
Review Article

Role of the Breast Cancer Resistance Protein (BCRP/ABCG2) in Drug Transport—an Update

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Received 20 July 2014; accepted 3 September 2014; published online 19 September 2014

Abstract. The human breast cancer resistance protein (BCRP, gene symbol *ABCG2*) is an ATP-binding cassette (ABC) efflux transporter. It was so named because it was initially cloned from a multidrug-resistant breast cancer cell line where it was found to confer resistance to chemotherapeutic agents such as mitoxantrone and topotecan. Since its discovery in 1998, the substrates of BCRP have been rapidly expanding to include not only therapeutic agents but also physiological substances such as estrone-3-sulfate, 17 β -estradiol 17-(β -D-glucuronide) and uric acid. Likewise, at least hundreds of BCRP inhibitors have been identified. Among normal human tissues, BCRP is highly expressed on the apical membranes of the placental syncytiotrophoblasts, the intestinal epithelium, the liver hepatocytes, the endothelial cells of brain microvessels, and the renal proximal tubular cells, contributing to the absorption, distribution, and elimination of drugs and endogenous compounds as well as tissue protection against xenobiotic exposure. As a result, BCRP has now been recognized by the FDA to be one of the key drug transporters involved in clinically relevant drug disposition. We published a highly-accessed review article on BCRP in 2005, and much progress has been made since then. In this review, we provide an update of current knowledge on basic biochemistry and pharmacological functions of BCRP as well as its relevance to drug resistance and drug disposition.

KEY WORDS: ABCG2; ATP-binding cassette; BCRP; drug transport; transporter.

INTRODUCTION

The human ATP-binding cassette (ABC) proteins belong to a large protein superfamily that now comprises 48 members (<http://nutrigene.4t.com/humanabc.htm>). Many of the human ABC proteins are efflux transporters, and three of them, namely P-glycoprotein (P-gp, gene symbol *ABCB1*), the multidrug resistance protein 1 (MRP1, gene symbol *ABCC1*), and the breast cancer resistance protein (BCRP, gene symbol *ABCG2*), have been implicated to be the major efflux transporters responsible for multidrug resistance in cancer cells.

Human BCRP is encoded by the *ABCG2* gene which is located on chromosome 4q22. Like P-gp and MRP1, BCRP possesses a very broad substrate and inhibitor specificity that is different from, but substantially overlaps with that of P-gp or MRP1. The role of BCRP in drug disposition has now also been appreciated because it highly resembles P-gp in tissue distribution and expression as well as the broad substrate and inhibitor specificity. Much progress has been made on substrates, inhibitors, and physiological and pharmacological roles of BCRP since we published the highly accessed review article on this subject in 2005 in the AAPS Journal (1). In the

present review, we will provide an update of current knowledge on this topic.

BCRP IN HUMAN CANCERS

The majority of the work in this area has been done with leukemia, particularly acute myeloid leukemia (AML). Since this topic has been extensively reviewed elsewhere (2), here we only provide updates of most important findings. Several studies have shown a positive correlation between high levels of BCRP expression and poor clinical outcomes in AML, e.g., a relapsed or refractory disease state, lower response rate, shorter overall survival, and/or no complete remission; however, other studies reported no correlation of BCRP expression with clinical outcomes or no expression of BCRP in AML (see references provided in review by Natarajan *et al.* (2)). This discordance may be attributed, at least in part, to methodologies used to detect BCRP expression. Some studies analyzed BCRP messenger RNA (mRNA) expression, but others detected BCRP protein levels. A general caution is that mRNA levels may not reflect levels of protein expression or activity. Also, BCRP protein expression was generally detected by flow cytometry using BCRP-specific monoclonal antibodies, and BCRP activity was measured using a flow cytometric efflux assay. Such BCRP expression and activity assays may not be sensitive and accurate enough to quantify small differences in patient samples. Careful validation of BCRP expression and activity data is necessary. Another

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striking observation is that, even in some studies that showed a correlation between BCRP expression and clinical outcomes, the anticancer drugs used (e.g., anthracyclines and cytarabine) are generally poor substrates or even not substrates of BCRP. It has therefore been argued that BCRP could be a biomarker for, but not a mechanism of drug resistance in at least some AML patients (2). Lastly, characterization of the role of BCRP in clinical drug resistance of AML is further complicated by frequent co-expression with P-gp and MRP1 (3) which confounds the interpretation of the data.

The development of tyrosine kinase inhibitors (TKIs) such as imatinib, nilotinib, and dasatinib to inhibit the oncogenic tyrosine kinase BCR-ABL has revolutionized the therapy for chronic myeloid leukemia (CML). However, clinical resistance to these TKIs has already emerged. *In vitro* studies have demonstrated that these TKIs are substrates and/or inhibitors of the efflux transporters P-gp and BCRP (4) as well as the uptake transporter OCT1 (5). Therefore, contributions of these transporters to drug resistance in CML patients with clinical outcomes of TKI therapy were evaluated in several clinical studies. A recent study investigated the correlation between mRNA expression of various transporters (P-gp, BCRP, OCT1, and OATP1A2) in peripheral blood leukocytes and clinical outcomes (e.g., major and complete molecular responses as well as drug resistance) in 118 chronic-phase CML patients receiving a standard dose of imatinib mesylate (6). They found that BCRP mRNA expression in non-responders was higher than that in responders before and during imatinib therapy. Furthermore, BCRP was overexpressed in those who did not achieve major molecular response. In the responder group, patients who achieved major molecular response had higher mRNA expression of OCT1. These data suggest that higher BCRP expression may be associated with imatinib resistance, and higher OCT1 expression could be associated with a successful imatinib therapy, in CML patients.

