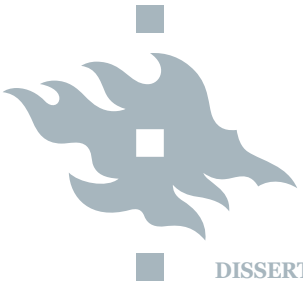

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In Vitro Evaluation of the Pharmacokinetic Effects of BCRP Interactions

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Centre for Drug Research
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

IN VITRO EVALUATION OF THE PHARMACOKINETIC EFFECTS OF BCRP INTERACTIONS

Noora Sjöstedt

ACADEMIC DISSERTATION

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Supervisors: Academy Research Fellow Heidi Kidron, Ph.D.
Centre for Drug Research
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Professor Arto Urtti, Ph.D.
Centre for Drug Research
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Reviewers: Docent Mikko Koskinen, Ph.D.
Drug Disposition and Pharmacometrics
Chemistry and Safety Sciences, R&D
Orion Corporation ORION PHARMA, Espoo
Finland

Professor Mikko Niemi, Ph.D.
Department of Clinical Pharmacology
Faculty of Medicine
University of Helsinki
Finland

Opponent: Professor Peter W. Swaan, Ph.D.
Department of Pharmaceutical Sciences
University of Maryland, Baltimore
USA

Custos: Professor Arto Urtti

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ABSTRACT

Transport proteins embedded in the cell membranes of many organs can affect the absorption, distribution and elimination of numerous drugs. This can lead to the enhanced or restricted uptake or distribution of the drugs, nonlinear pharmacokinetics, transporter-mediated drug-drug interactions (DDIs) and inter-individual variability. Transporters may therefore alter the safety and efficacy of drugs, thus it is important to study drug-transporter interactions in drug development.

The breast cancer resistance protein (BCRP, ABCG2) is one of the transporters involved in drug disposition. It belongs to the ATP-binding cassette (ABC) transporter family and uses ATP to expel drugs and other substrates out from cells. BCRP was initially found to cause drug resistance in cancer cell lines, but it is also expressed in healthy tissues such as the intestine, liver and blood-brain barrier, where it is one of the transporters limiting the uptake of many structurally diverse compounds. Despite interest in BCRP and other ABC transporters, it remains poorly understood how they recognize their substrates and which chemical structures are liable to interaction. In this thesis, a vesicle-based *in vitro* method was used to study the ligand preferences of BCRP. The results were compared to those obtained for the multidrug resistance associated protein 2 (MRP2, ABCC2), which is also implicated in drug transport. The results show that a range of natural compounds and their derivatives are able to inhibit BCRP transport and among these, flavonoids were identified as the most important group for inhibition. Conversely, MRP2 transport was affected by only few of the tested compounds. However, a more similar pattern of inhibition was seen for the two transporters when selected food additives were studied, where several food colourants were identified as inhibitors. In addition, the effect of one assay component (bovine serum albumin, BSA) on the *in vitro* transport kinetics of BCRP and MRP2 was evaluated. The inclusion of BSA in the vesicle assay lead to moderate changes (up to 2-fold) in transport activity, but the effects on *in vitro – in vivo* extrapolation are expected to be minor, at least based on the tested compounds. Finally, the vesicle assay was used to study the functionality of selected BCRP variants with polymorphisms in the transmembrane helices and they were found to have significantly impaired transport activity and expression compared to wild type BCRP.

In summary, the vesicle-based transport assay was applied to identify and evaluate the effects that BCRP interactions may have on the pharmacokinetics of BCRP substrates.

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Noora Sjöstedt
May 2017, Helsinki

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This Ph.D. dissertation is based on the following publications, which are referred to in the text by their Roman numerals:

I Deng F *, **Sjöstedt N** *, Kidron H: The Effect of Albumin on MRP2 and BCRP in the Vesicular Transport Assay. PLoS One Oct 5; 11(10):e0163886, 2016

II **Sjöstedt N**, Holvikari K, Tammela P, Kidron H: Inhibition of Breast Cancer Resistance Protein and Multidrug Resistance Associated Protein 2 by Natural Compounds and Their Derivatives. Mol. Pharm. 14(1):135-146, 2017

III **Sjöstedt N**, van den Heuvel JJMW, Koenderink JB, Kidron H: Transmembrane Domain Single-Nucleotide Polymorphisms Impair Expression and Transport Activity of ABC Transporter ABCG2. Pharm. Res. (*In press*)

IV **Sjöstedt N** *, Deng F *, Kidron H: Food Colorants Disturb the Transport Activity of Breast Cancer Resistance Protein (BCRP/ABCG2) and Multidrug Resistance Associated Protein 2 (MRP2/ABCC2). *(Manuscript)*

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* Authors contributed equally to the work

AUTHOR CONTRIBUTION

Publication I
The author participated in designing and performing the transport and inhibition experiments. The author analysed the data together with the co-authors. The author wrote the paper with the co-authors.

Publication II
The author designed the experiments with the co-authors. The author performed the fluorescence and nephelometry studies, the screening and IC_{50} determinations for BCRP and some of the MRP2 IC_{50} studies. The author participated in data analysis. The descriptor calculations and further structural analysis of BCRP inhibitors was performed by the author. The author wrote the paper with the help of the co-authors.

Publication III
The author designed the experiments with the co-authors. The author performed site-directed mutagenesis and baculovirus expression work, vesicle preparation and assays, immunoblotting and immunofluorescence studies. The author wrote the paper with the co-authors.

Publication IV
The author designed the studies with the co-authors, supervised experimental work and helped perform some of the experiments. The author analysed the data and wrote the paper with the help of the co-authors.

ABBREVIATIONS

ABC	ATP-binding cassette
ADME	absorption, distribution, metabolism and elimination
BCRP	breast cancer resistance protein
BSA	bovine serum albumin
CDCF	5(6)-carboxy-2',7'-dichlorofluorescein
CYP	cytochrome P450
DDI	drug-drug interaction
E_1S	estrone-3-sulphate
$E_2$17G	estradiol-17β-glucuronide
EMA	European Medicines Agency
ER	efflux ratio
FDA	United States Food and Drug Administration
IC_{50}	concentration required for 50% inhibition
ITC	International Transporter Consortium
IVIVE	*in vitro – in vivo* extrapolation
K_i	inhibition constant
K_m	concentration required to reach 50% of V_{max}
KO	knockout
LC-MS/MS	liquid chromatography–tandem mass spectrometry
$LogD_{7.4}$	water-octanol partition coefficient at pH 7.4
LTC_4	leukotriene C_4
LY	Lucifer yellow
MRP2	multidrug resistance associated protein 2
NBD	nucleotide-binding domain
OATP	organic anion transporting polypeptide
P_{app}	apparent permeability
PBPK	physiologically based pharmacokinetics
PET	positron emission tomography
PK	pharmacokinetic
QSAR	quantitative structure-activity relationship
SAR	structure-activity relationship
SCH	sandwich-cultured hepatocytes
SNP	single-nucleotide polymorphism
SNV	single-nucleotide variant
TMD	transmembrane domain
UGT	UDP-glucuronosyltransferase
V_{max}	maximum velocity of transport
VT	vesicular transport
WT	wild type

1 INTRODUCTION

The development of a new drug is an immensely time-consuming and costly process with a high likelihood of failure. This can be explained partly by the many different requirements that a successful drug candidate must fulfil. In addition to pharmacological potency, a drug must have sufficient exposure at its target site to be able to exert its effects (Morgan et al., 2012). For an oral drug, this means that the drug must be first absorbed and then distributed in the body to reach its target tissue. After eliciting its desired effect, the drug must be eliminated.

Drugs (and other molecules) distribute to tissues from the blood circulation by passing through cell membranes. One factor affecting the absorption and distribution of drugs are transport proteins embedded in the cell membranes of tissues throughout the body (Giacomini et al., 2010). The involvement of transporters in drug disposition may lead to nonlinear pharmacokinetics (PK), transporter-mediated drug-drug interactions (DDIs) as well as inter-individual variability. Thus, transporters, much like metabolic enzymes, can alter the safety and efficacy of drugs. Therefore, the interactions between new drug molecules and selected transporters should be studied during drug development (European Medicines Agency, 2012; US Food and Drug Administration, 2012). Understanding of transporter interactions may also facilitate the elucidation of the mechanisms of DDIs between drugs that are already on the market.

Despite the increasing number of drug–transporter interactions being recognized *in vitro,* and to a smaller extent *in vivo*, the interaction potential of a compound as well as its consequences are difficult to predict. Additionally, there are still many unanswered questions about the physiological functions and genetic variability of the identified drug transporters. These transporters exhibit broad and often overlapping capabilities to interact with different compounds, which increases the challenge of elucidating the properties of a single transporter on observed *in vivo* PK events.

This thesis describes the evaluation of the interactions of the drug efflux transporter ABCG2, known as the breast cancer resistance protein (BCRP), using a vesicle-based *in vitro* technique. The technique was applied to study the inhibition of BCRP and the multidrug resistance associated protein 2 (MRP2, ABCC2) by natural compounds and their derivatives as well as selected food additives. Genetic variants of BCRP were also generated and the same method was used to investigate the effects of the polymorphisms on BCRP transport activity. The utility of the vesicle-based assay to study transporter interactions and the implications of the *in vitro* findings to the clinical setting are discussed.

2 REVIEW OF THE LITERATURE

Transport proteins, or transporters, can be categorized into two major families, the solute carrier (SLC) family and the ATP-binding cassette (ABC) family. SLC transporters mediate the movement of molecules across the cell membrane, either by facilitated diffusion or by using ion or electrochemical gradients (Lin et al., 2015). ABC transporters, on the other hand, use ATP to drive transport irrespective of concentration gradients. Transporters are also characterised by the direction of transport that they mediate: influx transporters pump their substrates into cells, whereas efflux transporters expel molecules out from cells.

This thesis concentrates on two efflux transporters of the ABC family. ABC transporters are structurally characterized by nucleotide-binding domains (NBDs) that contain the so called Walker A and Walker B motifs and a signature C motif (Dean et al., 2001). Typically two of these domains are present and they form the basis for ATP hydrolysis. In addition to these, the basic structure also includes two transmembrane domains (TMDs), each with typically six transmembrane helices. The composition of these transmembrane domains are responsible for determining the substrate specificity and function of the transporters. Some members deviate from the typical TMD-NBD-TMD-NBD structure and have an additional transmembrane domain (e.g. ABCC1) or may completely lack these domains (ABCE and ABCF family members)(Dean et al., 2001). Others, such as the ABCG family members, consist of only half of the required elements and must, thus, form homodimers or heterodimers to function.

The ABC transporter family consists of 48 members in 7 subfamilies named from A to G. Most of the human ABC proteins are efflux transporters, but their roles and localisation in the cell are diverse (Dean et al., 2001). ABC transporters are involved in the transport of cholesterol, vitamin A, bile acids, long chain fatty acids as well as drugs, to name a few. In addition to this, some family members function as receptors or ion channels. ABC transporters are important for homeostasis and mutations in several ABC transporters have been linked to disease. For example, some ABCB variants are associated with progressive familial intrahepatic cholestasis, whereas variants of ABCA4 can cause visual impairment due to disrupted transport in photoreceptor cells. Moreover, cystic fibrosis is caused by the dysfunction of ABCC7, the cystic fibrosis transmembrane conductance regulator (CFTR).

Regarding drug transport, the most important subfamilies are ABCB, ABCC, and ABCG. Members from these families, especially efflux transporters ABCB1, ABCC2, and ABCG2, interact with a wide variety of drugs and drug-like compounds (Matsson et al., 2009). Other ABCC transporters, termed multidrug resistance associated proteins (MRPs), such as MRP1, MRP3, and MRP4, as well as ABCB11 (bile salt export pump, BSEP) which is involved in

bile salt extrusion, can also interact with xenobiotics, and might thus affect drug disposition or toxicity (Hillgren et al., 2013; Pedersen et al., 2013).

ABCB1, known as the multidrug resistance protein 1 (MDR1) or P-glycoprotein, was the first to be cloned and is the most exhaustively studied of the ABC drug transporters. The persisting, though unofficial, nomenclature still reflects the history of these drug transporters that were initially identified in multidrug resistant cancer cell lines. This is despite the fact that these interactions may be less important than their role in overall drug disposition and PK in the body. The influence of ABC transporters on drug PK is a result of their expression in tissues that are important for absorption and elimination, namely the intestine, liver, and kidney. In these tissues, ABC transporters work together with metabolic enzymes and contribute to the transport of drugs and other compounds that have undergone Phase I and/or Phase II metabolism (Doring & Petzinger, 2014).

In the following review, the two ABC transporters that are the main focus of this thesis, breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance associated protein 2 (MRP2, ABCC2) will be described in further detail.

2.1 BREAST CANCER RESISTANCE PROTEIN (BCRP)

The ABC family submember G2 was originally identified in multidrug-resistant cell lines of breast cancer and colon carcinoma (Doyle et al., 1998; Miyake et al., 1999), and in human placenta (Allikmets et al., 1998). This led to heterogeneous nomenclature, including breast cancer resistance protein (BCRP), mitoxantrone resistance protein (MXR) and ABC placenta protein (ABCP). BCRP has become the most commonly used non-standard name for ABCG2, however, and will be used from here on throughout this thesis.

The structure of BCRP, like other ABCG transporters, deviates from the basic ABC transporter structure, which consists of two TMDs and two NBDs. BCRP is often referred to as a "half-transporter", because the *ABCG2* gene only encodes for a 655 amino acid long (72 kDa) protein that has six transmembrane helices in one TMD and only one NBD (Figure 1A). Additionally, the order of the elements is reversed; the NBD at the N-terminus precedes the TMD (Doyle et al., 1998). Due to its half-structure, BCRP must form oligomers, either homodimers or higher order homomeric complexes, to be able to perform its transport function (Xu et al., 2004; Ni et al., 2010; Wong et al., 2016).

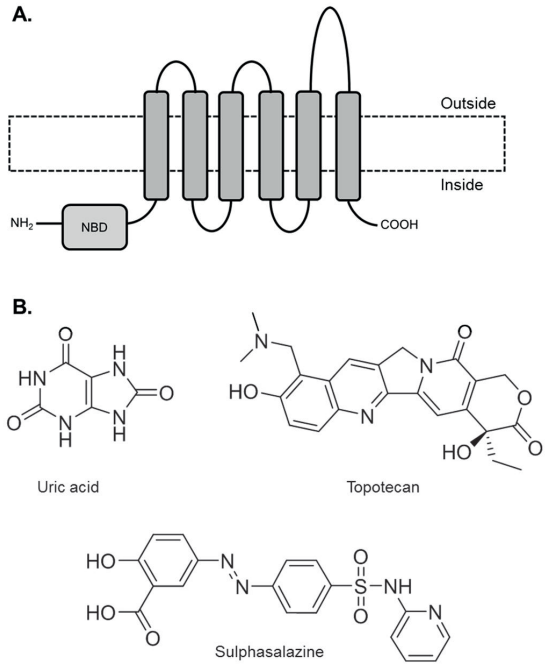


Figure 1 (A) Membrane topology of BCRP and (B) molecular structures of selected substrates

Despite originally being identified in cancer cell lines, BCRP is expressed ubiquitously in the body on the apical membranes of cells (Maliepaard et al., 2001), much like P-glycoprotein. BCRP is found in the canalicular membranes of the liver and proximal tubules of the kidney (Maliepaard et al., 2001; Huls et al., 2008), where it enhances the excretion of its substrates into the bile and urine, respectively. In barrier tissues such as the intestinal epithelium, placental syncytiotrophoblastic cells, and the blood-brain barrier, BCRP limits the entry of molecules (Maliepaard et al., 2001; Cooray et al., 2002). BCRP may also have a protective role in undifferentiated human embryonic stem cell lines, although the expression is decreased and lost during differentiation (Apati et al., 2008).

