, mainly the CYP3A4 enzyme, which is involved in the clearance of many antineoplastic agents [140]. However, a good estimate of the *in vivo* selectivity for efflux transporter inhibition is difficult, due to the lack of standardized methodology applied *in vitro* for these newer MDR modulators. For example, the inhibition constant (K_i) for ABCB1 mediated daunorubicin transport in rat liver canalicular membrane vesicles was 300 nM [126] while rhodamine efflux from human MDA adenocarcinoma cells was inhibited at 2.8 nM [119]. Similar for tariquidar, the EC_{50} for accumulation of paclitaxel in ABCB1 overexpressing CH^rB30 cells was 25.4 nM and 487 nM with vinblastine as a substrate [141]. For elacridar the EC_{50} values in the same assay were 109 and 512 nM for paclitaxel and vinblastine accumulation, respectively. In the absence of a useful *in vitro* to *in vivo* extrapolation, good *in vivo* markers for the desired effect become very important and for efficacy, the *ex vivo* determination of rhodamine accumulation in $CD56^+$ T cells and accumulation of Technecium-90m Sestamibi by scintigraphy are most important. To distinguish drug interactions related to drug elimination from the body, however, the effect of the MDR modulator on two frequently used anticancer agents i.e. doxorubicin and paclitaxel may be a good indicator. Doxorubicin is eliminated to a significant extent unchanged and possibly by ABCB1 while paclitaxel is almost exclusively eliminated as metabolites.

Biricodar inhibits both ABCB1 and ABCC1 but not ABCG2 [137, 142]. Although no study has directly addressed the effect of biricodar on P450 activity, biricodar reduced paclitaxel clearance by about 50% [142, 143] indicating inhibition of either CYP3A or CYP2C8 the two major clearance pathways of paclitaxel. This effect is similar to CsA and valspodar, which also reduce paclitaxel clearance by about 50% [123, 144, 145]. In contrast to CsA and valspodar, biricodar did not influence doxorubicin clearance [138], which is thought to be in part via ABCB1 efflux, in spite of clear effects on Technetium-99m Sestamibi concentrations in liver and tumor tissue [138].

Another newer generation compound is elacridar (GF-120918), an acridone carboxamide derivative. Elacridar is a very active MDR reverser *in vitro*, with ABCB1 and ABCG2 inhibition at nanomolar concentrations [146, 147], while ABCC1 is not inhibited by elacridar [136]. *In vivo* inhibition of ABCB1 by elacridar may be however less pronounced as evidenced by only a modest kinetic interaction with doxorubicin, data with Technetium-99m Sestamibi are not available [148]. The observed increase in systemic exposure to oral paclitaxel following coadministration with elacridar has been attributed to ABCB1 mediated transport inhibition [149], however, *in vitro* data indicate that elacridar is also capable of inhibiting CYP3A with a K_i value of 10.9 μM [150] an enzyme, which also metabolizes paclitaxel. No clinical studies have been reported which address P450 interactions.

ONT-093 is another promising MDR reversing agent, which inhibits ABCB1 at nanomolar concentrations [151]. ONT-093 does not appear to inhibit P450-mediated reactions at therapeutic concentrations. *In vitro* studies have indicated a relatively high K_i value of 39.8 μM for CYP3A4, which is clearly above the achieved plasma concentrations of 3 μM following a 400 mg oral dose in phase 1 studies [152, 153]. ONT-093 increases the oral bioavailability of paclitaxel possibly through inhibition of ABCB1 [154], however no human data on the effect of ONT-093 on paclitaxel intravenous clearance are available at this time.