BCRP expression has also been detected in a variety of solid tumors (7). The correlation between BCRP expression and clinical outcomes has primarily been evaluated in breast cancer and non-small cell lung cancer (NSCLC). In breast cancer, only one study reported a correlation between BCRP mRNA expression and response in a subgroup of patients receiving anthracycline-based chemotherapy (5-fluorouracil, adriamycin/epirubicin, and cyclophosphamide), and such a correlation did not exist in the cyclophosphamide, methotrexate, and 5-fluorouracil-treated group of patients (8). However, whether BCRP plays a role in drug resistance in these breast cancer patients is not known because anthracyclines are poor substrates of wild-type BCRP that is detected in cancer patients. A more recent study examined BCRP expression (mRNA and immunohistochemistry) and resistance to 5-fluorouracil (a BCRP substrate) in 140 breast cancer tissues specimens, and found that resistance to 5-fluorouracil was significantly correlated with the levels of BCRP expression; however, no outcome data were reported (9). In NSCLC, one earlier study reported a strong correlation between BCRP expression in tumor samples from 72 untreated stage IIIB or IV NSCLC patients and the response rate to platinum-based chemotherapy, and expression of other transporters including P-gp, MRP1, MRP2, and MRP3

was not significantly associated with response or survival (10). A more recent study showed that high BCRP expression determined by immunohistochemistry in biopsy specimens predicts short survival for advanced NSCLC patients treated with platinum-based chemotherapy (11). Since platinum compounds are not known to be BCRP substrates, the mechanisms by which BCRP expression is associated with clinical outcomes in lung cancer patients are not clear. Most recently, BCRP expression in 67 surgically resected pancreatic ductal adenocarcinoma samples determined using immunohistochemistry was reported to be a significant prognostic factor for early tumor recurrence and poor survival (12). Overall, the role of BCRP in drug resistance in cancers has not been well established. There are currently no clinical studies aimed at overcoming cancer drug resistance by inhibiting BCRP.

BCRP SUBSTRATES

Substrates of BCRP initially were reported to be a wide range of chemotherapeutics such as mitoxantrone, camptothecin derivatives, flavopiridol, and methotrexate (1). Notably, several TKIs such as imatinib, gefitinib, and nilotinib are BCRP substrates (1,13). A variety of photosensitizers including pheophorbide A, protoporphyrin IX, and related compounds are also BCRP substrates, suggesting that BCRP is a possible cause of cellular resistance to photodynamic therapy (14).

Other classes of anticancer drugs including vinblastine, cisplatin, and paclitaxel are not BCRP substrates (13). BCRP substrates are not limited to chemotherapeutics. Drugs that have been shown to be BCRP substrates include, among others, prazosin, glyburide, cimetidine, sulfasalazine, and rosuvastatin (1,13). Nucleoside and nucleotide analogs such as AZT and lamivudine are also BCRP substrates (1).

Fluorescent probes have proven to be useful reagents for analysis of cellular expression and function of ABC transporters. Fluorescent compounds that are commonly used as BCRP probe substrates include BODIPY-prazosin, Hoechst 33342, and pheophorbide A (1). Rhodamine 123 and Lyso-Tracker Green are substrates of the mutants, R482G and R482T, but not substrates of wild-type BCRP (15).

BCRP also transports conjugated organic anions, particularly sulfated and glucuronide conjugates, such as estrone-3-sulfate, dehydroepiandrosterone (DHEAS), and 17 β -estradiol 17-(β -D-glucuronide) (1). Organic conjugates of drugs, xenobiotics, and endogenous substances all could be BCRP substrates (1). In general, sulfated conjugates seem to be better BCRP substrates than glutathione and glucuronide conjugates. In addition, phosphorylated nucleosides and nucleotides, particularly their monophosphates such as AZT 5'-monophosphate, are also BCRP substrates (1).

Other BCRP substrates include chemical toxicants such as the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), phototoxic compounds such as protoporphyrin IX, the lipid phosphatidylserine, flavonoids such as genistein, uric acid, and vitamins (1,13). Collectively, BCRP has a very broad substrate specificity that is substantially overlapping, but distinct from that of P-gp or MRP1 (1,13). Selected substrates of wild-type BCRP that are therapeutic agents are shown in Table I. Although the number of known

Table I. Selected Drugs That Are Substrates of BCRP

Drug	Reference
Anthracenes	
Mitoxantrone	(16,17)
Bisantrone	(17)
Aza-anthracycline (BBR3390)	(18,19)
Camptothecin derivatives	
Topotecan	(17)
SN-38	(20)
Irinotecan	(21,22)
Diflomotecan	(23)
Polyglutamates	
Methotrexate	(24,25)
Methotrexate-Glu ₂	(24,26)
Methotrexate-Glu ₃	(24,26)
Nucleoside analogs	
AZT	(27,28)
AZT 5'-monophosphate	(27,28)
Lamivudine (3TC)	(27,28)
Other drugs	
Prazosin	(17)
Indolocarbazole	(29)
Flavopiridol	(30)
Canertinib (CI1033)	(31)
Imatinib mesylate (STI571)	(32)
Gefitinib (ZD1839)	(33)
Nilotinib	(34)
Glyburide	(35)
Cimetidine	(36)
Sulfasalazine	(37)
Nitrofurantoin	(38)
Rosuvastatin	(39)
Pantoprazole	(40)

Substrates listed in this table are only for wild-type BCRP

BCRP substrates is now over 200, not much work has been done to analyze structure-activity relationship (SAR). The only SAR study was for camptothecin analogs, and the authors found that BCRP preferentially transports the camptothecin analogs with high polarity at carbon positions 10 and 11 over those with low polarity (41). We have recently developed a predictive model for BCRP substrates using a support vector machine (SVM) method based on 263 known BCRP substrates and non-substrates (42). This SVM model has an overall prediction accuracy of ~76%, and thus would be useful for prediction and screening of new BCRP substrates.