Many proteomic studies have concentrated on quantifying drug transporters in the intestine and liver, due to the importance of these tissues to the overall PK of drugs. According to the study by Drozdzik et al. (2014) BCRP contributed 4% to the total expression of 10 clinically relevant transporters studied in human intestinal samples. Conversely, BCRP contribution to the total transporter expression of 11 studied transporters in the liver was merely 1% as reported by Wang et al. (2015) and even lower (0.34%) according to a meta-analysis of proteomics data from Caucaisians by Burt et al. (2016). In contrast, the meta-analysis evaluated the contribution of MRP2 and P-glycoprotein in the liver to be around 2% each (Burt et al., 2016).

In the intestine and colon, the absolute protein abundance of BCRP has been measured to be roughly between 0.1 and 0.5 pmol/mg membrane protein by Groer et al. (2013) and Drozdzik et al. (2014), whereas some studies report values around 2.5 pmol/mg membrane protein (Harwood et al., 2015; Harwood et al., 2016a; Nakamura et al., 2016). This more than five-fold difference could be due to the applied analytical methods or the inherent variability in samples. BCRP expression appears to decrease slightly along the length of the intestine (Groer et al., 2013; Drozdzik et al., 2014; Harwood et al., 2015), but this finding may be obscured by the small number of samples analysed in the studies. BCRP tends to be expressed at similar or slightly lower levels compared to the other intestinal drug efflux transporters, P-glycoprotein and MRP2 (Groer et al., 2013; Drozdzik et al., 2014; Harwood et al., 2016a; Nakamura et al., 2016). In the liver and kidney, the expression of BCRP is lower than P-glycoprotein and MRP2 (Fallon et al., 2016). BCRP expression in the liver showed approximately 3-fold interindividual variability, which is less than in the intestine (Tucker et al., 2012; Prasad et al., 2013). The average expression of BCRP in liver samples from 50 donors was 0.14 pmol/mg membrane protein and did not show any dependency on sex, age or condition of the liver (fatty vs. non-fatty) (Prasad et al., 2013).

The closest human protein to BCRP, with approximately 30% sequence identity, is ABCG1, the human homologue of the *Drosophila* White protein. While ABCG1 and other ABCG family transporters are mainly involved in cholesterol transport (Velamakanni et al., 2007), the role of BCRP in cholesterol transport is unclear. Cholesterol is, however, important for BCRP function. In the cell membrane, BCRP is located in lipid rafts, which are areas of the membrane that are enriched in cholesterol (Storch et al., 2007). BCRP shows decreased activity after cholesterol depletion (Storch et al., 2007; Telbisz et al., 2007) and in *Spodoptera frugiperda* (Sf9) insect cell based preparations that have lower endogenous membrane cholesterol content than mammalian cells (Pal et al., 2007; Telbisz et al., 2007). On the other hand, the cholesterol-loading of BCRP-overexpressing Sf9 membrane vesicles significantly increases the drug-stimulated ATPase activity and transport rate of BCRP substrates (Pal et al., 2007; Telbisz et al., 2007).

BCRP is involved in the transport of endogenous compounds such as uric acid (Matsuo et al., 2009; Woodward et al., 2009), and estrogen conjugates, including estrone-3-sulphate (E_1S) (Imai et al., 2003). BCRP is also able to transport estradiol-17β-glucuronide ($E_2$17G), although in general, it tends to favour the transport of sulphate conjugates (Chen et al., 2003; Imai et al., 2003; Suzuki et al., 2003). Based on *in vitro* studies in Bcrp or BCRP overexpressing cell lines and *in vivo* studies in mice, BCRP protects the body against dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (van Herwaarden et al., 2003; Pavek et al., 2005), photosensitiser pheophorbide A and protoporphyrin IX (Jonker et al., 2002).

A variety of structurally unrelated drugs are transported by BCRP. Notably, BCRP can confer resistance to chemotherapeutics including mitoxantrone

(Doyle et al., 1998; Maliepaard et al., 1999), methotrexate (Chen et al., 2003), tyrosine kinase inhibitors, such as gefitinib and erlotinib (Elkind et al., 2005; Li et al., 2007), as well as topoisomerase I inhibitors topotecan and SN-38, a metabolite of irinotecan (Maliepaard et al., 1999; Jonker et al., 2000; Jonker et al., 2002; Nagashima et al., 2006). On the other hand, P-glycoprotein substrates paclitaxel and vincristine are not related to drug resistance in BCRP-overexpressing cells (Doyle et al., 1998). BCRP substrates that are not cancer drugs include, but are not limited to, cimetidine (Pavek et al., 2005), sulphasalazine (Zaher et al., 2006; Jani et al., 2009) and statins such as rosuvastatin and atorvastatin (Kitamura et al., 2008; Keskitalo et al., 2009). The structures of selected BCRP substrates are shown in Figure 1B.

In addition to BCRP substrates, a large number of drugs and drug-like compounds have been shown to inhibit BCRP and there is considerable overlap in inhibitors especially between BCRP and P-glycoprotein (Matsson et al., 2007; Matsson et al., 2009). Standard BCRP inhibitors used in research are fumitremorgin C (FTC), a fungal toxin, (Rabindran et al., 2000) and Ko143, its more potent and less toxic derivative (Allen et al., 2002). Other inhibitors include steroid hormones and drugs (Imai et al., 2003; Pavek et al., 2005; Dankers et al., 2012), immunosuppressants (Gupta et al., 2006), HIV protease inhibitors (Gupta et al., 2004), and calcium channel blockers (Zhang et al., 2005b), as well as many natural compounds, especially flavonoids (Zhang et al., 2004; Tan et al., 2013).

The binding site and transport mechanism of BCRP are poorly defined, because there is no available high-resolution crystal structure of the protein (Gandhi & Morris, 2009). Based on the analysis of ATPase stimulation data of 39 compounds, Xu et al. (2015) proposed that BCRP binds its substrates in the cytosolic leaflet, after they have partitioned into the lipid bilayer. A similar mechanism was suggested by Matsson et al. (2007), who found that membrane partitioning and the presence of π-electron systems were important characteristics of BCRP inhibitors in a cell-based assay. The presented pharmacophore, that includes a hydrogen bond acceptor, is also in line with the proposed role of transmembrane helix hydrogen bond donors for the extraction of ligands from the lipid bilayer by BCRP (Xu et al., 2015). The specific residues that are involved in the interaction of BCRP with its ligands are yet to be elucidated. However, studies on the spontaneous R482 variants of BCRP found in cell lines indicate that this residue in transmembrane helix 3 is important for the interaction of substrates with BCRP. A change at this position to glycine or threonine abrupts the transport of methotrexate and folic acid, but results in the ability to transport daunorubicin, rhodamine 123 and Lyso Tracker Green, which are not transported by wild type (WT) BCRP (Chen et al., 2003; Robey et al., 2003).

Quantitative structure-activity relationship (QSAR) models and structure-activity relationship analyses, that relate molecular properties to biological activity, have been made mainly for BCRP inhibitors and on fairly narrow datasets of related compound structures (Gandhi & Morris, 2009). Many of

these studies are not aimed at predicting drug-drug interactions, but at finding potent and selective BCRP inhibitors to overcome multidrug resistance. Therefore, the datasets typically contain analogues of know BCRP inhibitors such as fumitremorgin, tariquidar, and flavonoids (Gandhi & Morris, 2009). Although it is difficult to draw overall conclusions from the (Q)SAR studies, it seems that lipophilicity, described by either water-lipid partitioning coefficient LogP or $LogD_{7.4}$, and the planarity of the molecule are important features of BCRP inhibitors (Gandhi & Morris, 2009). Additionally, aromaticity was identified alongside $LogD_{7.4}$ as a determinant of BCRP inhibition (Matsson et al., 2009).

2.1.1 PHARMACOGENETICS OF BCRP

Several hundred single-nucleotide polymorphisms (SNPs) or variants (SNVs) have been identified in the *ABCG2* gene (1000 Genomes Project Consortium, 2015). These include non-synonymous polymorphisms, which result in a change of the coded amino acid, and could, therefore, affect protein structure and function. The most common non-synonymous genetic variants of BCRP are c.34G>A (rs2231137) and c.421C>A (rs2231142), which cause the amino acid changes p.V12M and p.Q141K, respectively. The frequency of these variants varies between populations of different ethnicity. The c.421C>A variant is present at a minor allele frequency of 29% in East Asians, 9% in Europeans, and only 1% in Africans (1000 Genomes Project Consortium, 2015). In the Finnish population, the c.421A allele is reported to have a frequency of 9.5% (Keskitalo et al., 2009). A similar trend in frequency is seen for c.34A allele, which is most prevalent in East Asians (33%), but only present in 6% of Europeans and Africans (1000 Genomes Project Consortium, 2015). Other variants occur at considerably lower frequency and are thus rarely represented in clinical studies. Of note is that null alleles of *ABCG2* define the blood group phenotype Jr(a-), which can result in serious transfusion reactions (Saison et al., 2012; Zelinski et al., 2012).

The p.Q141K polymorphism appears to impair BCRP transport activity and it seems to be a risk factor for hyperuricemia and gout due to the decreased excretion of uric acid (Matsuo et al., 2009; Woodward et al., 2009). The risk of gout is also increased in subjects with the p.Q126X variant, a nonsense mutation in the intracellular region of BCRP (Matsuo et al., 2009). The observed change in the activity of the p.Q141K variant can be explained, at least partly, by the reduced expression of BCRP. A proteomics study on human liver samples showed that patients harbouring the p.Q141K polymorphism had significantly lower BCRP expression levels than those with WT BCRP (Prasad et al., 2013). This is in line with findings from *in vitro* studies, which show that the expression of p.Q141K is ≤ 50% of WT BCRP expression, probably due to the proteasomal degradation of the variant (Kondo et al., 2004; Tamura et al., 2006; Tamura et al., 2007b; Urquhart et al., 2008; Furukawa et al., 2009;

Skoglund et al., 2014). However, no change in expression related to genotype was seen in human intestinal biopsy samples (Urquhart et al., 2008). Contrary to the p.Q141K variant, p.V12M does not appear to affect expression levels *in vitro* and it has limited effects on transport activity (Tamura et al., 2007a; Yamasaki et al., 2008; Matsuo et al., 2009; Deppe et al., 2014; Skoglund et al., 2014; Kim et al., 2015).

Due to its fairly high frequency, the p.Q141K variant has been the focus of many pharmacogenetics studies, which have mainly concentrated on anti-cancer agents and statins. These studies have been covered extensively in a review by Mao and Unadkat (2015). Their compilation of the research indicates increases of exposure up to 3.5-fold in the subjects with the p.Q141K variant compared to WT subjects. However, the consequences of the polymorphism were not found to be significant in all studies and appear to depend on the drug, its dosing route as well as the genotype (heterozygous or homozygous) of the study population (Mao & Unadkat, 2015). More recent reports suggest that bicalutamide, a prostate cancer drug, is influenced by the p.Q141K polymorphism and homozygotes may experience a 60% increase in exposure (Kim et al., 2015). Moreover, Bauer et al. (2016) reported an effect of p.Q141K on [^{11}C] tariquidar brain distribution in a positron emission tomography (PET) study, but only in the presence of P-glycoprotein inhibition.

Information about the effects of polymorphisms is important, since increased plasma concentrations and exposure may manifest as an increased risk for side effects, as seen for gefinitib induced diarrhoea in patients with the p.Q141K variant (Cusatis et al., 2006). For the low frequency variants, *in vivo* data on their functional consequences is generally unavailable. *In vitro* data, however, suggests that several naturally occurring BCRP polymorphisms decrease either (cell surface) expression or transport activity, or both, of the variant proteins (Kondo et al., 2004; Tamura et al., 2007a; Tamura et al., 2007b; Yoshioka et al., 2007; Kawahara et al., 2010; Deppe et al., 2014; Skoglund et al., 2014).

2.1.2 CLINICAL SIGNIFICANCE OF BCRP

The influence of a transporter on the overall permeability of a compound is dependent not only on the affinity and transport rate of the transporter, but also the passive permeability of the compound (Giacomini et al., 2010; Poirier et al., 2014). Highly lipophilic and thus, highly permeable compounds are less dependent on active mechanisms to cross cell membranes. On the other hand, the role of a single transporter will be downplayed if several parallel active routes exist. Moreover, when evaluating clinical significance, the effects of altered PK must always be evaluated within the context of the so called therapeutic window of the drug (Poirier et al., 2014). For some drugs, even large fluctuations in drug concentrations do not cause changes in efficacy or

safety, but for others, such as P-glycoprotein substrate digoxin, even a slight increase in concentrations can cause adverse effects.

In 2010, the International Transporter Consortium (ITC) highlighted BCRP as one of the important transporters to be considered during drug development (Giacomini et al., 2010). BCRP interaction studies have since been included in the drug-drug interaction (DDI) guidance documents of both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA)(European Medicines Agency, 2012; US Food and Drug Administration, 2012). Drugs should be studied to determine whether they are substrates of BCRP, and thus possible victims of DDIs, or whether they are inhibitors, meaning that they could be perpetrators of DDIs. Substrate studies may, however, be waived in the case of highly permeable and soluble drugs, which are unlikely to be greatly influenced by active transport (Tweedie et al., 2013). Despite the recognition of BCRP as significant factor in drug PK, clinical data on BCRP-mediated DDIs is rare compared, for example, to the data available for P-glycoprotein, that includes over 60 documented DDIs where P-glycoprotein is implicated (Poirier et al., 2014). To date, the strongest data supporting the clinical importance of BCRP on the PK of drugs comes from the pharmacogenetics studies in subjects harbouring the p.Q141K variant of BCRP.

Studies in Bcrp knockout (*Bcrp1-/-*) mice have been important in recognizing the potential significance of BCRP transport on drug PK and disposition into protected tissue compartments such as the brain, placenta, and testis (Vlaming et al., 2009). They have shown, for example, that Bcrp can limit the brain entry of tyrosine kinase inhibitors imatinib and sorafenib, but the effects of Bcrp at the blood-brain barrier are generally fairly modest (Agarwal et al. 2010, Kalvass et al. 2013). It should be kept in mind that data from knockout animal models can be confounded by expressional changes of other transporters and the interpretation of data may be difficult if the disposition and elimation routes are very different in the preclinal species compared to humans. Knockout models also serve as a kind of 'worst-case' model for BCRP inhibition and can thus overpredict the magnitude of DDIs. For example, studies in *Bcrp1-/-* mice show a 6-fold increase in the bioavailability of oral topotecan compared to WT mice (Jonker et al., 2000). In cancer patients, however, GF120918 increased oral topotecan bioavailability by only 2.4 fold (Kruijtzer et al., 2002). Exposure to sulphasalazine in *Bcrp1-/-* mice was 111-fold higher compared to WT mice when dosed orally (Zaher et al., 2006), but the increase in oral sulphasalazine AUC was less pronounced (8-fold) when Bcrp was inhibited with curcumin in mice and smaller still (2.0 – 3.2 fold) in human volunteers (Kusuhara et al., 2012). The data from the curcumin inhibition study in humans is similar to changes observed in clinical studies with subjects with the BCRP p.Q141K polymorphism, which showed up to a 3.5 fold increase in the exposure of sulphasalazine (Urquhart et al., 2008; Yamasaki et al., 2008). In addition to these drug interactions/knockout effects, it was recently reported that the

investigational tyrosine kinase inhibitor fostamatinib, a BCRP inhibitor, increased rosuvastatin exposure by almost 2-fold (Martin et al., 2016).