Zosuquidar (LY335979) inhibits ABCB1 with an IC_{50}-value of 59 nM but does not modulate ABCC1 and ABCG2

activity [155]. To completely reverse doxorubicin, etoposide, and irinotecan drug resistance in the ABCB1 and ABCC2 overexpressing Caco-2 colon cancer cell line, a concentration of 0.5 μM of zosuquidar was needed [156]. This concentration is similar to the concentrations achieved in clinical trials [157] and below the concentrations needed to competitively inhibit CYP3A (K_i = 3.8 μM) [158]. In patients with advanced malignancies doxorubicin pharmacokinetics were unaffected when doxorubicin was coadministered with oral zosuquidar (40-75 mg/m^2) [157]. However, intravenous zosuquidar (75 mg/m^2) resulted in a 1.3-fold increase in doxorubicin plasma exposure (AUC) and a 2-fold increase in doxorubicinol exposure [159]. The specificity of zosuquidar for ABCB1 may be responsible for the small increase of doxorubicin exposure as compared to other MDR modifiers. ABCB1 is inhibited in a concentration-dependent manner as determined by inhibition of rhodamine efflux in CD56$^+$ cells in patients [157, 160]. Also, some inhibition of P450 mediated paclitaxel metabolism is indicated by a minor reduction in paclitaxel clearance following oral dosing of zosuquidar and intravenous paclitaxel. Clinical pharmacokinetic data for the combination of docetaxel and zosuquidar in patients with advanced malignancies are not yet available [161].

Tariquidar, (XR-9576) is another potent MDR modulator. Tariquidar inhibits ABCB1 possibly by binding to a site other than the active site and does not appear to be a substrate [141]. Due to the noncompetitive nature of the inhibition, tariquidar has a long duration of action even when it is removed from the tissue culture media [162]. Tariquidar is specific for ABCB1 in that it does not inhibit ABCC1 [163] and it is also a very weak inhibitor of cytochrome P450 [164]. The *in vivo* inhibition of ABCB1 has been demonstrated by *ex vivo* inhibition of rhodamine efflux by CD56$^+$ lymphocytes [165] and in cancer patients through functional imaging using Technetium-99m Sestamibi [166, 167]. Coadministration with tariquidar increased the plasma AUC of doxorubicin and paclitaxel by 44% and 26%, respectively [168].

SUMMARY

In summary, efflux proteins play a key role in the disposition of endogenous compounds and xenobiotics, including drugs. As such, these transporters are involved in drug-drug interactions and their expression and function is associated with disease and resistance to drug treatment. The use of probe substrates, which undergo little biotransformation, used in low doses (sensitive to modulation), and can be relatively transporter specific, such as talinolol and digoxin, appear to be optimal substrates, at this time, to assess drug interactions involving ABCB1. Specific probe substrates with minimal metabolic clearance for other efflux transporters will necessitate further development. The association of efflux transporter SNPs or haplotypes with variation in drug disposition in humans remain ongoing for most of the transporters mentioned in this review, however, this information is invaluable to assess the role of specific transporters in drug disposition *in vivo.*

Several modulators of MDR are currently in development, however, mainly data from phase I and II studies are available and frequently the publications are in form of abstracts. The potency of these modulators could be demonstrated through surrogate endpoints such as *ex vivo* accumulation of rhodamine in CD56$^+$ and through functional imaging with agents such as Technecium-99m Sestamibi. The anticancer agents most frequently studied were paclitaxel and doxorubicin and many of the modulators are also inhibiting cytochrome P450 mediated clearance of e.g. paclitaxel. Overall higher potency, greater selectivity and surrogate endpoints should allow for better dosing regimens and provide an optimistic scenario for phase III clinical trials.

LIST OF ABBREVIATIONS

ABC = ATP-binding cassette

ABCB1 = P-glycoprotein (Pgp) or multi-drug resistance protein 1 (MDR1)

ABCC1 = multi-drug resistance associated protein 1 (MRP1)

ABCC2 = multi-drug resistance associated protein 2 (MRP2) or canalicular multi-specific organic anion transporter (cMOAT)

ABCG2 = breast cancer resistance protein (BCRP) mitox-anthrone resistance protein (MXR) or ABCP

AUC = area under the curve

b. i. d. = twice a day

CYP = cytochrome P450

EIAs = enzyme-inducing anticonvulsants

i. v. = intravenous

GS-X = glutathione conjugate

MDR = multi-drug resistance

OATP = organic anion transporting polypeptide

PXR = pregnane X receptor

q. d. = once a day

SJW = St. John's wort

SNPs = single nucleotide polymorphisms

XREM = xenobiotic response enhancer module.

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