BCRP INHIBITORS

A large number of BCRP inhibitors with diverse chemical structures have been identified. Some selected BCRP inhibitors that are therapeutics are shown in Table II. First of all, many P-gp inhibitors are also excellent BCRP inhibitors. The first example of such BCRP inhibitors is GF120918 with an IC₅₀ value of ~50 nM for BCRP (58). We were the first to report that the HIV protease inhibitors ritonavir, saquinavir, and nelfinavir are effective BCRP inhibitors (48). TKIs such as imatinib, nilotinib, and apatinib (4) and inhibitors for other type kinases such as the serine/threonine Polo-like kinase 1 inhibitor BI2536 (59) are also potent dual P-gp/BCRP inhibitors.

Table II. Selected Drugs That Are Inhibitors of BCRP

Drug	IC ₅₀ (nM)	Reference
Tyrosine kinase inhibitors		
Gefitinib	300	(43)
Imatinib mesylate	170	(44)
Erlotinib	ND	(45)
Nilotinib	ND	(4,46)
Lapatinib	ND	(47)
HIV protease inhibitors		
Ritonavir	19,500	(48)
Saquinavir	19,500	(48)
Nelfinavir	12,500	(48)
Lopinavir	7660	(49)
HCV protease inhibitors		
Boceprevir	81,000	(50)
Telaprevir	30,000	(51)
Calcium channel blockers		
Dipyridamole	6400	(52)
Nicardipine	4800	(52)
Nimodipine	13,700	(52)
Nitrendipine	ND	(52)
Antifungal azoles		
Ketoconazole	15,300	(53)
Itraconazole	ND	(53)
Fluconazole	ND	(53)
Immunosuppressants		
Cyclosporin A	4300	(53)
Tacrolimus	3600	(53)
Sirolimus	1900	(53)
Other drugs		
Novobiocin	50–100	(54,55)
Tamoxifen	ND	(56)
Reserpine	ND	(57)
Omeprazole	10,000–50,000	(40)
Pantoprazole	ND	(40)

ND not determined

There are highly selective BCRP inhibitors. The typical example is fumitremorgin C (FTC) secreted from the fungi *Aspergillus fumigatus* with an IC₅₀ value of ~1 μM. FTC did not inhibit P-gp or MRP1 (21). Neurotoxicity of FTC precludes its use in *in vivo* studies. Several FTC analogues including Ko132, Ko134, and Ko143 with a much more potent inhibitory effect (with IC₅₀ values of 100–200 nM) and a high selectivity while displaying low *in vivo* neurotoxicity have been developed (60).

Other notable BCRP inhibitors include, among others, novobiocin (54), tamoxifen, and its derivatives TAG-11 and TAG-139 (56), reserpine (57), the pipercolinate derivatives VX-710 (Bircodar) (61), tryprostatin A (a *A. fumigatus* second metabolite) (62), and dietary flavonoids such as chrysin and biochanin A (63).

Besides the abovementioned inhibitors, derivatives of a variety of known BCRP inhibitors such as resveratrol, tariquidar, chromone, and chalcone were synthesized and tested in the past 5 years. Several of the derivatives have been shown to be potent and highly specific BCRP inhibitors. For example, placement of the quinolone-2-carboxamido group to position 3 of the benzamide moiety of tariquidar resulted in a highly potent and selective BCRP inhibitor with an IC₅₀ value of 60 nM for BCRP, of >29,000 nM for P-gp, and of >20,000 nM for MRP2 (64). Likewise, a chromone derivative was found to be one of the most active, selective, and non-

toxic BCRP inhibitors reported ever (with an IC_{50} value of 110 nM) (65).

The molecular mechanisms of BCRP inhibition have not been fully understood, but could be diverse. For example, some inhibitors such as FTC and Ko143 are considered “general” inhibitors as they inhibit ATPase activity of BCRP. Other inhibitors are BCRP substrates, and as such, can act as competitive inhibitors. In this regard, some inhibitors may interact with BCRP on binding sites of one class of substrates but not others, and hence only inhibit efflux of one particular class of substrates. It is also possible that some inhibitors interact with BCRP on sites other than substrate binding sites and induce conformational changes in the large binding pocket, and thus allosterically affect transport of some substrates. All these can result in substrate-dependent inhibition. For example, Giri *et al.* (66) performed transport inhibition studies and found that nelfinavir effectively inhibited efflux of the nucleoside substrates zidovudine and abacavir, but had no effect on efflux of prazosin and imatinib, suggesting that zidovudine and abacavir possibly interact with BCRP at sites that do not overlap with those for prazosin and imatinib. We therefore hypothesize that BCRP possesses multiple substrate sites (see details in the “**STRUCTURE AND FUNCTION**” section), and as such, inhibition of BCRP can be substrate-dependent. Such complex inhibition mechanisms remain a challenge for predicting and screening BCRP inhibitors in drug discovery. To address this issue, it would be highly valuable to develop ligand-based computational methods for predicting general and substrate-dependent inhibitors of BCRP. A recent study using the Bayesian classification method developed predictive models for BCRP inhibitors with an overall prediction accuracy of ~70% (67), suggesting that development of classification methods for virtual screening of novel BCRP inhibitors is possible. Other classification methods such as the support vector machine (SVM) may prove valuable too. We used the SVM method to predict P-gp and BCRP substrates (42,68). Such classification methods would predict BCRP inhibitors purely based on structural features of known BCRP inhibitors and non-inhibitors, regardless of their mechanisms of inhibition.