The challenge in ascribing the observed DDIs to BCRP is the distinct overlap of substrate and inhibitor specificities not only between ABC transporters, but also the influx transporters of the organic anion transporting polypeptide (OATP) family as well as cytochrome P450 enzymes (Poirier et al., 2014). For example, several clinical DDI studies performed with rosuvastatin as the victim drug show a significant increase in peak plasma concentration or exposure when co-administered with a BCRP inhibitor (Mao & Unadkat, 2015). However, since rosuvastatin is also a substrate of several OATP transporters (Kitamura et al., 2008), the inhibition of these transporters may also contribute to the observed changes. The same applies for the interaction between topotecan and GF120918 described by Kruijtzer et al. (2002); although the contribution of P-glycoprotein to topotecan kinetics is considered low, it cannot be ruled out as a component of the observed interaction.

The need for *in vivo* DDI studies to clarify the role of a new drug as a BCRP inhibitor can be evaluated by looking at the ratio of expected plasma or intestinal concentrations and an *in vitro* derived IC_{50} value, which is the concentration required to inhibit 50% of transport. The ITC recommends an *in vivo* DDI study to be conducted if this ratio is above 0.1 for plasma (i.e. $[I]_1/IC_{50} \geq 0.1$) and 10 for intestinal concentrations (i.e. $[I]_2/IC_{50} \geq 10$) (Tweedie et al., 2013). Optimal practices for the *in vivo* study are, however, still debated. Recently, Lee et al. (2015) proposed sulphasalazine as a probe substrate for BCRP-mediated intestinal DDIs and rosuvastatin for both intestinal and hepatic DDIs. To study whether a new drug will suffer from BCRP inhibition, curcumin and lapatinib are recommended as inhibitors (Lee et al., 2015). The need for more than one substrate and inhibitor points to the complexity of BCRP interactions due to its localization in different tissues as well as the overlap in inhibitor specificity with other transporters. It should be noted, for example, that the inhibition of hepatic BCRP transport may not result in altered plasma concentrations, but exposure in the liver could be evaluated by monitoring the cholesterol-lowering effects of rosuvastatin (Lee et al., 2015).

2.2 MULTIDRUG RESISTANCE ASSOCIATED PROTEIN 2 (MRP2)

The multidrug resistance associated protein 2 (MRP2), previously also known as the canalicular multispecific organic anion transporter (cMOAT), is encoded by the *ABCC2* gene. This protein, composed of 1545 amino acids, belongs to the ABCC family, which is often referred to as the multidrug resistance associated protein (MRP) family, due to their presumed role in resistance to cancer treatment. In contrast to BCRP, which is smaller than typical ABC transporters, MRP2 has altogether 17 transmembrane helices in

three TMDs (Nies & Keppler, 2007)(Figure 2A). The five helices that make up the so called TMD0 in the N-terminus are important for the trafficking of the protein to its correct apical location in cells (Fernandez et al., 2002). MRP2, like BCRP, is expressed at apical membranes in the intestine, liver, and kidney (Sandusky et al., 2002). According to a proteomic analysis by Drozdzik et al. (2014), the expression of MRP2 accounts for up to 10% and 25% of the total transporter abundance of clinically relevant multidrug transporters in the small intestine and colon, respectively. A similar analysis of liver samples suggests that MRP2 contributes as much as 9% to the expression of quantified, potentially clinically significant, transporters in the liver (Wang et al., 2015). However, based on a meta-analysis of proteomic studies, MRP2 seems to contribute roughly 2% to hepatic drug transporters in Caucasians (Burt et al., 2016).

In the liver, MRP2 is involved in the biliary excretion of bilirubin conjugates. It was, in fact, originally cloned from rats and was found to be absent in two mutant strains of hyberbilirubinemic rats (Buchler et al., 1996). In humans, a similar phenotype of conjugated hyperbilirubinemia is related to the absence of MRP2 at the canalicular membrane in the liver (Kartenbeck et al., 1996). This syndrome, called the Dubin-Johnson syndrome, can be caused by several sequence variants of the *ABCC2* gene that lead to a dysfunctional MRP2 protein (Nies & Keppler, 2007). Despite jaundice and the pigmentation of the liver, Dubin-Johnson syndrome appears benign (Dubin & Johnson, 1954).

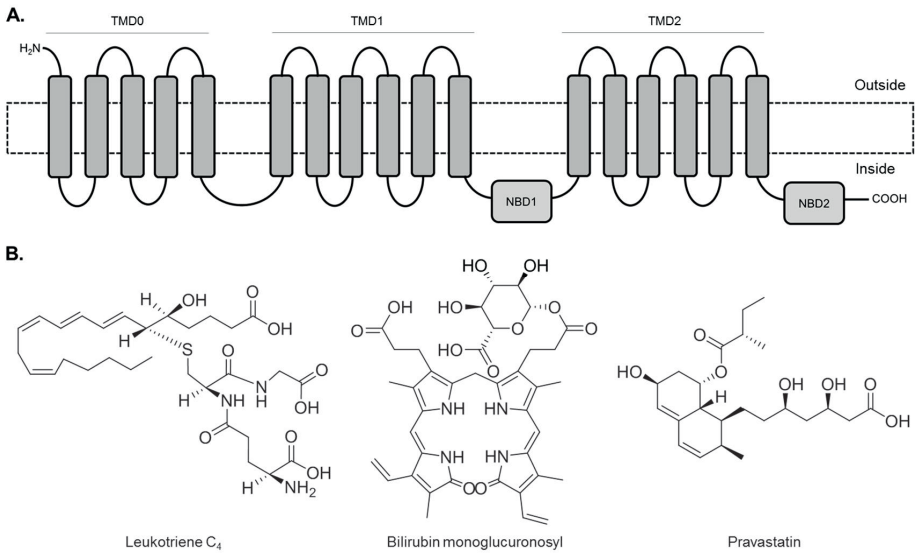


Figure 2 (A) Membrane topology of MRP2 and (B) molecular structures of selected substrates

In addition to bilirubin conjugates, MRP2 translocates a variety of endogenous and exogenous organic anions and anionic conjugates, mainly glucuronides and glutathione conjugates (Nies & Keppler, 2007). The endogenous substrates of MRP2 include leukotriene C_4 (LTC_4) and $E_2$17G (Cui et al., 1999). MRP2 can confer resistance to chemotherapeutics such as vincristine, etoposide, doxorubicin, and epirubicin (Cui et al., 1999) as well as methotrexate (Hulot et al., 2005) and irinotecan (Chu et al., 1997). In addition, MRP2 contributes to the intestinal and biliary efflux of other drugs, such as statins (Matsushima et al., 2005). The structures of selected MRP2 substrates are presented in Figure 2B.

MRP2 is inhibited by many drugs, for example benzbromarone, cyclosporine A, lansoprazole, and lopinavir (Pedersen et al., 2008). The leukotriene D4 receptor antagonist MK-571 is a typically used *in vitro* MRP2 inhibitor, which was originally considered MRP-specific, but has since been shown to inhibit other ABC transporters as well (Wang et al., 2008; Matsson et al., 2009). As is the case for many transporters, there is a lack of specific MRP2 inhibitors available (Hillgren et al., 2013). SAR studies on MRP2 suggest that lipophilicity, aromaticity, and hydrogen bonding are important features for MRP2 interaction (Pedersen et al., 2008; Zhang et al., 2009; Wissel et al., 2015; Wissel et al., 2017). However, substrates and specific inhibitors of MPR2 tend on average to be less lipophilic than BCRP or P-glycoprotein inhibitors and substrates (Matsson et al., 2009). Anionic charge seems to be more important for MRP2 substrate transport and stimulation than for inhibition (Pedersen et al., 2008). Although the presence of a negative charge may improve inhibitory activity, it is not a requirement for inhibition (Wissel et al., 2017).

MRP2 is characterized by higher selectivity for interaction than BCRP and P-glycoprotein as highlighted by Matsson et al. (2009). Out of 122 studied compounds, 26 (21%) inhibited MRP2, whereas the number was almost double for P-glycoprotein and BCRP. Notably, 73% of MRP2 inhibitors were also inhibitors of either P-glycoprotein or BCRP or both. Furthermore, MRP2 was much more selective than its family member MRP1 (ABCC1) in a structure-activity analysis of flavonoids; out of the 29 studied compounds, most inhibited MRP1, but only two compounds inhibited MRP2 (van Zanden et al., 2005).

The transport of $E_2$17G exhibits sigmoidal kinetics in MRP2 vesicles, which suggests that MRP2 has at least two ligand binding sites, one of which may be involved directly in transport and the other in its modulation (Bodo et al., 2003; Zelcer et al., 2003). The presence of an allosteric binding site makes the inhibitory and stimulatory effects of compounds on MRP2 complex and substrate-dependent (Zelcer et al., 2003; Kidron et al., 2012). For example, diclofenac and E_1S stimulate MRP2-mediated $E_2$17G transport, but inhibit the transport of LTC_4 and the fluorescent probe 5(6)-carboxy-2,7-dichlorofluorescein (CDCF) (Kidron et al., 2012).

There are few *in vivo* studies that link MRP2 function to altered pharmacokinetics of drugs in humans. In a case-study, Hulot et al. (2005) reported that a loss-of-function MRP2 variant (R412G) was linked to methotrexate overdose and nephrotoxicity. Additionally, a SNP in the 5'-flanking region of MRP2 is suggested to be associated with a decrease in irinotecan-induced diarrhoea, but only in patients where the risk was already decreased by a particular UGT1A1 genotype (de Jong et al., 2007). Furthermore, a synonymous polymorphism (T482T) appears to decrease pravastatin exposure, possibly due to an increase in expression levels of MRP2 (Niemi et al., 2006).

To date, there are no clear clinical examples of MRP2-mediated DDIs (Hillgren et al., 2013), although it has been proposed that the induction of intestinal MRP2 expression by rifampicin could be one factor in the DDIs of rifampicin with morphine and propafenone (Fromm et al., 2000). One explanation for the lower number of *in vitro* identified interactions compared to other ABC drug transporters could be the preference of MRP2 for conjugated metabolites. Early *in vitro* interaction studies typically focus on the parent compounds and not on the metabolites that may be formed *in vivo*. It is, however, possible that the inhibition of hepatic MRP2 could lead to toxicity caused by the intracellular accumulation of MRP2 substrates. The inhibition of MRP2 is therefore recommended to be studied if clinical or preclinical data point to drug–induced conjugated hyperbilirubinemia (Hillgren et al., 2013).

2.3 *IN VITRO* METHODS AND EXTRAPOLATION TO *IN VIVO*

The interactions of drugs with ABC transporters can be studied with several *in vitro* methods. The rationale for choosing a certain method should be based on the properties of the compound being evaluated as well as the research question (Brouwer et al., 2013; Zamek-Gliszczynski et al., 2013). Since each method has its own limitations, the complementary use of different methods is often beneficial for elucidating transporter interactions (Glavinas et al., 2008). The *in vitro* methods can be categorized roughly into cell-based and vesicle-based methods, and they can be applied to either study whether a compound is a substrate of a transporter or whether it is an inhibitor. The principles and main properties of the *in vitro* methods typically used to study BCRP and MRP2 are described below. Emphasis is given to techniques, from which the output data can be used to extrapolate *in vitro* findings to *in vivo* situations.

2.3.1 *IN VITRO* METHODS FOR EVALUATING EFFLUX AND INHIBITION

The permeability of a drug across a polarized cell membrane can be studied in confluent monolayers grown on permeable membrane supports. Cell lines that are commonly used include the human colorectal adenocarcinoma cell line Caco-2, which is recommended to be used as an intestinal model, as well as kidney derived cell lines from pig (LLC-PK1) and dog (Madin-Darby canine kidney, MDCK) (European Medicines Agency, 2012; US Food and Drug Administration, 2012; Brouwer et al., 2013). These kidney cell lines form tight monolayers and they can be transfected with the cDNA of the transporter of interest (Brouwer et al., 2013). To evaluate whether a compound is a substrate for a certain transporter, in a typical setting, the apparent permeability (P_{app}) of a compound in the apical to basolateral (A-B) and in the basolateral to apical (B-A) directions is determined (Figure 3). With this information, the efflux ratio (ER) can be calculated:

(1) $$ER = \frac{P_{app\ B-A}}{P_{app\ A-B}}$$

The ER is a measure of how much influence a transporter has on overall drug permeability. Efflux transporters on the apical membrane hinder the A-B permeability, but enhance permeation in the B-A direction. Compounds with ER values above two are usually considered to be transporter substrates (Brouwer et al., 2013). The ER in control cells should be around one, since the permeability is expected to be equal in both directions in the absence of transporters. The ER ratio is influenced not only by active transport, but also passive diffusion across the cell monolayer. Therefore, the monolayer integrity and leakiness should be carefully monitored using passive permeability markers, for example mannitol and by measuring transepithelial resistance (TEER) (Hubatsch et al., 2007).

Using the monolayer assay, the effects of efflux transporters may be underestimated for low permeability compounds that rely on active influx to enter cells *in vivo*. For these compounds, it may be beneficial to use an organotypic cell line, such as Caco-2, instead of an overexpression system, or to perform studies in double-transfected cell lines. For example, the combination of hepatic influx transporter OATP1B1 with MRP2 in MDCKII cells was needed to identify the contribution of MRP2 to $E_2$17G transport (Matsushima et al., 2005). Without the uptake transporter, no directional permeability could be observed. Appropriate controls and non-transfected cell lines should always be used to rule out any interference of endogenous transporters expressed by the cell line. It should, however, be noted that the endogenous transporter expression may vary between transfected cells and the parent cell line (Kuteykin-Teplyakov et al., 2010). For organotypic cell lines, the use of inhibitors is usually required to clarify the role of a single

transporter. This approach is, however, complicated by the lack of specific transporter inhibitors as described earlier.

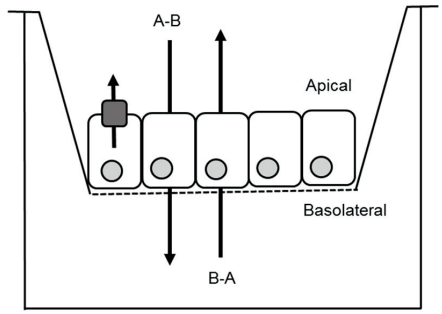


Figure 3 Scheme of the transcellular transport assay. A monolayer of cells is grown on a permeable membrane support and the permeability of a compound across the cell membrane is measured. Measurements are typically performed in both the apical to basolateral (A-B) and the basolateral to apical (B-A) directions. The presence of apical efflux transporters (dark grey square), such as BCRP or MRP2, will increase permeability in the B-A direction and decrease the apparent A-B permeability of substrate drugs.

Cell monolayers can also be used to study the kinetics of transport and calculate the maximum velocity of transport (V_{max}) and the K_m, which describes affinity and corresponds to the concentration required to reach 50% of V_{max}. However, these calculations require the application of compartmental models to the data to derive reliable and system-independent parameter values (Tachibana et al., 2010; Harwood et al., 2013; Zamek-Gliszczynski et al., 2013). Inhibition in cell monolayers can be studied either at a single concentration or over a concentration range to determine the IC_{50} value or the inhibition constant K_i. Whereas the IC_{50} is related to specific assay conditions, such as substrate concentration, the K_i is a more robust parameter (Burlingham & Widlanski, 2003). However, the workload required to determine the K_i value is significantly higher than that required for the IC_{50}, which is why the latter is often favoured. Several calculation methods can be applied to estimate IC_{50} values and it should be noted that they can lead to slightly different results (Bentz et al., 2013). This may have an impact when comparing the results to cut-off IC_{50} and expected *in vivo* inhibitor concentration ratios set by the regulatory authorities. Although kinetic parameters and IC_{50} values can be measured with the monolayer experiments, the drawback of the system, especially for these types of experiments, is that they are fairly labour intensive and costly. Experiments are usually performed manually in a 12- or 24-well plate format, which results in low throughput and limits the amount of different concentrations to be tested, for example. Automation may serve as a way to circumvent this disadvantage (Larson et al., 2012), especially in an industrial setting.

Besides transcellular permeability studies, inhibition may also be studied by measuring the accumulation of a probe substrate, typically a fluorescent compound, in cells overexpressing the transporter of interest (Hegedus et al., 2009). The inhibition of efflux is seen as an increase in the intracellular accumulation of the probe compared to the control with probe only and based on this data, IC_{50} values can be calculated. Although it is possible, substrates are rarely studied with these accumulation studies (Hegedus et al., 2009). On the other hand, sandwich-cultured hepatocytes (SCH) are a specialized system to study hepatic processes in detail. In this system, primary hepatocytes grown between two collagen layers polarize and start to display canalicular networks typical to the liver (Swift et al., 2010). Because primary hepatocytes are used, both uptake and efflux transporters, as well as metabolic enzymes, are expressed in the system. Transport studies are performed in the presence and absence of calcium ions. The removal of Ca^{2+} ions leads to the opening of the tight junctions of the bile canaliculi. The resulting data can be used to calculate a biliary excretion index, apparent biliary clearance or intrinsic biliary clearance, of which the latter parameter can be further utilised in pharmacokinetic models (Brouwer et al., 2013).

In addition to whole cells, the transport activity of ABC transporters can be evaluated in inside-out oriented membrane vesicles that are formed when the cell membrane is extracted (Glavinas et al., 2008). Vesicles can be produced in principle from any cell types expressing the transporter of interest including tissue preparations. Commonly used transporter-transfected cell lines include human embryonic kidney (HEK293) cells and Chinese hamster ovary (CHO) cells as well as *Spodoptera frugiperda* derived insect cells (Sf9, Sf21, or High-five) (Glavinas et al., 2008; Brouwer et al., 2013). Transport proteins can also be isolated, purified, and reconstituted into proteoliposomes to enable high control over the membrane composition and ensure the absence of endogenous transport (Glavinas et al., 2008).

In the inside-out vesicles, efflux transporters, such as BCRP and MRP2, pump substrates into, rather than out of the vesicles in the presence of ATP. Passive permeability and unspecific binding to the vesicles is accounted for by performing assays in the absence of ATP and subtracting this value from transport observed with ATP. The possible presence of endogenous transport should be evaluated in vesicles prepared from parent cell lines. A schematic illustration of the vesicular transport (VT) assay is shown for BCRP in Figure 4A. The amount of substrate accumulated into the vesicle can be determined using fluorescence measurement, mass spectrometry, or scintillation counting depending on the substrate. False negative results may be obtained for compounds with high passive permeability that can diffuse out of the vesicles or that produce a high background signal from nonspecific binding (Brouwer et al., 2013; Tweedie et al., 2013). On the other hand, the VT assay is especially suitable for studying low permeability compounds, such as the hydrophilic conjugates that are substrates of MRP2 (Glavinas et al., 2008; Hillgren et al., 2013). The assay benefits from higher throughput than cell-based assays as it

is easily performed in a 96-well plate format, and vesicles can be prepared in large batches and preserved at – 80 °C. Michaelis-Menten type enzyme kinetic calculations can directly be applied to the data, because the test compound has direct access to the intracellular domains of the transporter (Zamek-Gliszczynski et al., 2013). The compound concentration in assay buffer is thus a surrogate for the intracellular concentration.

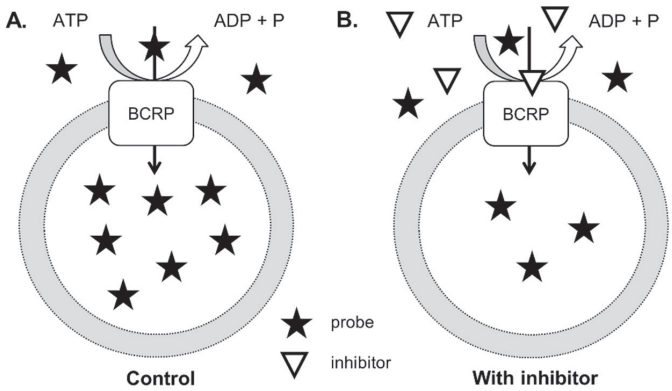


Figure 4 Scheme of the vesicular transport assay for BCRP. In inverted membrane vesicles BCRP transports its probe substrate into the vesicles (A). The amount of accumulated probe can be measured. In the indirect application of the assay, the effect of test compounds on BCRP transport activity can be evaluated (B). The effect of an inhibitor is seen as a reduction in uptake.

The inhibition or stimulation of transport by a compound can be studied using an indirect application of the assay (Figure 4B). A probe substrate is incubated in the presence and absence (control) of test compound. A decrease in probe accumulation indicates inhibition, whereas stimulation results in an increased amount of accumulated substrate. The requirement for inhibition assays is a suitable probe compound that shows active high transport compared to the background. This can be a problem for some transporters, for example P-glycoprotein, that favour hydrophobic compounds as substrates. For BCRP and MRP2, however, several suitable probes exist including methotrexate, E_1S, and Lucifer yellow for BCRP and E_217G, LTC_4 and CDCF for MRP2 (Heredi-Szabo et al., 2008; Hegedus et al., 2009; Brouwer et al., 2013). A drawback of the vesicle assay is that it is rarely possible to use the victim drugs of the potential DDIs as probe substrates, although this would be preferred due to the possible substrate-dependency of inhibition (Tweedie et al., 2013). Examples of substrates and inhibitors that may be used in whole cell-based and vesicle-based studies are compiled in Table 1 and Table 2.

Table 1 *Examples of in vitro BCRP and MRP2 substrates.*

	Compound	Km (μM)	ER	Test system	Ref.
BCRP	E_1S	7.4	-	Sf9-BCRP vesicles	Elsby et al. 2011
		16.6	-	BCRP-P388 vesicles	Suzuki et al. 2003
		-	4.5	MDCKII-BCRP cells	Xia et al. 2007
	Methotrexate	681	-	MCF7/MX vesicles	Volk and Schneider 2003
	Prazosin	-	4.5	MDCKII-BCRP cells	Poirier et al. 2014
	Rosuvastatin	2.02 & 60.9[a]	-	HEK-BCRP vesicles	Kitamura et al. 2008
		10.8 & 307[a]	-	BCRP vesicles	Huang et al. 2006
		-	≈ 5	MDCKII-BCRP cells	Deng et al. 2008
	Sulphasalazine	0.7	-	BCRP vesicles	Jani et al. 2009
		-	≈ 19[b]	Caco-2 cells	Dahan and Amidon 2009
MRP2	CDCF	12.3	-	Sf9-MRP2 vesicles	Heredi-Szabo et al. 2008
	$E_2$17G	120	-	Sf9-MRP2 vesicles	Zelcer et al. 2003
		99	-	HEK-MRP2 vesicles	Elsby et al. 2011
	Methotrexate	-	≈ 2-4	MDCK-MRP2 cells	Jia et al. 2016
		480	-	MDCKII-MRP2 vesicles	El-Sheikh et al. 2006
	Vinblastine	137	36	MDCK-MRP2 cells	Tang et al. 2002

[a] Values for high and low affinity binding sites

[b] Efflux partially mediated by MRP2

Table 2 *Examples of in vitro BCRP and MRP2 inhibitors.*

	Compound	IC_{50} or K_i (μM)	Test system (probe)	Ref.
BCRP	Cyclosporin A	6.7	BCRP vesicles (E_1S)	Xia et al. 2007
		< 50 μM	MDCKII-BCRP cells (E_1S)	Xia et al. 2007
	Ko143	0.013	BCRP vesicles (E_1S)	Xia et al. 2007
		0.026	Sf9-BCRP vesicles (methotrexate)	Tan et al. 2013
		0.01	MDCKII-BCRP cells (pheophorbide A)	Weiss et al. 2007
	Sulphasalazine	0.74	Sf9-BCRP vesicles (E_1S)	Elsby et al. 2011
MRP2	Benzbromarone	1.2	MDCK-ABCC2 vesicles (CDCF)	Colombo et al. 2013
		36	Sf9-MRP2 vesicles ($E_2$17G)	Elsby et al. 2011
		4.4	Sf9-MRP2 vesicles (CDCF)	Heredi-Szabo et al. 2008
	Cyclosporin A	45.3	MDCKII-ABCC2 vesicles (CCK-8)	Letschert et al. 2005
		21	HEK-MRP2 vesicles (monoglucuronosyl bilirubin)	Kamisako et al. 1999
		8.11	MDCK-MRP2 cells (vinblastine)	Tang et al. 2002
	MK-571	10	Sf9-MRP2 vesicles ($E_2$17G)	Pedersen et al. 2008
		7.6	MDCK-ABCC2 cells (CDCF)	Colombo et al. 2012
		28	Sf9-MRP2 vesicles ($E_2$17G)	Elsby et al. 2011
		4.1	Sf9-MRP2 vesicles (CDCF)	Heredi-Szabo et al. 2008
		26.4	MDCK-MRP2 cells (vinblastine)	Tang et al. 2002
	Probenecid [a]	580	Sf9-MRP2 vesicles (CDCF)	Heredi-Szabo et al. 2008
		2300	Sf9-MRP2 vesicles (LTC_4)	Heredi-Szabo et al. 2008

[a] Stimulation of MRP2-mediated $E_2$17G transport also observed (Zelcer et al. 2003).

2.3.2 UTILISATION OF *IN VITRO* DATA IN MODELLING

Although *in vitro* data such as the ER and IC_{50} values can be used by themselves, for example to prioritise compounds for further stages of drug development, the clinical significance of these interactions is difficult to understand based on the *in vitro* kinetic or inhibition data alone (Brouwer et al., 2013). The incorporation of *in vitro* derived data in mechanistic, physiologically-based pharmacokinetic (PBPK) models can serve as a 'bottom up' approach to understand the contributions of specific processes in complicated systems (Rowland et al., 2011; Zhao et al., 2011). It also enables the generation of dynamic time-course data instead of static values (Rowland et al., 2011). Additionally, *in vitro* data can be used to refine covariate models generated from *in vivo* data using the classical 'top down' approach to modelling (Jamei et al., 2009). The complexity of *in silico* (PBPK) models should always be based on the question that the model is built to answer. Usually, the simplest model that can answer the proposed question is the most suitable choice. In principle, there is no limit for detail in PBPK model structure, but it quickly becomes limited by the vast amount of data required for the detailed description of biological events.

In PBPK modelling, the body is represented as distinct tissue compartments that are connected to each other by blood flow. Depending on the complexity and purpose of the model, tissue compartments may be divided into subcompartments or lumped together. The key idea in PBPK modelling is the separation of physiological data, the so-called system-related parameters, and drug-related parameters from each other in the model (Jamei et al., 2009; Rowland et al., 2011). The systems-related data includes, for example, the volumes and weights of organs as well as blood flow and the abundance of metabolic enzymes and transporters in these organs. The drug-related parameters include physicochemical properties such lipophilicity and ionisation, which are important for predicting distribution into tissues, permeability properties, and metabolic and transporter-mediated clearances. Additionally, interindividual variability and population differences can be included by combining demographic data and variability to the physiological parameters. This means that instead of simulating PK events in the average man, simulations can be performed with virtual patients that represent the extremes of the likely patient populations (Rowland et al., 2011).

An integrated part of mechanistic PBPK modelling, is the *in vitro – in vivo* extrapolation (IVIVE) of metabolic and transporter data. There are several basic steps in the IVIVE of transport data (Harwood et al., 2013). First, robust data must be available to describe the passive permeability, as well as the kinetic transport parameters, K_m and V_{max}, of the drug to be modelled. Second, the V_{max} value should be scaled. V_{max} is an inherently system-based parameter which is dependent on the expression level of the transporter in the system. Thus, appropriate scaling procedures must be applied to account for the difference in expression levels *in vitro* and *in vivo*. The importance of taking expression levels into account during IVIVE was recently demonstrated by

Olander et al. (2016). In their Caco-2 cell permeability study, the influx transporter OATP2B1 contributed roughly 60% to pitavastatin uptake, but when human intestinal proteomic data was used to scale the transport, the contribution of OATP2B1 was only 5%.Without the scaling, the *in vitro* data would have resulted in a gross overestimation of the contribution of OATP2B1 to pitavastatin transport *in vivo*.

The evaluation of transporter expression has previously relied mainly on mRNA data, which sometimes shows low correlation with actual protein expression (Vogel & Marcotte, 2012). Immunoblotting, can also be used, but it is more a qualitative than a quantitative method. Therefore, the advancement of mass spectrometric methods for protein quantification and the increasing amount of data on transporter abundance in human tissues are key factors for improving the quantitative IVIVE of transporters (Harwood et al., 2013). For example, Harwood et al. (2016c) demonstrated that BCRP protein abundance was better correlated with the observed transport of E_1S than was mRNA expression.

Proteomic analysis is not without its challenges, however. Different procedures in plasma membrane extraction and purification, as well as peptide selection, can result in inter-laboratory differences as shown, for example, by Harwood et al. (2016b). In a comparison of scaling between Caco-2 and intestinal samples, they found no significant difference in relative expression for P-glycoprotein between two laboratories, but a 2-fold difference was observed for BCRP. Although the difference in this case was fairly moderate, combining proteomic data from different sources might lead to inaccurate conclusions due to this variability. Recently, Vrana et al. (2017) published a paper which describes the generation of a database of methods for the targeted proteomic quantitation of 284 proteins related to pharmacokinetic processes. Efforts like this are likely to facilitate the acquisition of high-quality proteomic data for PBPK modelling.

An assumption of proteomic data used to scale transport activity, is that all of the detected protein is active. For vesicles, this assumption must be refined by correcting the protein abundance with the proportion of vesicles that is in the active, inverted conformation (Harwood et al., 2013; Vildhede et al., 2016). Additionally, it should be kept in mind that *in vitro* derived parameters are dependent on assay conditions and calculation methods. Even a factor that may seem minor, such as shaking, can have a clear effect on the observed passive permeability of high permeability compounds (Artursson & Karlsson, 1991). Therefore, rigorous validation and optimisation of best practices are required to provide robust estimates for *in silico* modelling. Due to the complexity of whole-body PBPK systems, the extrapolation principles described above have been used for *in vitro – in vitro* extrapolation as a proof of concept. Recently, Vildhede et al. (2016) combined efflux transporter data generated in VT assays with uptake and passive diffusion data from accumulation studies to model the kinetics of pitavastatin in sandwich-cultured human hepatocytes. Transporter expression data from each system

was used to scale parameters to the hepatocytes. Out of the nine transporters studied, the simulations identified BCRP as the main transporter mediating canalicular efflux and OATP1B1 as the main uptake transporter of pitavastatin. In this way, the bottom-up *in vitro* simulation model presented by Vildhede et al. (2016) was able to provide information about the relative contribution of the different transporters in hepatocytes and increase confidence in the bottom-up approach.

It should be noted that, in addition to permeability and transport related parameters, models often require a parameter to describe intracellular binding. Unbound intracellular concentrations may be of interest, because many drug targets reside inside the cell and therefore this concentration is the driving force for the pharmacological or toxicological effects. For example, the IC_{50} concentrations from vesicle studies should be related to the unbound intracellular concentrations in PBPK models and therefore intracellular binding must be accounted for. Equilibrium dialysis of the test compound with cell lysates has been proposed as a relatively simple way of measuring the binding to cellular components (Mateus et al., 2013). Although it has its limitation of disrupting cellular organelle integrity, this technique may prove useful for estimating intracellular binding.