A large number of structure-activity relationship (SAR) and quantitative SAR (QSAR) studies have been done for structurally related or diverse BCRP inhibitors to understand the structural features of compounds critical for an inhibitor. This topic has been extensively reviewed elsewhere (69–71). Lipophilicity seems to be a significant determinant for BCRP inhibition for some compounds including flavonoids and FTC analogs (60,72), but not for other compounds such as the tariquidar analogs (73). Planar structure, amine bonded to a carbon of a heterocyclic ring, and hydrogen bonding potential may also be important for certain inhibitors (13,71). At present, SAR and QSAR models based on one set of inhibition data generally cannot be extrapolated to a different set of data. This limitation cannot be resolved until we fully understand the mechanism by which BCRP interacts with substrates and inhibitors.

STRUCTURE AND FUNCTION

To understand the mechanism by which BCRP interacts with substrates and inhibitors, it is essential to first

understand the structure and function of the transporter. BCRP is a polytopic transmembrane (TM) protein with 655 amino acids. It is the second member of the subfamily G of the large human ABC transporter superfamily, and hence also named as ABCG2. Two unique features in BCRP distinguish it from most other ABC transporters including P-gp and MRP1. First, BCRP is a half ABC transporter with only one nucleotide-binding domain (NBD) and one membrane-spanning domain (MSD) (2,74). In comparison, P-gp and MRP1 contain two tandem repeated halves. Second, the NBD in BCRP precedes the MSD, a domain organization that is opposite to that of P-gp and MRP1 (2,74). Such unique structural features imply that BCRP may operate quite differently in transport mechanism compared to P-gp and MRP1. Key knowledge and recent progress regarding structure and function of BCRP are summarized below.

Membrane Topology

An accurate membrane topology is essential for reliable homology modeling and mechanistic understanding of drug transporters. For example, a recent three-dimensional (3D) model of BCRP predicted based on evolutionary sequence information cannot be correct because the topology of BCRP (with 7 TM α -helices) that these authors used is incorrect (75). We have recently determined the topology of BCRP using HA epitope insertion and immunofluorescence (76) (Fig. 1). This topology suggests that BCRP contains 6 TM α -helices; however, the computer-predicted TM2 and TM5 are shifted to the extracellular and intracellular loops, respectively, in the experimental topology (76). Such a significant shift of TM helices would result in a drastic change in helical packing of BCRP in its 3D structure. It is worth noting that the only known *N*-glycosylation site at position 596 (Asn⁵⁹⁶) (77) in the experimental topology is located in the extracellular loop connecting TM5 and TM6. According to the so-called “12+14 rule”; that is, the acceptor site Asn in extracellular loops must be spaced at least 12 residues from the proximal and 14 residues from the distal TM segments to be efficiently glycosylated (78), Asn⁵⁹⁶ in the experimental topology can be glycosylated, which is consistent with experimental findings. While this is a novel topology, people continue debating on whether this topology is accurate due to the concern that insertion of HA tags may distort helical packing and hence overall structure of BCRP. Therefore, this new experimental topology requires further validation using other appropriate biochemical and biophysical methods.

Homodimerization or Homooligomerization

Using sucrose density gradient sedimentation and non-denaturing gel electrophoresis, Xu *et al.* (79) demonstrated that detergent-solubilized BCRP may form homotetramers. Likewise, the electron microscopy (EM) analysis revealed that BCRP existed as a tetramer of dimers in detergent solutions (80) or a tetramer in 2D crystals (81). Recently, evidence that BCRP can form homodimers or homooligomers in intact cells has been obtained by using techniques such as fluorescence resonance energy transfer (FRET) (82) or bimolecular fluorescence complementation (83). The mechanism by which BCRP forms homodimers or homooligomers is not clear. The Cys residue at position 603 seems

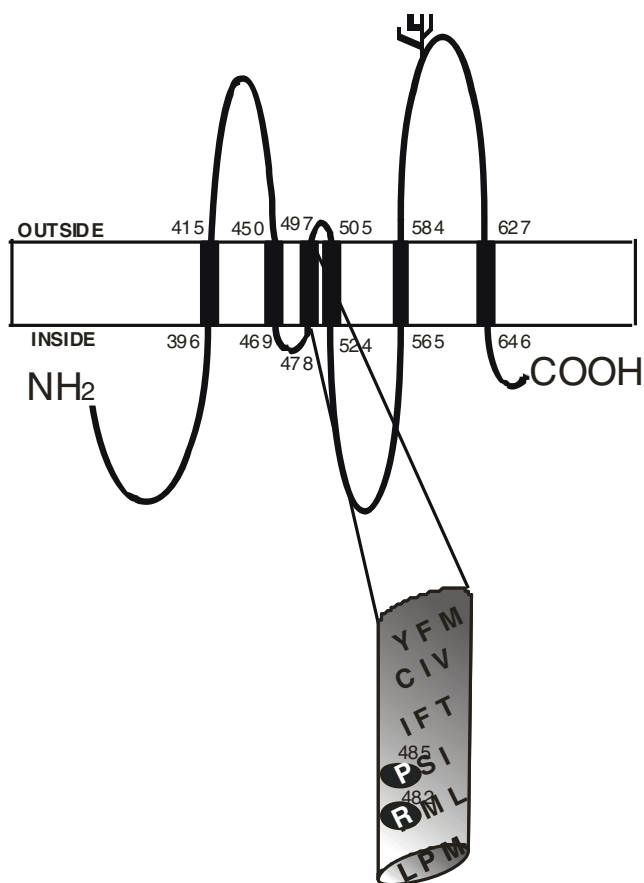


Fig. 1. A membrane topology model of BCRP. BCRP contains one NBD and one MSD with six TM α -helices. The boundary of TM α -helices is approximate and based on our experimentally determined membrane topology. The N-glycosylation site (Asn⁵⁹⁶) is indicated in the extracellular loop connecting TM5 and TM6. The putative TM3 is shown in an expanded view. Two residues in TM3 important for substrate selectivity (Arg⁴⁸² and Pro⁴⁸⁵) are indicated by shaded circles in the expanded view of TM3