An advantage of PBPK models is that they allow us to simulate concentrations in tissues that cannot be accessed in humans, such as the liver. This is useful, because hepatic intracellular concentrations are important in understanding drug-induced liver toxicity (Chu et al., 2013). Moreover, distribution into tissues such as the brain cannot be resolved from plasma concentration data. The challenge with the validation of detailed PBPK models is the obvious lack of clinical data on tissue concentrations. Although PET studies are emerging as tool for studying the tissue distribution of transporter substrates (Kusuhara, 2013), preclinical animal models are still an important stepping stone toward better predictive models. The benefit of PBPK modelling is that it can take into account the physiological differences of preclinical animals and humans. Proteomic data that describes interspecies variability in transporter expression may help to explain observed differences in PK between species (Wang et al., 2015; Fallon et al., 2016) and serve as a link between preclinical species and clinical observations. For instance, cross-species differences in BCRP expression levels have been found, with BCRP expression in the human liver being almost 10-fold lower than in dogs and the expression in the human kidney 50-fold lower than in rats (Fallon et al., 2016). This magnitude of variability can be expected to influence the importance of these routes in the elimination of BCRP substrate drugs and can be taken into consideration in PBPK modelling. Possible species differences in substrate and inhibitor specificity can be incorporated based on *in vitro* data. However, according to Bakhsheshian et al. (2013), BCRP substrate and inhibitor specificities seem to be similar at least between human and mouse, but are more variable for MRP2 (Zimmermann et al., 2008).

3 AIMS OF THE STUDY

The general aim of this Ph.D. thesis was to understand which compounds are liable to interact with BCRP and how BCRP function and expression can change with genetic variation. How one experimental factor (the inclusion of bovine serum albumin) could affect the observed transport in an established assay system was also investigated. Natural and natural-like compounds, as well as food additives, were used in the studies, since information on these interactions is interesting in itself for identifying food-drug interactions. The results obtained for BCRP were compared with MRP2 results, to better understand differences and similarities between these two ABC transporters.

The specific aims of the thesis were:

1. To study the effect of bovine serum albumin on the measured transport kinetics of BCRP and MRP2 in the vesicular transport (VT) assay (I).

2. To study and compare the inhibition of BCRP and MRP2 by natural compounds and their derivatives, and by selected food preservatives, colorants and sweeteners (II, IV).

3. To identify important molecular properties of BCRP inhibitors (II).

4. To evaluate the effects of naturally occurring, transmembrane domain single-nucleotide polymorphisms on the expression and the function of BCRP (III).

4 MATERIALS AND METHODS

4.1 MATERIALS

The key reagents and materials used in the studies are listed in Table 3. The human *ABCC2* (MRP2) and *ABCG2* (BCRP) cDNAs were kindly provided by Dr. Piet Borst (Netherlands Cancer Institute, the Netherlands) and Dr. Douglas Ross (University of Maryland School of Medicine, MD, USA), respectively. The in-house library used in publication II, contains 124 natural compounds and natural compound dervatives from various commercial sources. The major compound subgroups in the library are flavonoids, coumarins, and benzoic acid derivatives. In publication IV, the studied food additives were selected from the list of food additives permitted for use in the European Union and were obtained from Sigma-Aldrich (USA).

Table 3 *Key reagents and materials used in the studies*

Reagent/material	Use	Supplier	Publication
Lucifer yellow CH dipotassium salt (LY)	BCRP probe	Sigma-Aldrich (USA)	I-IV
Estrone-3-sulphate (E_1S)	BCRP probe	Sigma-Aldrich (USA)	III
^{3}H-E_1S (54 Ci/mmol)	BCRP probe	Perkin Elmer (USA)	III
5(6)-carboxy-2',7'-dichlorofluorescein (CDCF)	MRP2 probe	Sigma-Aldrich (USA)	I,II,IV
Estradiol-17β-glucuronide ($E_2$17G)	MRP2 probe	Sigma-Aldrich (USA)	I
Randomly methylated β-cyclodextrin cholesterol (Cholesterol-RAMEB) complex	Cholesterol-loading of BCRP vesicles	Cyclolab Ltd (Hungary)	I-IV
Bio-Rad protein assay dye	Protein concentration measurement	Bio-Rad (USA)	I-IV
Essentially fatty acid free bovine serum album	Studies on albumin effects	Sigma-Aldrich (USA)	I
Sf9 *(Spodoptera frugiperda*) cell line	Expression of BCRP and MRP2	ATCC (USA)	I-IV
HyClone SfX insect cell medium	Sf9 cell culture	ThermoFisher Scientific (USA)	I-IV
Foetal bovine serum	Sf9 and HEK293 cell culture	Gibco (USA)	I-IV
HEK293 cell line (CRL-1573)	Expression of BCRP	ATCC (USA)	III

Reagent/material	Use	Supplier	Publication
Dulbecco's Modified Eagle Medium (DMEM) (#32430)	HEK293 cell culture	Gibco (USA)	III
Bac-to-bac protocol	Generation of recombinant baculovirus	Invitrogen (USA)	I-IV
Cellfectin II reagent	Sf9 cell transfection	Invitrogen (USA)	III
Q5 site-directed mutagenesis kit	Incorporation of SNVs	New England Biolabs (USA)	III
Gateway LR clonase II enzyme mix	Gateway cloning	ThermoFisher Scientific (USA)	III
10 % Mini-PROTEAN TGX Stain-Free gel	SDS-Page	Bio-Rad (USA)	III
Anti-BCRP/ABCG2 antibody BXP-21 (ab3380)	Western blot, immunofluorescence	Abcam (UK)	III
Goat Anti-Mouse IgG Antibody, (H+L) HRP conjugate (AP308P)	Western blot	Millipore (USA)	III
Goat anti-Mouse IgG (H+L)-Alexa Fluor 488 Antibody (A-11001)	Immunofluorescence	ThermoFisher Scientific (USA)	III
Amersham ECL Prime Western blotting detection reagent	Western blot detection	GE Healthcare (UK)	III

4.2 METHODS

4.2.1 EXPRESSION OF BCRP AND MRP2 TRANSPORT PROTEINS

Human MRP2 cDNA was subcloned from pGEM3-MRP2 into the pFastBac1 vector using BamHI/HindIII sites. BCRP cDNA was subcloned from pcDNA3-BCRP into the BamHI/XhoI sites of the pFastBac1 plasmid. The QuikChange Lightning Site-directed mutagenesis kit was used to convert the BCRP cDNA to the WT BCRP (Uniprot Q9UNQ0). The pENTR221-hBCRP was constructed as described in Dankers et al. (2012).

BCRP and MRP2 were expressed in Sf9 (*Spodoptera frugiperda*) insect cells (I-IV) and in human embryonic kidney 293 (HEK293) cells (III) using the Bac-to-bac baculovirus expression system. A baculovirus containing the enhanced yellow fluorescent protein (eYFP) gene and an empty bacmid served as controls for the HEK293 and Sf9 systems, respectively.

Sf9 cells were cultured as a suspension at 27°C in HyClone SfX insect cell culture medium supplemented with 5% FBS. HEK293 cells were grown as an adherent culture in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco #32430) with 10 % FBS at 37°C, 5% CO_2. Cells were harvested 55 – 72 h after the addition of baculovirus by centrifugation at 1000 g, 10 min (Sf9 cells) or 3000 g 15 min (HEK293 cells) at 4°C.

4.2.2 SITE-DIRECTED MUTAGENESIS

The BCRP variants (III) were produced using the Q5 site-directed mutagenesis kit. Non-synonymous SNVs were incorporated into the ABCG2 gene in either the pFastBac1-BCRP or the pENTR221-hBCRP plasmids. The 1582 G>A (A528T) variant was made using Q5 polymerase with overlapping primers and template DNA was degraded using the DpnI enzyme. All resulting plasmids were sequenced for the whole *ABCG2* gene to verify the presence of the SNVs using the GATC Biotech sequencing service (Germany). The BCRP gene from the pENTR221-hBCRP vector was further transferred to a modified Bac-to-bac vector using the Gateway cloning system (Invitrogen, USA).

4.2.3 PRODUCTION OF MEMBRANE VESICLES

Sf9-BCRP and Sf9-MRP2 vesicles (I, II, and IV) were produced using a modification of the method presented by Chu et al. (2004). All steps were performed at 4°C. First, cells were washed twice with buffer containing 50 mM Tris and 300 mM mannitol (pH 7.0) and centrifuged at 800 g for 5 min. Cells were then resuspended in membrane buffer (50 mM Tris, 50 mM mannitol, 2 mM EGTA, pH 7.0) and homogenized by making 40 strokes with a tight fitting Dounce homogenizer. Lysates were incubated on ice for 1 h. Cell debris was removed by centrifugation at 800 g, 10 min, 4°C and the supernatant was subsequently centrifuged at 100 000 g for 1 h 15 min. Finally, the resulting pellet was resuspended in membrane buffer and passed 20 times through a 27G needle.

The Sf9-BCRP vesicles were loaded with cholesterol to improve the dynamic range of the assay (Telbisz et al., 2007)(I, II, IV). Membranes were incubated for 20 min on ice in membrane buffer with a water-soluble randomly methylated β-cyclodextrin cholesterol complex at a final cholesterol concentration of 2.5 mM. Excess cholesterol/cholesterol complex was removed by repeating centrifugation at 100 000 g, after which the pellet was resuspened in membrane buffer and homogenized with a needle. The final concentration of cholesterol in the membrane vesicles was approximately 100 µg cholesterol/mg total protein according to the Amplex Red assay kit.

The HEK293-BCRP vesicles used in publication III were prepared as follows. Cells were resuspended in Tris-sucrose (TS) buffer (10 mM Tris, 250 mM sucrose, pH 7.4) and homogenized with the Dounce homogenizer as were the Sf9 cells. The lysate was centrifuged for 20 min at 4 000 g, 4°C. The resulting supernatant was subjected to further centrifugation for 1 h 30 min, 21 000 g at 4°C. The supernatant was discarded and the remaining pellet was resuspended in TS buffer and passed 20 times through a 27G needle to homogenize the suspension and form vesicles.

The protein concentration of all vesicle preparations was assayed using the Bio-Rad protein assay which is based on the Bradford method (Bradford, 1976). Vesicles were diluted to a stock concentration of 5 mg/ml (Sf9 vesicles, I-IV) and 1.5 mg/ml (HEK293 vesicles, III).

4.2.4 VESICULAR TRANSPORT ASSAY

The activity of BCRP and MRP2 was studied in all publications using the vesicular transport assay. The probe substrates used in this study were 5(6)-carboxy-2',7'-dichlorofluorescein (CDCF) and estradiol-17β-glucuronide ($E_2$17G) for MRP2 and Lucifer Yellow (LY) and estrone-3-sulphate (E_1S) for BCRP (Figure 5).

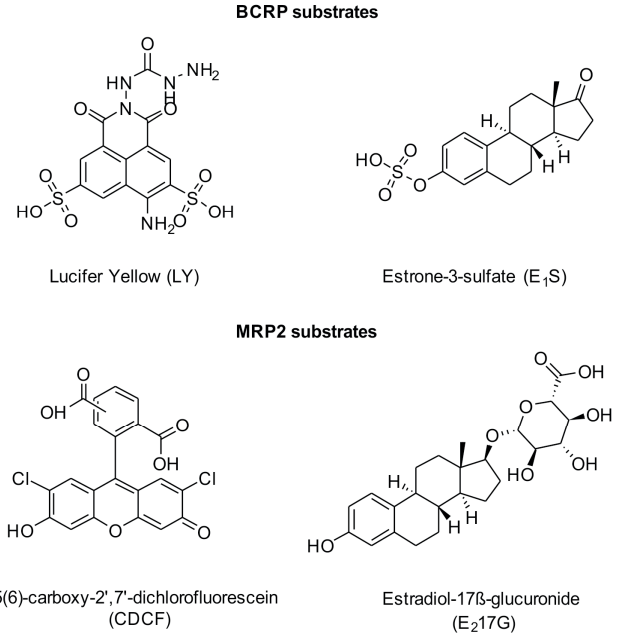


Figure 5 Molecular structures of the compounds used as BCRP and MRP2 substrates in the vesicular transport assay.

The Sf9 transport assays were performed as a modification of the PREDIVEZ Vesicular Transport Assay kit protocol (Solvo, Hungary). In the assay, vesicles are diluted in buffer containing 40mM MOPS-Tris (pH 7.0), 60 mM KCl, 6 mM $MgCl_2$ and transporter substrate. In MRP2 assays, 1.9 mM glutathione was included in reactions. In inhibition assays, probe substrates LY (50 μM) and CDCF (5 μM) were used and test compounds (dissolved in DMSO) were included in the mixture at 50 μM in the initial screening (II, IV). The aggregation of selected test compounds in assay conditions was studied with nephelometry (Nepheloscan Ascent, Thermo Fisher Scientific, USA)(II, IV). The DMSO concentration in the assays did not exceed 2%. In publication I, 0.1% or 1% essentially fatty acid free bovine serum albumin was included in assays and the kinetics of BCRP-mediated LY transport as well as the MRP2-mediated transport of CDCF and $E_2$17G was studied.

In the assays, vesicles were first preincubated at the appropriate temperature, then Mg-ATP (4 mM final concentration of ATP) was added to

start the reaction. Reactions were stopped with cold washing buffer (40 mM MOPS-TRIS (pH 7.0) and 70 mM KCl), filtered on Multiscreen$_{HTS}$ glass fiber filter plates (Millipore, USA) and washed five times with the buffer. The key assay parameters for each of the substrates are indicated in Table 4. The protocol was slightly modified for the studies performed with HEK293-BCRP vesicles (III). Vesicles (15 µg) were incubated in TS buffer with substrate, 10 mM $MgCl_2$ and 4 mM ATP or AMP (background control). A mixture of ^{3}H-labelled and unlabelled E_1S was used in E_1S assays with a final concentration of 1 µM. Reactions were terminated at appropriate times with ice-cold TS buffer, filtered and washed twice.

Table 4 *Assay parameters of the substrates used in the VT assay*

	BCRP substrates		MRP2 substrates	
	LY	E_1S	CDCF	$E_2$17G
Concentration in inhibition assays	50 µM	N.A.	5 µM	N.A.
Preincubation time *	5 min	5 min	10 min	15 min
Incubation time with ATP	10 min	2 min	30 min	8 min
Assay temperature	37°C	32°C	37°C	37°C
Analysis	Fluorescence	Scintillation counting	Fluorescence	LC-MS/MS

* Preincubation not used in HEK293 vesicle assays. N.A. = not applicable

After drying, the LY and CDCF samples were eluted with 0.1 M NaOH and collected on a 96-well plate. Before fluorescence detection, LY samples were treated with an equal volume of 0.1 M HCl. Fluorescence was measured with Varioskan Flash (ThermoFisher Scientific, Finland). Excitation and emission wavelengths were 510 and 535 nm for CDCF and 430 and 538 nm for LY, respectively. $E_2$17G samples were eluted with 0.1 M ammonium hydroxide and subjected to mass spectrometric analysis with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (for details, see publication I). E_1S samples were measured with a Wallac 1450 Microbeta Trilux scintillation counter (Perkin Elmer, USA) after addition of 50 µl of OptiPhase HiSafe 2 scintillation cocktail to each well (III).

4.2.5 INTERFERENCE ASSAYS

There is a possible caveat in using fluorescence based assays, especially when working with coloured test compounds. Many compounds possess the ability to either quench (i.e. extinguish) probe fluorescence or produce their own fluorescence which may disturb the probe signal. This can result in the false interpretation of the data. For example, a highly quenching test compound may appear to decrease uptake since the fluorescence signal is decreased, but may in reality have little effect on transport.