to be involved in intermolecular disulfide bond formation (84); however, substitutions of Cys⁶⁰³ had no effects on either dimer/oligomer formation or activity of BCRP in intact cells (82,83). It is possible that, in addition to intermolecular disulfide bonds formed by Cys⁶⁰³, intermolecular disulfide bonds formed by other Cys residues and/or non-covalent protein-protein interactions could also be crucial for BCRP dimer/oligomer formation. Indeed, Mitomo *et al.* (85) found that BCRP fully retained transport activity in the presence of 2-mercaptoethanol at 10 mM, a concentration that is sufficient to break down disulfide bonds between BCRP monomers. These data suggest that intermolecular disulfide bonds alone are not essential for BCRP dimer/oligomer formation and function. One possibility is that BCRP dimers are assembled in the membrane also through protein-protein interactions, and therefore can still be fully maintained even if disulfide bond formation is diminished.

Structure Determination and Homology Modeling

The first structural study by McDevitt *et al.* (80) illustrated that BCRP protein particles in a detergent solution formed a higher order oligomeric complex that was organized as a tetramer of BCRP dimers. A 3D structure at 18-Å

resolution was constructed, which allowed visualization of an overall shape and an oligomeric state for BCRP. More recently, Rosenberg *et al.* (81) reported the first projection structures of BCRP determined by cryo-EM of well-diffracting 2D crystals. The 2D crystals showed a $p121_b$ symmetry and the projection structures were determined to 5-Å resolution. At this resolution, ring-shaped high-density features in the projection maps were visualized, probably representing TM α -helices. There were four BCRP monomers (two BCRP dimers) in one unit cell of the 2D crystals, indicating the existence of an oligomeric complex of BCRP. This study also illustrated a significant conformational change upon mitoxantrone binding; that is, BCRP had a more closed and symmetric configuration in the presence of mitoxantrone than that with no mitoxantrone bound. A 3D structure of BCRP at a medium resolution based on these 2D crystals has now been constructed (Rosenberg *et al.*, manuscript submitted).

Without high-resolution 3D structures, homology models play an important role in interpretation of experimental data and in providing guidance for future studies. Several earlier studies developed homology models of BCRP based on computer-predicted topology which is now known to be different from the experimentally determined topology (76). Here, we briefly discuss the homology models of BCRP developed in our laboratory based on the experimental topology. We first refined sequence alignment for TM segments between BCRP and the templates (MsbA, the first and second halves of mouse P-gp and Sav1866) by comparing the experimentally determined TM segments of BCRP with those of the templates observed in crystal structures. Next, the templates were edited to reflect the same domain organization in BCRP by “cutting” the linker regions between the MSD and the NBD of the templates. Three homology models of BCRP representing different conformational states have been generated. The first model based on MsbA (PDB code 3B5W) represents the substrate-unbound nucleotide-free inward-facing open apo conformation. The second model based on mouse P-gp (PDB code 3G60) represents the substrate-bound nucleotide-free inward-facing closed apo conformation (Fig. 2). The third model based on Sav1866 (PDB code 2HYD) represents the nucleotide-bound outward-facing conformation. The inward-facing states display a wide separation of the two NBDs that is open to the intracellular side of the plasma membrane (Fig. 2). The distance between the two NBDs in the open apo state is greater than that in the closed apo state. On the other hand, the extracellular side of the inward-facing conformations is closed by joining together of two large extracellular loops connecting TMs 1 and 2 and TMs 5 and 6. In contrast, the outward-facing conformation is exactly opposite with the extracellular side open and the intracellular side closed. These atomic homology models have already been published (74,81).

The homology models are consistent with biochemical data published to date. First, the intracellular entry of the inward-facing models is spacious enough to allow access of a bulk of BCRP substrates from the inner lipid leaflet of the plasma membrane or cytoplasm. Molecular docking of several BCRP substrates to the closed apo model indeed suggests the existence of multiple binding sites in the large central pocket primarily

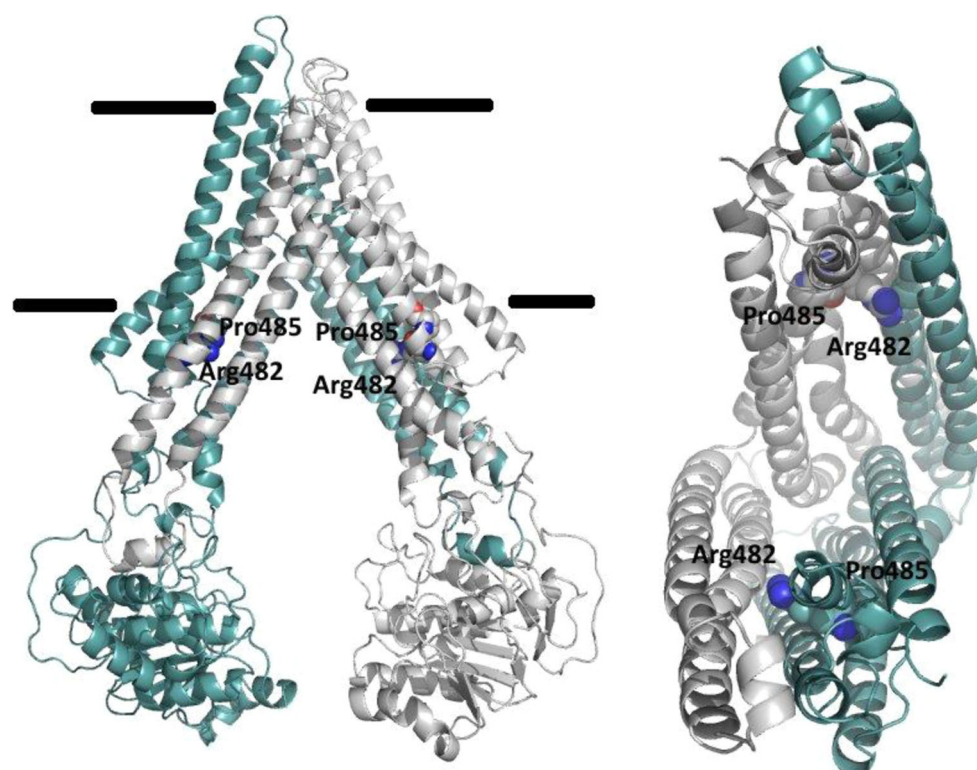


Fig. 2. A homology model of BCRP based on the mouse P-gp structure representing a nucleotide-free inward-facing “closed apo” conformation. Two BCRP monomers in a dimer are shown in different colors. The internal cavity formed by TMs is open to the intracellular space. Arg⁴⁸² and Pro⁴⁸⁵ in TM3 are shown in blue and red colors. The right panel only shows TM helices

formed by TM α -helices (86). Second, Arg⁴⁸² has been extensively studied by site-directed mutagenesis and found to be crucial for substrate specificity and transport activity (see details in Mutagenesis analysis below). In the models, Arg⁴⁸² in TM3 is located in the central binding pocket at a position near the cytosolic membrane interface (Fig. 2). Docking calculations indicate that Arg⁴⁸² directly interacts with mitoxantrone and Hoechst33342, but not with prazosin and SN-38 (86). This is consistent with previous findings that resistance to mitoxantrone was increased, but resistance to SN-38 or efflux of prazosin was not affected, by mutations of Arg⁴⁸² (16,87). This also is in agreement with the studies showing that prazosin binds to a site that does not fully overlap with that for mitoxantrone or Hoechst33342 (88) and the binding of a prazosin derivative to BCRP was relatively unaffected by mutations of Arg⁴⁸² (89). Molecular docking also has provided explanations for the unique role of Arg⁴⁸² in determining transport selectivity for methotrexate and derivatives. Molecular docking using the closed apo model suggests that methotrexate can directly associate with the positively charged Arg⁴⁸² by direct salt-bridge interactions via their negatively charged carboxylate or sulfate groups (90). However, such electrostatic interactions do not occur for mitoxantrone, prazosin, or Hoechst33342, explaining why mutations of Arg⁴⁸² do not significantly affect efflux of the three substrates (16), but abolish transport of methotrexate (24). In summary, the homology models could be used to interpret biochemical data. More studies are needed to further refine and validate these models, given the slow pace in determining high-resolution 3D structures of BCRP.

Mutagenesis Analysis

Spontaneous mutations of Arg⁴⁸² were initially discovered in BCRP from drug-selected drug-resistant cancer cell lines (91). This residue was found to be a critical determinant of substrate selectivity and immediately became the subject of extensive mutagenesis studies. Wild-type BCRP with Arg⁴⁸² does not transport daunorubicin, rhodamine 123, and Lyso-Tracker Green; however, these compounds are excellent substrates of the BCRP mutants R482T and R482G (16,91). Methotrexate is a substrate of wild-type BCRP only (24). Mitoxantrone, BODIPY-prazosin, and Hoechst 33342 are substrates of both wild-type BCRP and the two mutants (16). Arg⁴⁸² is predicted to be located in TM3 α -helix near the cytoplasmic interface (Fig. 1), and is likely part of the large drug binding pocket. As discussed above, Arg⁴⁸², a positively charged residue, is possibly involved in salt-bridge interactions with some substrates (90). It should be emphasized that mutations of Arg⁴⁸² have never been identified in human subjects or in DNA samples from cancer patients.

The realization that Arg⁴⁸² is located in a TM α -helix triggered mutagenesis studies on other residues in TM α -helices (Fig. 2). We identified a polar residue, Thr⁴⁰² in TM1, which is important for overall transport activity. Ala or Arg substitution of Thr⁴⁰² caused a significant reduction by 50–90% in efflux of mitoxantrone, BODIPY-prazosin, and Hoechst33342 as well as its ability to confer resistance to mitoxantrone and SN-38 (92). According to the closed apo model, Thr⁴⁰² is not directly involved in substrate binding, but

participates in interhelical interactions that are functionally important. Consistent with this observation, Thr⁴⁰² was proposed to be near or part of the GXXXG motif that may play an important role in dimerization or helical interactions (93). Pro residues in TM α -helices often form flexible hinges and can play a key role in dynamic conformational changes. We found that Ala substitution of Pro⁴⁸⁵ in TM3 significantly reduced efflux of BODIPY-prazosin by 70%, but had no effect on efflux of mitoxantrone and Hoechst 33342 (94). Homology modeling suggests that Pro⁴⁸⁵ can introduce a flexible hinge in TM3, and as such, making the drug binding pocket more structurally dynamic. Mutation of Pro⁴⁸⁵ would eliminate conformational flexibility of the drug binding pocket, thus affecting binding of some drugs, but not others. Thus, structural flexibility introduced by Pro⁴⁸⁵ in TM3 may contribute to substrate specificity. Notably, both Arg⁴⁸² and Pro⁴⁸⁵ are located within TM3 (Figs. 1 and 2). Based on these findings, we hypothesize that TM3 is part of the translocation pathway that encompasses multiple substrate binding sites, and as such, TM3 is critical for conformational dynamics of the binding pocket and plays a crucial role in determining substrate specificity.