The natural compound library used in Publication II, and the food additives studied in Publication IV, contained several coloured and potentially interfering compounds. To rule out any interference, the effect of the test compounds on the fluorescence of the assay probes (CDCF and LY) was studied as detailed below. Test compounds were diluted in the solvent used in the measurement step of the transport assay, i.e. 0.1 M NaOH (CDCF samples) or 1:1 0.1 M NaOH/0.1 M HCl (LY samples). Due to the nature of the assay, the concentration of the test compound in the final measurement step is unknown. Therefore the testing was performed at the highest possible concentration, assuming that all of the test compound was retained in the final elute. Wells were spiked with an appropriate amount of LY or CDCF that resulted in a control signal corresponding to the fluorescence signals seen in the control wells in the vesicle assays. Fluorescence was measured as in the vesicular transport assay. The obtained fluorescence values in the presence of the test compounds were compared to values in the control wells (DMSO instead of test compound). Further testing at lower test compound concentrations was performed for selected compounds in publication IV.

4.2.6 IMMUNOBLOTTING

The relative expression level of BCRP in the vesicle preparations was studied with immunoblotting to see whether altered expression could explain the observed differences in activity (III). Samples for western blotting were prepared by mixing vesicles with 2 x Laemmli sample buffer and separating them on a commercial 10 % SDS-Page gel (Mini-PROTEAN TGX Stain-Free gel). Samples were blotted to a 0.45 µm nitrocellulose membrane and then blocked with 5% (w/v) skimmed milk solution. Membranes were probed for 1 h with the anti-human BCRP mouse monoclonal antibody BXP-21 (1:5000 in 5% w/v skimmed milk in TBS-0.1% Tween 20) for BCRP detection. After washing with TBS-Tween, the secondary antibody, goat Anti-Mouse IgG antibody (H+L) HRP conjugate (1:10 000) was applied in 2.5% (w/v) skimmed milk in TBS-Tween for 1 h. The membrane was finally washed with TBS-Tween and the Amersham ECL Prime Western blotting detection reagent was used to visualize the bands. The membrane was imaged using the ChemiDoc XRS+ imager from Bio-Rad. The total protein content in each lane was visualized using the Stain-free technology from Bio-Rad. This data was used to normalise the band intensity of the BCRP specific signal according to the Stain-free workflow (Bio-Rad) using the Image Lab software v. 5 (Bio-Rad). The final data was presented as a relative band intensity (%) compared to the WT BCRP.

4.2.7 IMMUNOFLUORESCENCE MICROSCOPY

In publication III, the localization of transduced BCRP in HEK293 cells was studied using immunofluorescence microscopy. LabTek 8-well chamber slides (Nunc, Denmark) were coated with 0.1 mg/ml poly-D-lysine solution to

improve the adhesion of HEK293 cells, which were seeded at 0.2 x 10^5 cells/chamber and allowed to attach for 24 h. Cells were then transduced using BCRP variant baculovirus. Transduction efficiency was improved by adding 5 mM sodium butyrate to the medium. Cells were fixed with 4% paraformaldehyde solution 24 h after transduction. Cells were permeabilised with 0.5% saponin in PBS before labelling BCRP with the BXP-21 antibody (1:2000). Goat anti-mouse IgG (H+L)-Alexa Fluor 488 secondary antibody (1:200) was used as the secondary antibody. 25 µg/ml DAPI was used to stain the nuclei of the cells. Normal mouse IgG (1:400) was applied as a control in one well. A Leica DM6000B wide-field microscope (Leica Microsystems, Germany) was used for imaging with 40x magnification. The experiments were performed on two separate batches of transduced HEK293 cells.

4.2.8 DATA ANALYSIS

In all vesicle assays, reactions were performed in triplicate in the presence and the absence of ATP (I-IV). ATP-dependent transport was calculated as the difference between these two values. The dynamic range of the assay was evaluated based on the ratio of observed transport in the presence and the absence of ATP. In inhibition experiments, data was normalised to the ATP-dependent uptake in the vehicle control. Compounds that decreased transport to ≤ 50% at 50 µM were regarded as potential inhibitors (II, IV).

Curve fitting was performed using GraphPad Prism version 6.05 (GraphPad Software Inc., USA). The kinetic parameters of transport for CDCF and LY were calculated by fitting the Michaelis-Menten equation (I, III):

$$v = \frac{V_{max} \times C}{K_m + C} \quad (2)$$

where v is the measured ATP-dependent transport, C is the concentration of the substrate, V_{max} is the maximal transport and K_m is the substrate concentration at which the transport is 50% of V_{max}. For $E_2$17G, the allosteric sigmoidal model of kinetics was used (I):

$$v = \frac{V_{max} \times C^h}{K_{half}^h + C^h} \quad (3)$$

where K_{half} is the substrate concentration required for 50% V_{max}, h is the Hill slope and other parameters are the same as in the basic Michaelis-Menten equation.

In the inhibition assays (I, II, IV), the concentration required for 50% inhibition (IC_{50}) was calculated using the four parameter logistic curve:

$$Response = Bottom + \frac{Top - Bottom}{1 + (\frac{[I]}{IC_{50}})^h} \quad (4)$$

where Response is the ATP-dependent transport normalized to the control, Bottom and Top are the plateaus of the maximal and minimal inhibition, [I] is the inhibitor concentration and h is the Hill slope.

Molecular descriptors were calculated with PaDEL-Descriptor (Yap, 2011) and ACD/Labs version 8.0 (Advanced Chemistry Development, Inc., Canada) (II, IV). In Publication II, principal component analysis (PCA) for the library compounds was performed with Simca-P version 10.5 (Umetrics, Sweden). The consequences of the BCRP polymorphisms were predicted using online tools PolyPhen2 (Adzhubei et al., 2010) and SIFT Human Protein service (Kumar et al., 2009). The Uniprot entry Q9UNQ0, corresponding to WT BCRP, was used as a template for the predictions.

4.2.9 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 6.05 in all cases. The extra-sum-of-squares F-test was used to evaluate whether there was a significant difference in calculated kinetic parameters (K_m and V_{max}) in publications I and III. The statistical significance of differences between transport in the presence of 0.1% and 1 % BSA was analysed using the unpaired t-test in publication I. Finally, in publication III, one-way ANOVA with the Dunnett's *post hoc* test was applied to verify the statistical significance of differences in ATP-dependent transport or BCRP expression between the variant and WT forms of BCRP. In all cases, p values below 0.05 were considered significant.

5 SUMMARY OF THE MAIN RESULTS

5.1 THE EFFECT OF ALBUMIN ON MRP2 AND BCRP IN THE VESICULAR TRANSPORT ASSAY (I)

The addition of albumin is known to affect the kinetic parameters of metabolic enzymes (UGTs and CYP450s) measured *in vitro* in human liver microsomes or membrane preparations of recombinant enzymes. The mechanism behind the 'albumin effect' has been proposed to be inhibition of UGTs and CYPs by fatty acids released from the membrane preparations. Metabolic enzymes and transporters have an overlap in substrate and inhibitor specificity and similar membrane preparations are used to study transport by BCRP and MRP2 using the vesicular transport (VT) assay. Therefore, it was investigated whether albumin could affect the kinetic parameters of transport in this assay. The inclusion of 0.1% bovine serum albumin in the VT assay increased the maximal transport activity of CDCF and $E_2$17G by MRP2 by 110% and 55%, respectively (Figure 6A). Only a 28% increase of LY transport was seen in BCRP vesicles (Figure 6B). The K_m of transport was not significantly altered in any of the cases. The albumin effect was sensitive to protein amount, but not to the cholesterol loading of the BCRP vesicles. Using 1% BSA resulted in a larger albumin effect than 0.1% BSA in one case only. Finally, whether fatty acids also inhibit MRP2 or BCRP transport was investigated. The results indicate that BCRP can be inhibited by oleic acid, the most abundant fatty acid in Sf9 cells, and the apparent calculated IC_{50} was 28 μM. Results for MRP2, however, were inconclusive, because oleic acid severely compromised the integrity of the vesicles.

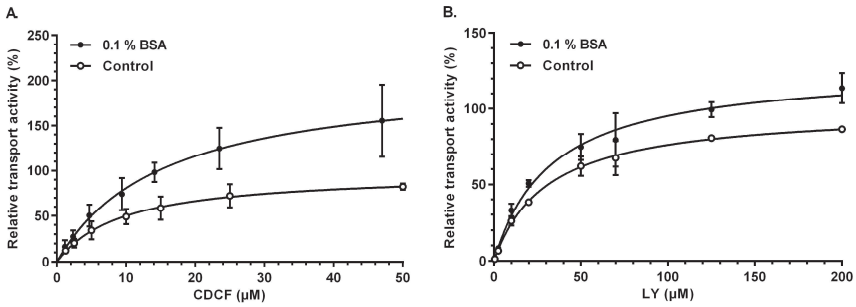


Figure 6 The albumin effect on the transport activity of MRP2 and BCRP. The ATP-dependent transport of 5(6)-carboxy-2',7'-dichlorofluorescein (CDCF) in MRP2 vesicles (A) and Lucifer yellow (LY) in BCRP vesicles (B) was studied in the prescence and absence (control) of 0.1% BSA. Data points are normalized to the calculated V_{max} of the control and represented as mean ± SD (n=3-4). Curves represent fitting to the Michaelis-Menten equation. Modified from Deng et al. 2016.

5.2 INHIBITION OF BCRP AND MRP2 BY NATURAL COMPOUNDS AND THEIR DERIVATIVES (II)

The interaction of 124 natural compounds and natural compound derivatives with BCRP and MRP2 was studied using the VT assay. In the initial screening at 50 µM, 45 inhibitors of BCRP were identified (36% of all library compounds), whereas only four MRP2 inhibitors were found (Figure 7). By broadening the criteria, altogether six potential dual inhibitors of BCRP and MRP (gossypin, myricetin, morin, nordihydroguaiaretic acid, octyl gallate, and silybin) were selected for IC_{50} studies. The range of determined IC_{50} values was 0.642 – 13.1 µM for BCRP and 19.2 – 49.8 µM for MRP2 (Table 5). Flavonoids were the most important compound group for BCRP inhibition: 86% of all flavonoids inhibited BCRP. The C-ring 2,3 double bond and C4-ketone seemed to be beneficial for inhibition since many flavones and flavonols, flavonoids with these elements, were among the most potent inhibitors. Molecular weight, $LogD_{7.4}$, the number of aromatic atoms, and the number of rings were identified as descriptors that distinguish BCRP inhibitors from non-inhibitors. Analysis of MRP2 inhibitor properties was not possible due to the small number of identified inhibitors.

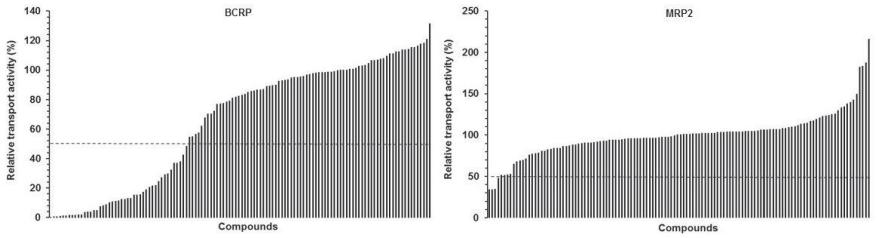
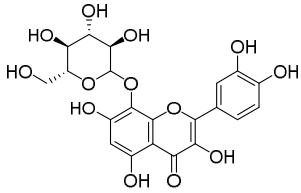
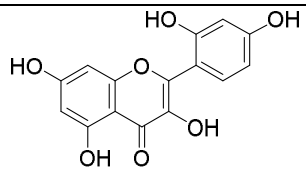
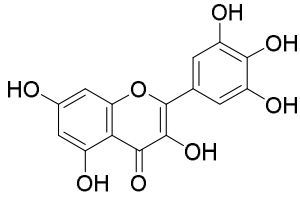
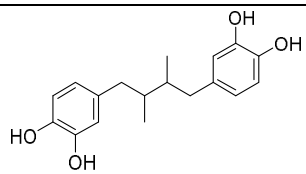
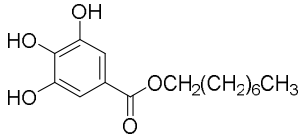
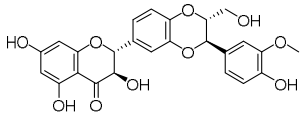


Figure 7 Results of the natural compound library screening. Bars represent the relative transport activity in the presence of the test compounds normalised to the control (vehicle only). Compounds are sorted according to their activity to illustrate the number of hits (relative transport activity < 50%) identified in the screens for each transporter and are not in comparable order between graphs. Error bars are omitted for clarity.

Table 5 *Dual inhibitors of BCRP and MRP2 identified in the natural compound library. The IC_{50} values were calculated from dose-response curves using GraphPad Prism v.6.05 and are presented with the 95% confidence interval (95% CI).*

Inhibitor	Structure	IC_{50} (μM) (95% CI)	
		BCRP	MRP2
Gossypin		9.66 (6.50 - 14.4)	30.3 (21.9 − 41.9)
Morin		0.642 (0.533 - 0.773)	25.1 (19.6 − 32.6)
Myricetin		0.835 (0.739 - 0.946)	47.5 (37.5 − 60.2)
Nordihydro-guaiaretic acid		13.1 (10.0 - 17.0)	39.9 (34.6 − 46.0)
Octyl gallate		10 (6.38 - 15.7)	19.2 (14.8 − 24.9)
Silybin		1.45 (1.08 - 1.99)	49.8 (42.4 − 58.5)

5.3 EFFECTS OF TRANSMEMBRANE DOMAIN VARIANTS ON BCRP EXPRESSION AND ACTIVITY (III)

The effects of nine naturally occurring, non-synonymous transmembrane polymorphisms (G406R, F431L, S441N, P480L, F489L, M515R, L525R, A528T, and T542A) on BCRP activity and expression were studied. BCRP variants were produced using site-directed mutagenesis and then expressed in insect-derived Sf9 and human-derived HEK293 cells using the baculovirus expression system. The transport activity of the variants was studied using the VT assay with probe substrates LY and E_1S. Reduced transport compared to the WT was seen for all variants. For eight out of nine variants, LY transport was less than 25% of WT in both Sf9 and HEK293 vesicles, whereas the A528T variant decreased transport by approximately 50% in both systems (Figure 8). Similar results were obtained with the physiological BCRP substrate E_1S.

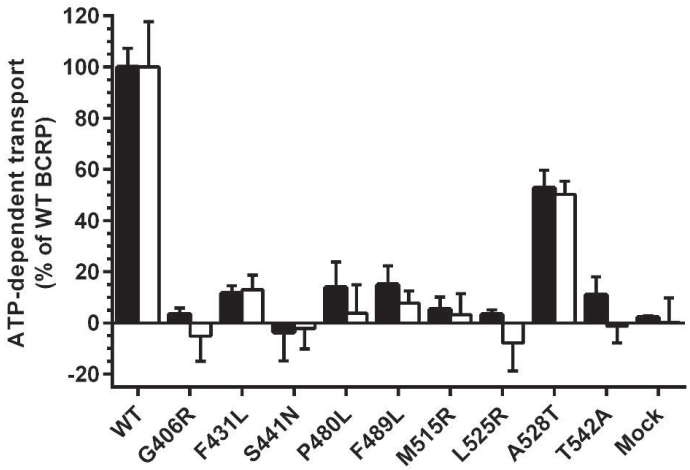


Figure 8 Relative ATP-dependent transport of Lucifer yellow (LY) in BCRP variant vesicles. The transport of 50 µM LY by the BCRP variants was studied using Sf9 (solid bars) or HEK293 (open bars) veicles. The data is normalised to the transport activity of wild type (WT) BCRP and presented as the mean with standard deviation from two separate experiments performed in triplicates. According to one-way ANOVA with the Dunnett's *post hoc* test, the transport activity in all preparations was significantly different from WT BCRP ($p < 0.0001$).