Other residues that are functionally important include Glu⁴⁴⁶ in the extracellular loop connecting TMs 1 and 2 (87), Leu⁵⁵⁴ in the intracellular loop connecting TMs 4 and 5 (95), and Lys⁸⁶ and Glu²¹¹ in the NBD (96–98). Mutations of these residues resulted in either significantly impaired or no transport activity. Mutations of Lys⁸⁶ and Glu²¹¹ have been shown to cause a complete loss of ATPase activity (96,98). However, another study showed that the activity loss caused by mutations of Lys⁸⁶ was possibly due to altered subcellular localization and cell surface targeting of BCRP (97). More information about mutations and their effects on function and expression of BCRP can be found in an open access database (<http://abcmutations.hegelab.org/>). More mutagenesis studies should be carried out, particularly in the MSD, to expand the scope of our understanding of critical residues important for substrate selectivity and overall transport activity.

Multiple Substrate Binding Sites

It is generally believed that ABC transporters possess multiple drug binding sites in a large pocket formed by TM α -helices. Although high-resolution 3D structures of BCRP have not been available, several lines of biochemical evidence support the existence of multiple binding sites. First of all, as we discussed earlier, mutations of some residues such as Arg⁴⁸² and Pro⁴⁸⁵ that are likely located in the drug binding pocket affect efflux of some substrates, but not others. Whether these residues directly interact with substrates or play an indirect but critical role in maintaining the architecture or inducing conformational changes of binding sites requires further investigation. Additional evidence comes from direct binding or transport studies. For example, photolabeling of BCRP with the substrate [¹²⁵I]iodoaryl azidoprazosin was inhibited by some compounds, but not others (99). Clark *et al.* conducted direct binding kinetic studies and showed that there are possibly two distinct binding sites in BCRP, one for mitoxantrone and Hoechst33342 and another for prazosin (88). Giri *et al.* performed transport inhibition studies and

found that the nucleoside analog substrates zidovudine and abacavir seem to interact with BCRP at sites that do not overlap with those for prazosin or imatinib (66). Lastly, our homology models have been used to interpret mutagenesis and transport data of our own (92,94) and others studies (90). The models suggest a large internal cavity formed by two bundles of six TMs that is spacious enough to accommodate multiple drugs. The exact locations of binding sites in BCRP are still not known, and this awaits the determination of high-resolution 3D structures of the transporter complexed with one or more substrates or inhibitors.

SINGLE NUCLEOTIDE POLYMORPHISMS

A large number (>80) of single nucleotide polymorphisms (SNPs) of the *ABCG2* gene have been identified in DNA samples of ethnically diverse origins. Of these SNPs, 34G>A (V12M) and 421C>A (Q141K) occur most frequently in East Asians (~30–60%) and with relatively low allele frequencies in Caucasians and African-American populations (~5–10%). All other SNPs generally have allele frequencies of ~1% or less. Notably, two SNPs, 376C>T (Q126stop) and 1000G>T (E334stop) result in substitutions of a stop code in the *ABCG2* gene. *In vitro* expression and functional studies generally support the conclusion that the Q141K variant resulting from the 421C>A SNP has reduced cell surface expression in transfected cells and therefore cells expressing Q141K display lower efflux activities compared to those expressing wild-type BCRP (100). V12M resulting from the 34G>A SNP and other variants (e.g., I206L, F208S, N590Y, and D620N) display expression levels and drug resistance profiles comparable to wild-type BCRP (100,101). The SNPs 114T>C, 369C>T, 474C>T, 564A>G, 1098G>A, and 1425A>G do not cause amino acid changes; but whether they can alter BCRP expression and activity by affecting protein translation is not known. SNPs in the promoter region such as -15622C>T and -1379A>G have also been reported and may affect transcriptional regulation of the *ABCG2* gene (102).

TISSUE LOCALIZATION AND ROLE IN DRUG DISPOSITION

Among normal human tissues, the highest expression of BCRP was observed on the apical membrane of the placental syncytiotrophoblasts (103). In addition, BCRP is prominently expressed on the apical membrane of the epithelium in the small intestine and colon and on the canalicular membrane of hepatocytes (103). BCRP is also expressed on the apical membrane of human kidney proximal tubular cells (104); however, its level of expression in human kidney is relatively low compared to that in the liver and small intestine. BCRP is also on the apical (luminal) membrane of the microvessel endothelial cells in human brain (105) and the retinal capillary endothelial cells (106) as well as in the blood-testis (107) and blood-spinal cord (108) barriers. The tissue localization of Bcrp1, the rodent homolog of human BCRP in mice and rats, is similar to that in humans.

This strategic localization and substantial expression of BCRP in human and rodent tissues implies that BCRP can play a crucial role in limiting absorption (in the small

intestine), mediating distribution (e.g., in the blood–brain and blood–placental barriers), and facilitating biliary and renal elimination (in the liver and kidney) of drugs or xenobiotics that are BCRP substrates. The role of BCRP in drug disposition was first appreciated in studies using Bcrp1-knockout mice and will be briefly discussed below.