BCRP was detected in Western blots of all BCRP vesicle preparations, but the variant's expression levels tended to be lower than WT (Table 6). Relative expression levels were lower in HEK293 than Sf9 vesicles. Only negligible amounts of G406R, S441N, and M515R could be detected in HEK293 vesicles.

Immunofluorescence studies showed that some of the observed results in function and expression could be explained by improper localization of the variants inside the cell (Table 6).

Table 6 *Effects of studied transmembrane region polymorphisms on BCRP expression*

Nucleotide change	Amino acid change	Relative expression (± SD)		Localization
		Sf9	HEK293	
1216G>A	G406R	42 (± 3.2)	1.1 (± 0.57)	Intracellular
1291T>C	F431L	110 (± 9.6)	54 (± 14)	Cell surface
1322G>A	S441N	49 (± 17)	1.9 (± 2.4)	Intracellular
1439C>T	P480L	81 (± 8.4)	19 (± 7.2)	Cell surface
1465T>C	F489L	84 (± 15)	18 (± 11)	Cell surface
1544T>G	M515R	45 (± 13)	1 (± 0.82)	Intracellular
1574T>G	L525R	78 (± 20)	25 (±24)	Intracellular
1582G>A	A528T	110 (± 35)	49 (± 11)	Cell surface
1624A>G	T542A	120 (± 22)	28 (± 9.8)	Cell surface

5.4 INHIBITION OF BCRP AND MRP2 BY FOOD ADDITIVES (IV)

The interaction of food additives with ABC transporters remains an overlooked area of possible food-drug interactions. In this study, 25 food additives, including preservatives, colourants, and sweeteners were tested for BCRP and MRP2 inhibition using the VT assay. None of the preservatives inhibited either transporter, but several colourants inhibited both BCRP and MRP2 (Figure 9). All azo dyes inhibited one or both of the transporters. Allura red AC, brilliant black BN and carmoisine inhibited BCRP and MRP2, sunset yellow FCF and tartrazine inhibited BCRP. Additionally, brilliant blue FCF, a triarylmethane dye, inhibited MRP2 transport and natural-based dyes chlorophyllin sodium copper complex and curcumin inhibited both transporters. The only sweetener with inhibitory activity was neohesperidin dihydrochalcone, which was identified as the most potent BCRP inhibitor in dose-response studies (IC_{50} = 0.86 μM). Brilliant blue was the most potent MRP2 inhibitor with an IC_{50} of 3.22 μM.

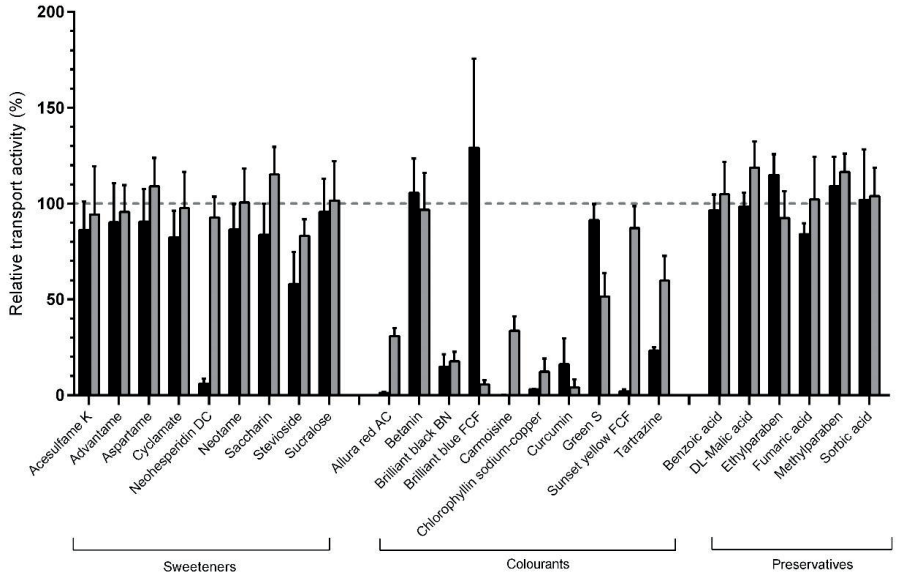


Figure 9 Relative transport activity of BCRP and MRP2 in the presence of food additives. Twenty five food additives were tested at 50 µM for their potential to inhibit BCRP (black bars) and MRP2 (grey bars) in the vesicular transport assay. The probe substrates used were 50 µM Lucifer yellow and 5 µM CDCF for BCRP and MRP2, respectively. Bars show the mean ± SD of ATP-dependent transport normalised to the transport in the presence of vehicle (DMSO) only (n=3).

6 DISCUSSION AND FUTURE PROSPECTS

In this thesis, different compounds' manner of interaction with BCRP and MRP2 was studied by using the VT assay. The method was successfully applied to screen for inhibitors of BCRP and MRP2 and to evaluate the effects of transmembrane helix variants on BCRP transport activity. The effect of albumin on the kinetic parameters derived using the assay was also studied. The results of the thesis publications and the future prospects of transporter studies are discussed below.

6.1.1 'ALBUMIN' EFFECT AND IVIVE

In publication I, the effect of bovine serum albumin (BSA) in the VT assay was assessed to determine whether it would affect the observed transport and possibly influence IVIVE. A moderate, but significant increase was observed in V_{max} values, but this was partly counteracted by the slight increase in K_m values leading to minor changes in calculated clearance values. Unfortunately, unlike the metabolic studies, it was impossible to compare the kinetic parameters to *in vivo* data, since this was unavailable for the probe substrates used in the studies. *In vivo* validation data for transporter kinetics is generally scarce due to the interplay between transporters and their presence at several distinct barrier sites, simultaneously effecting many stages of disposition. This is in contrast to metabolic enzymes that are mainly restricted to either the liver or intestine or both (Zhang & Huang, 2013). In view of improvements in the validation of methods, studies showing extrapolation even between two different *in vitro* systems (Kunze et al., 2014; Vildhede et al., 2016) are likely to be of benefit.

The data suggests that albumin could affect the *in vitro – in vivo* extrapolation, but the effect on the transporters and substrates studied here seems to be small. A similar level of variability may also arise from inter-batch variation in vesicles or inter-, even intra-laboratory practices. Indeed, the loading of Sf9-BCRP vesicles with cholesterol has a greater effect on V_{max} values, although it did not influence the BSA effect in this study. For BCRP, the observations from this study, as well as from others (Pal et al., 2007; Telbisz et al., 2007), suggest that for the kinetic IVIVE of BCRP substrates, a mammalian system where BCRP is in its native environment should be favoured. Regarding the 'albumin effect', it should be noted that we only studied two transporters and three substrates altogether. It is known from studies with UGTs that the albumin effect is substrate and enzyme-dependent (Manevski et al., 2013). Taken together, it cannot be concluded whether or not the addition of BSA would improve IVIVE for other drugs, but rather that the possibility of BSA influence should be considered if BSA is included in assays.

6.1.2 INHIBITION OF BCRP AND MRP2

In publication II and IV, the inhibition of BCRP and MRP2 was investigated. There are several reasons why it is of interest to study the inhibitors of ABC transporters. Initially, after the identification of transporters in multidrug resistant cell lines, the main focus was on finding potent, non-toxic inhibitors that could be used *in vivo* to overcome multidrug resistance. Despite many efforts, this strategy has so far yielded disappointing results and finding ABC transporter inhibitors that are suitable for clinical use has been difficult (Chen et al., 2016). Moreover, the strategy can be criticized on the basis that alongside increased permeation into tumours, the systemic administration of inhibitors may cause adverse effects by increasing exposure in other tissues as well. Due to these limitations, a timelier, challenging question is the quest for specific inhibitors that could aid in elucidating the roles of specific transporters when several proteins are involved in drug PK. In addition to identifying useful inhibitors, inhibition studies can reveal information about the molecular properties that are needed for interaction with transporters. It should be kept in mind, however, that inhibitors may bind to transmembrane regions distinct from the transport binding site or even interact with the NBDs. Therefore, the properties of inhibitors may differ from those of substrates. Lastly, the inhibition of transporters might be studied to answer the question of whether there is a possibility for transporter-mediated DDIs or food-drug interactions. In publication II, a library of natural compounds and their derivatives was screened to answer these last two questions, i.e. identify the properties of inhibitors and discuss interaction potential with BCRP and MRP2. Similarly, in publication IV, it was hypothesised that food additives could inhibit these intestinal transporters and the VT assay was used to study this interaction.

In the natural compound library, a major compound group that contained 29% of compounds, was flavonoids. Flavonoids are abundant in our everyday diet and they are responsible for much of the taste and the colour of fruits and vegetables (Ross & Kasum, 2002). Although the interaction of BCRP with flavonoids has been studied previously (Cooray et al., 2004; Imai et al., 2004; Zhang et al., 2004; Ahmed-Belkacem et al., 2005; Zhang et al., 2005a; Katayama et al., 2007; Pick et al., 2011; Juvale et al., 2013), it was interesting to find that almost all of the studied flavonoids were able to inhibit BCRP. On the contrary, the amount of hits for MRP2 (3%) was much lower than expected based on previous screenings, where approximately 20% of tested compounds were identified as inhibitors (Pedersen et al., 2008; Wissel et al., 2015). The low number of hits could be due to the composition of the library, which consisted of distinct compound groups, including flavonoids, which are known to have low interaction with MRP2 (van Zanden et al., 2005). Interestingly, BCRP and MRP2 showed clear overlap of inhibition in the food additive study, whereas preliminary data on P-glycoprotein inhibition by the same compound set indicated inhibition by only one compound (data not shown).

Although it was not pursued in depth in this study, the data suggests that there is a potential for the analysis of the structure-activity relationships (SAR) of the flavonoids, since there were some trends noted even in the preliminary screening. From this data, we were able to identify some important structural features such as the C-ring ketone and 2-3 double bond. Further dose-response analysis and IC_{50} determination may reveal even greater differences between structural features, because several flavonoids were able to almost abolish transport at the 50 μM concentration used in the studies. The SARs of flavonoids have indeed been investigated by other groups in the search for potent BCRP inhibitors (Ahmed-Belkacem et al., 2005; Zhang et al., 2005a; Katayama et al., 2007; Pick et al., 2011).

To further understand flavonoid interactions, it would be extremely interesting to perform molecular modelling and docking studies to see which parts of the protein the flavonoids interact with. It has been proposed that certain flavonoids might interact with the NBDs of ABC transporters (Matsson et al., 2009), but this finding is not supported as a general mechanism by the results presented here due to the observed differences in BCRP and MRP2 inhibition patterns. The NBD interaction proposed by Matsson et al. (2009) was based on docking to a structure based on the NBD of MRP1, but docking studies in general have been limited by the lack of structural information on full proteins. Obtaining the high-resolution crystal structures of mammalian ABC transporters has been challenging, and until recently the structure of mouse P-glycoprotein, published by Aller et al. (2009), was the most relevant available structure for human ABC drug transporters. Since P-glycoprotein represents the typical ABC organization with two TMDs and two NBDs, this crystal structure has had limited use for the modelling of BCRP and MRP2 which deviate from the basic structure. The recently published structures of bovine ABCC1/MRP1 (Johnson & Chen, 2017) and human heterodimer transporter ABCG5/ABCG8 (Lee et al., 2016), however, are likely to greatly improve the quality of homology models for MRP2 and BCRP, respectively. In fact, a homology model of BCRP that is based on the ABCG5/ABCG8 structure has been published recently (Laszlo et al., 2016). Docking studies using this model support the conception that BCRP has several binding sites.

The existence of multiple binding sites is a challenge for SAR analyses of BCRP and MRP2, because it may result in probe-dependent effects of modulators. In this regard, docking analysis could aid in the interpretation of *in vitro* interaction data. Additional information about binding sites can also be gained from protein structures captured in the ligand-bound state, as has been done with the crystal structure of mouse P-glycoprotein (Aller et al., 2009). Based on structural data, it has recently been suggested that unlike P-glycoprotein and BCRP, MRP1 recognizes its substrates from the cytoplasm and not the lipid bilayer (Johnson & Chen, 2017). This may explain some of the differences observed between the interactions of compounds with BCRP and MRP2, if we consider MRP2 to resemble MRP1 in this respect. This is an example of how the increase in structural information can quickly provide a

better understanding of drug binding and transport. This knowledge could be further used in modifying and prioritising lead compounds in drug development. With BCRP though, it remains to be seen whether future protein crystallisation studies will shed light on the higher order oligomeric states proposed by other methods (Xu et al., 2004; Wong et al., 2016).

The fact that this and previous studies (Cooray et al., 2004; Imai et al., 2004; Zhang et al., 2004; Tan et al., 2013) suggest that many flavonoids can inhibit BCRP may be of importance, because they are abundant in our diet (Ross & Kasum, 2002; Manach et al., 2004). Based on *in vitro* data, it has been proposed, for example, that flavonoids in orange and grapefruit juice could alter dasatinib PK through the inhibition of BCRP (Fleisher et al., 2015). However, the challenge of extrapolating the *in vitro* findings from the vesicle studies to a clinical setting, is that some of the studied compounds are hydrophilic and may have limited access to the inside of the cell and the intracellular domains of the transporters *in vivo*. In this respect, the VT assay may overestimate the potency of the compounds, because the measured IC_{50} values are related to the intracellular concentrations. Indeed, for low permeability compounds, a shift is sometimes observed between IC_{50} values from vesicle- and cell-based assays (Szeremy et al., 2011; Poirier et al., 2014). Although the expected intestinal *in vivo* concentrations could easily exceed the determined IC_{50} values for the studied dyes and the natural compounds taken, for example, as herbal supplements, the intracellular levels may remain low enough not to result in significant inhibition. Therefore, the results should be verified in cell-based assays, before further conclusions on the possible *in vivo* significance can be made. On the other hand, our studies did not consider the interactions of metabolites that might be formed from the studied compounds *in vivo*. Flavonoids, for example, are good substrates of UGTs, because they typically contain several phenolic groups (Chen et al., 2014). Due to the increased hydrophilicity of metabolic conjugates, they often require active transport to escape cells, meaning that there is the potential for interaction with efflux transporters. MRP2, in particular, may be prone to interaction with these metabolites since it is known to favour glucuronide conjugates (Morris & Zhang, 2006; Nies & Keppler, 2007; Alvarez et al., 2010). In fact, the interaction of drug metabolites with transporters and their role in drug-drug interactions has been brought to attention by Zamek-Gliszczynski et al. (2014) as factor or drug efficacy and safety to be considered during drug development.

Based on the inhibition data from the natural compound library screening, we found that molecular weight, $LogD_{7.4}$, the number of aromatic atoms, and the number of rings were important features separating for BCRP inhibitors and non-inhibitors. These descriptors were not, however, enough to construct a successful classification model of this data using partial least squares regression (data not shown). On the other hand, the identified descriptors are in line with those reported by others (Zhang et al., 2005a; Matsson et al., 2007; Matsson et al., 2009). As expected based on these findings, the low molecular weight preservatives and the sweeteners that did not contain many (aromatic)

rings were not identified as BCRP inhibitors in the food additive studies. On the contrary, the colourants that were found to inhibit BCRP are higher in molecular weight (>350 g/mol) and contain two or more ring structures. However, the calculated $LogD_{7.4}$ values of the colourants were lower than expected for BCRP inhibitors, with the average and median $LogD_{7.4}$ being -2.2 and -2.6. In the natural compound screening, the $LogD_{7.4}$ of BCRP inhibitors was generally > 0. The reason for this discrepancy is not known.

6.1.3 PHARMACOGENETICS

In addition to studying inhibition, the VT assay was used in this thesis to evaluate changes in the transport activity of naturally occurring genetic variants of BCRP. In publication III, nine naturally occurring genetic variants of BCRP were expressed in both Sf9 insect cells and human-derived HEK293 cells. A significant decrease in transport activity was observed for the two tested substrates, LY and E_1S, for all variants. The results were the same irrespective of the expression system. There was, however, a clear difference between the relative expression levels of the BCRP variants between the vesicles from Sf9 and HEK293 according to western blot results. Relative expression levels in Sf9 cells were higher than in HEK293, implying that the amino acid changes led to both altered expression and function. The low expression, especially at the cell membrane, was verified with immunofluorescence microscopy in the transduced HEK293 cells. Why the studied SVPs lead to this impaired expression on the cell membrane is unknown, but it has been previously reported that the p.Q141K, p.F208S, and p.S441N variants suffer from ubiquitin-mediated proteasomal degradation (Nakagawa et al., 2008; Furukawa et al., 2009). Ubiquitination is a regulatory process in the cell, which targets damaged or misfolded proteins and signals them to be degraded. Incorrect folding and subsequent ubiquitination could explain the low expression of p.G406R, p.M515R, and p.L525R, as observed previously for p.S441N (Nakagawa et al., 2008). This may also explain the higher relative expression in Sf9 cells, which are grown at 27°C. It has been shown that lower temperature can enhance the cell surface expression of the CFTR (ABCC7) variant p.ΔF508 that suffers from misfolding (Denning et al., 1992; Heda & Marino, 2000). However, the expression of p.Q141K, which is located next to the amino acid corresponding to the amino acid 508 in CFTR, was not significantly improved in HEK293 cells grown at 28°C whereas in Sf9 cells the expression was similar to WT BCRP (Saranko et al., 2013).

Although the studied variants are rare, the results indicate that patients carrying these forms, could be at risk for aberrant effects of BCRP substrate drugs. The p.A528T variant had approximately 50% reduced transport activity, which is similar to the reported effects of the fairly common p.Q141K variant (Kondo et al., 2004; Tamura et al., 2006; Matsuo et al., 2009). Therefore, it would be expected that subjects with this SNP would be at risk for increased exposure to, for example, rosuvastatin and sulphasalazine as the

p.Q141K subjects (Urquhart et al., 2008; Yamasaki et al., 2008; Keskitalo et al., 2009). Furthermore, the eight other variants showed even higher impairment of activity, suggesting that their effects *in vivo* could be more drastic. However, it is not known whether the decrease in activity would be as significant for the heterozygous carriers of the SNVs or what kind of consequences the heterozygous combinations of the SNVs would have. For the p.Q141K variant, for example, significant changes in atorvastatin exposure compared to WT were seen only for the subjects homozygous for p.Q141K polymorphism (Keskitalo et al., 2009). Nevertheless, the heterozygous combination of the fairly common c.421C>A variant allele with one of the more rare variant alleles studied in this thesis could cause significant changes in exposure. Indeed, in a case-report by Gotanda et al. (2015), the combination of p.Q126X polymorphism with p.Q141K led to a more pronounced change in sulphasalazine kinetics than heterozygous p.Q141K alone.

This type of data can help to explain interindividual variability in drug exposure or response, and the genetic information could be utilized for personalized drug dosing to decrease adverse effects as has been proposed for rosuvastatin (DeGorter et al., 2013). Vast developments in genome sequencing techniques over the last 20 years have reduced the time and the cost of sequencing, meaning that the availability of genetic data has increased immensely. The techniques have become so mundane, that genome sequencing is even marketed directly to consumers who are curious about their ancestry and health-related risks (Zettler et al., 2014). Despite these advances, the implementation of pharmacogenetic testing in the clinic is not widespread (Dias et al., 2017). This is explained by the gaps in knowledge regarding the clinical validity and utility of testing, i.e. the difficulty of linking specific genetic changes to outcomes as well as demonstrating the benefit of using this genetic data, for example, for dose adjustments. Once the validity of testing is proved, further data is needed to indicate the cost-effectiveness of the testing. In spite of these hurdles and the need for further research, genetic testing and personalized medicine hold promise in providing each patient with the right drug at the right dose for the optimal therapeutic effect and decreased adverse events.

In considering the potential consequences of altered transporter function, it is imperative to keep in mind that the observed changes are always related to the importance of that particular transporter in relation to other excretion pathways. The theoretical maximal fold increase in drug exposure if, for example BCRP, is completely inhibited, can be calculated as $1/(1-f_e)$, where f_e is the fraction of the drug excreted via BCRP (Zamek-Gliszczynski et al., 2009). The examination of this relationship shows that only when a single transporter accounts for more than 50% of total excretion, can a more than 2-fold increase in exposure be expected when it is inhibited or impaired. For well-tolerated drugs with a wide therapeutic window, a 2-fold change is unlikely to have clinical significance. Therefore, the clinical significance of altered exposure

should always be considered with regard to the therapeutic window of the drug.

Due to the overlap in the substrate specificity of ABC transporters and their co-localisation in tissues, the dysfunction of BCRP, for example, may be compensated for by increased efflux by MRP2 or P-glycoprotein. It should be noted that although the inhibition/impairment of a single transporter may result in moderate changes in exposure, dual inhibition/impairment may have more dramatic effects. Using a hypothetical dual substrate of P-glycoprotein and BCRP, Kusuhara and Sugiyama (2009) demonstrated that the knockout of a single transporter would result in a maximum 3-fold increase in the brain exposure of the substrate, whereas the dual knockout would increase exposure 15-fold.

In addition to the apical ABC transporters, MRP3 and MRP4 are expressed on the sinusoidal membrane of hepatocytes (Hillgren et al., 2013). There is still a need to characterise the role of these basolateral transporters in drug and drug metabolite kinetics (Zamek-Gliszczynski et al., 2014). In some cases, they might compensate for impaired canalicular efflux, but this will result in altered pharmacokinetic consequences, because substrates are pumped back to the systemic circulation and not bile. On the other hand, MRP3 is expressed in the basolateral membranes of enterocytes in the intestine at levels comparable to the apical ABC transporters (Groer et al., 2013; Drozdzik et al., 2014; Nakamura et al., 2016). MRP3 may thus mediate the entry of drug metabolites into the systemic circulation via this route also.

6.1.4 UTILITY OF THE VESICULAR TRANSPORT ASSAY

The results of this thesis suggest that, despite its limitations, the vesicle-based assay can provide interesting information about compound interactions with BCRP and MRP2. The major benefits over cell-based assays are throughput and convenience, because after preparation, vesicles are preserved at -80°C and can used instantly after thawing. Compared to Caco-2 cells, which require at least three weeks to reach confluence (Hubatsch et al., 2007), this is a major advantage. The 96-well format allows higher throughput and requires less reagents than typical cell experiments. Therefore, the VT assay is useful for screening compound libraries when material is limited. Although it can be argued that the inside-out conformation of the vesicles makes this system artificial compared to whole cells, it is an exceptional method for studying transporter interactions without confounding elements such as membrane permeability. In this way, the VT assay is especially suited for generating data for SAR studies. For MRP2, having direct access to the intracellular regions is important, because many of its substrates are intracellularly formed, hydrophilic conjugates. On the other hand, for BCRP, interaction seems to require membrane partitioning in both the cellular and vesicle-based systems, since we identified lipophilicity as an important descriptor in the VT assay as was done before by Matsson et al. (2007) and Zhang et al. (2005a) in cell based

systems. Therefore, the disconnect between vesicle-based and cell-based data regarding the effective concentrations, as described above, may have less impact and cause for concern for BCRP. Still, the transcellular permeability assay with cell monolayers may give more information about the contribution of active transport in relation to passive permeability, but the vesicle system is of value when wanting to study these processes separately.

The baculovirus system used in this thesis to express BCRP and MRP2 is a reasonably fast and straightforward method that is widely used for protein expression in insect cells. The system has also been modified to allow expression in mammalian cell lines. Transient expression using the modified BacMam expression system has been reported to yield P-glycoprotein and BCRP cell surface expression that is comparable to levels in overexpression systems (Shukla et al., 2012). The transient system is convenient for studying variants, because there is no need for antibiotic selection and the maintenance of cell clones carrying the plasmid. Additionally, the combination of data from the Sf9 and HEK293 expression systems proved to be useful for explaining the observed transport defects. Based on the Sf9 data only, the significant changes in expression observed in the HEK293 system would not have been identifiable. On the contrary, based on the HEK293 data solely, too much emphasis may have been given to the expression levels as an explanation for the low activity observed in the VT assay.

Like in any other assay, appropriate controls are extremely important to ensure the high quality of data. When assessing the transport of a new compound, it is necessary to perform studies in both transporter overexpressing and mock infected vesicles to separate background endogenous transport. Although the endogenous transport in Sf9 vesicles is thought to be fairly low (Glavinas et al., 2008), endogenous ATP-dependent transport of some compounds is activated when vesicles are loaded with cholesterol (unpublished observation). However, when a suitable probe substrate with low background transport is fully characterized and validated, it can be used to screen for inhibitors without the interference of other transport (or metabolic) pathways. With fluorescent probes, additional testing for test compound interference should be considered, as was done in publications II and IV, because probe signals may suffer from quenching or the intrinsic fluorescence of the test compounds. Another cause for false positives in inhibition assays can be the ability of test compounds to disrupt the vesicle integrity, as observed with oleic acid in publication I.

6.1.5 TRANSPORTER STUDIES IN DRUG DEVELOPMENT

The efficacy of a drug is partly dictated by its ability to reach its target in the body (Morgan et al., 2012). The thorough understanding of the absorption, distribution, metabolism, and elimination (ADME) properties of candidates is needed for the assessment of the safety and the efficacy of new treatments, as well as decrease the risk of failure in clinical trials. Because transporters can

affect the ADME of drug molecules in the body and thus their exposure, the interactions between transporters and drug candidates may need to be evaluated during drug development. This view is supported by the current drug-drug interaction guidelines of both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (European Medicines Agency, 2012; US Food and Drug Administration, 2012). The increased appreciation of transporter involvement in PK is also evident in drug labelling: Agarwal et al. (2013) reported an increase from 10% in 2003 to 71% in 2011 of drug package insert leaflets containing transporter information.

Drug discovery and development is a process that proceeds according to a typical pipeline, from compound screening to clinical studies, but in the assay sense it is iterative and transporter studies can be useful for understanding PK events in all phases of the process. The timing and complexity of transporter studies should be decided on a project basis considering the indication of the drug (Taub et al., 2013). For example, studies on multidrug resistance transporters, such as BCRP and MRP2, might be prioritised when developing new anti-cancer agents to avoid resistance. In other cases, *in vitro* studies such as those recommended by the EMA and FDA, might be performed in the preclinical stage or during Phase I clinical studies. As the understanding of transporter interactions and primary excretion routes increases along the pipeline, more mechanistic *in vitro* and *in vivo* transporter studies can be designed to focus on these emerged concerns in Phases II and III. Transporter studies may even be the focus of postmarketing evaluation based on observations from clinical use (Tweedie et al., 2013). Vesicle-based assays can be useful during all of these phases, since they can be utilised for screening as well as more detailed interaction and kinetic studies to increase mechanistic understanding.

The current challenges regarding transporter studies are to find the best practices for performing assays and interpreting their results. This is especially true for vesicle-based assays, since transcellular permeability studies in monolayers still appear to be the gold standard. In this thesis, issues related to the VT assay, such as compound interference for detection and vesicle integrity, the inclusion of BSA as well as the aggregation of test compounds were considered. It is acknowledged by the authorities, that the field of drug transporter studies is constantly evolving and practices should not rely solely on written authority guidances, but on current literature (European Medicines Agency, 2012; US Food and Drug Administration, 2012; Tweedie et al., 2013). Reliable i*n vitro* data can help to cut costs and decrease the need for unnecessary (pre)clinical studies (Zhang & Huang, 2013). Further experience will help to refine proposed transporter decision trees and find optimal, possibly laboratory-specific, cut-off criteria that avoid false negative results, which might cause safety concerns, as well as false positives, which can lead to needless *in vivo* studies (Tweedie et al., 2013; Zhang & Huang, 2013).

It is recommended that IC_{50} values by themselves should not be used to evaluate the consequence of DDIs, but only evaluate the need for *in vivo*

studies (Tweedie et al., 2013). However, IC_{50} data, such as that generated with the VT assay, incorporated into PBPK models may provide insight into DDI risk. PBPK modelling may also help to design better *in vivo* studies for capturing DDIs, since the presence of the interaction may be dependent on the dosing scheme applied in the study (Zhao et al., 2011; Tweedie et al., 2013). Although the validation of *in vitro* transporter data is difficult, isolated *in vitro* systems are essential to be able to solve the roles of specific transporters. The complexity of BCRP interactions, for example, is evident by looking at the data presented by Lee et al. (2015), for compounds that might be considered as potential *in vivo* BCRP inhibitors: The involvement of other transporters or metabolic enzymes was reported for all of the eleven compounds. By combining data from different assays in PBPK models, the interplay and the roles of the different ADME processes might be resolved. Using the PBPK approach, *in vitro* data may be used to its full potential to help guide and streamline decision making (Jamei et al., 2009).

PBPK modelling has made advances in the past decades for a multitude of reasons such as the increased characterisation of *in vitro* systems, improvements in scaling paradigms, the development of computing power as well as the availability of commercial PBPK software (Jamei et al., 2009; Rowland et al., 2011). PBPK models of different complexity that incorporate transporter data can be used in many stages of drug development and refined during the process to provide more quantitative data (Rowland et al., 2011; Zhao et al., 2011). One of the advantages of PBPK simulations is that they can be used to study possible outcomes in specialized populations. For example, the data concerning BCRP variants, generated in publication III, could be incorporated into a PBPK model to understand *in vivo* consequences. PBPK modelling could also be used to optimise dosing in these patients. Simulations based on virtual populations can help to capture the variability in patients and predict PK in special populations such as children and the elderly. However, for this goal to be achieved, more information is needed on the maturation and the expressional changes of transporters and metabolic enzymes that occur with age (Zhao et al., 2011). This is another area in which the proteomic techniques will provide further insight.

7 CONCLUSIONS

BCRP is an ABC transporter that has the ability to interact with a wide range of structurally different compounds. The inhibition of BCRP *in vivo* can lead to increased drug exposure and adverse effects, which is why BCRP interaction should be explored. The same principle applies for MRP2, although the clinical significance of MRP2 in drug disposition is more elusive. Interactions with BCRP and MRP2 are difficult to predict based on molecular properties due to the existence of multiple binding sites and the limited knowledge of the protein structure. Because there is an overlap of transporter specificities and a lack of transporter-specific inhibitors, it is important to study the transporters in isolated systems such as the vesicle-based assay used in this thesis.

The publications have shown that although it is difficult to validate kinetic data from the vesicle-based assay, the assay can be beneficial for evaluating the ability of compounds to inhibit efflux transporters. The inclusion of BSA in the assay can increase transport rates, but the albumin effect may be either transporter- or substrate-dependent or both. Based on the results, BCRP is susceptible to inhibition by natural compounds, especially flavonoids, which can be present in our everyday diet. Although the interaction of these compounds with MRP2 was low, certain food colorants could inhibit both transporters. This suggests that the consumption of certain foods could cause inhibition of intestinal BCRP and MRP2 and result in the altered exposure of concomitantly administered substrate drugs. Based on the studies with natural compounds and derivatives, inhibitors of BCRP tend to show higher molecular weight, lipophilicity, aromaticity, and number of rings in their structure than non-inhibitors. Finally, the baculovirus-based expression system, combined with the VT assay proved to be a useful method for identifying significantly decreased transport activity and expression of nine genetic variants of BCRP, pointing to the functional importance of transmembrane residues in BCRP.

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