In the liver and kidney, BCRP facilitates biliary and renal elimination of drugs and xenobiotics. Breedveld *et al.* (40) demonstrated that the area under the concentration–time curve (AUC) of intravenously administered methotrexate in wild-type mice was increased ~twofold by co-administration of intravenous (IV) pantoprazole (a Bcrp1 inhibitor); however, the AUC of IV methotrexate in Bcrp1-knockout mice was not affected. IV pantoprazole also decreased the systemic clearance of IV methotrexate in wild-type mice to the same level as in Bcrp1-knockout mice, but had no effect on methotrexate clearance in Bcrp1-knockout mice. Further analysis confirmed that pantoprazole reduced methotrexate clearance by predominantly inhibiting biliary excretion of methotrexate mediated by Bcrp1 (40). Many similar studies can be found in an excellent review by Vlaming *et al.* (109).

In the blood–brain barrier, numerous studies have now confirmed that BCRP significantly limits brain penetration of drugs or xenobiotics, which is consistent with its high levels of expression on the luminal side of brain endothelial cells. For example, Agarwal *et al.* (110) showed that the steady-state brain-to-plasma concentration ratio of sorafenib in Bcrp1-knockout mice was increased ~fourfold compared to that in wild-type mice. Since P-gp and Bcrp1 are co-localized to the same site in the blood–brain barrier, a synergistic effect between the two transporters was observed in many studies, that is, the brain exposure (brain-to-plasma AUC or concentration ratio) of a P-gp/BCRP dual substrate in P-gp/Bcrp1 double-knockout mice is much greater than additive of brain exposure to the drug in P-gp and Bcrp1 single-knockout mice (111). This synergistic effect does not seem due to direct biological or physical interactions between the two transporters, and can be explained by pharmacokinetic theory (112). That is, such apparent “synergy” would be expected if P-gp and BCRP are the principal pathways of clearance of the drug from the brain. This situation is analogous to fraction of a drug metabolized by two enzymes. Theoretically, complete inhibition of both enzymes could lead to an infinite increase in the plasma concentration ratio (in the presence of inhibitor *vs.* in the absence of inhibitor) of the drug.

In the blood–placental barrier, BCRP expels drugs or xenobiotics from the fetal compartment back to the maternal circulation, thus limiting their fetal exposure. We found that fetal exposure (fetal-to-maternal plasma AUC ratios) to nitrofurantoin and glyburide in Bcrp1-knockout mice was increased ~five- and twofold, respectively, compared to those in wild-type mice (35,113). The role of BCRP in determining fetal exposure to drugs and xenobiotics can also be studied using other methods such as human placenta perfusion, and this topic has been extensively reviewed elsewhere (114).

BCRP is also expressed in the mammary gland and is strongly induced in the lactating breast of mice, cows, and humans (115). In the mammary gland, Bcrp1 has been shown to actively transport drugs (e.g., topotecan and cimetidine), xenobiotics (e.g., 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine or PhIP), and vitamins (e.g., riboflavin) into breast

milk (115,116). Although pharmacological or toxicological implications of BCRP-mediated milk secretion of drugs and xenobiotics are not known, caution should be taken when lactating women take medications that are BCRP substrates so that undesired side-effects or toxicity for their breast-feeding babies may be avoided.

The role of BCRP in drug disposition in humans has been demonstrated in clinical studies. One early study revealed that co-administration of oral GF120918 significantly enhanced oral bioavailability of topotecan in cancer patients from 40 to 97% (117), suggesting that inhibition of BCRP in the small intestine increased the absorption of topotecan. Subsequent studies investigated the correlation between *ABCG2* SNPs and pharmacokinetics (PK) of drugs that are BCRP substrates. The most extensively studied drugs are statins and anti-cancer drugs, and the most extensively analyzed SNP is *ABCG2* 421C>A. Most of the clinical studies published thus far investigating the impact of *ABCG2* 421C>A SNP on drug PK are summarized in Table III. Overall, the impact of the *ABCG2* 421C>A SNP on drug PK seems to depend on the drug tested, the genotype that the subjects carry (421CA heterozygous or 421AA homozygous), and the route of drug administration. As shown in Table III, even for well-established BCRP substrates such as topotecan, irinotecan, and nitrofurantoin, clinical studies have not revealed statistically significant effects of the *ABCG2* 421C>A SNP on PK of these drugs. For drugs, particularly the statins such as rosuvastatin, fluvastatin, simvastatin, and atorvastatin on which the *ABCG2* 421C>A SNP had an effect, it is generally the 421AA homozygous genotype, but not the 421CA heterozygous genotype, that was associated with significantly higher plasma AUC or C_{max} of orally administered drugs. Most of the clinical studies used oral administration with only a few that utilized intravenous (IV) administration. We notice that the route of drug administration could also have an effect. For example, the 421CA genotype was shown to be associated with significantly increased plasma AUC or C_{max} only after single IV administration of diflomotecan (23).

We see contradictory results from different studies for the same drugs. The typical example of such drugs is sulfasalazine. Sulfasalazine is a BCRP substrate and was suggested to be used as an *in vivo* BCRP probe based on the finding that the AUC of oral sulfasalazine in subjects carrying the 421CA genotype was 2.5-times greater than that in subjects carrying the 421CC genotype (121). The observation was initially confirmed by a second study with Japanese subjects showing that the AUC of oral sulfasalazine in subjects carrying the 421CA or 421AA genotype was 1.9- or 3.5-times greater, respectively, than that in subjects carrying the 421CC genotype (120). However, these findings were not reproduced in a third study with Chinese subjects (146). Reasons for such contradictory data are not known, but might be related to the relatively small sample size of these studies (usually <20 subjects for a specific genotype). On the other hand, three different studies have consistently shown that pitavastatin PK is not associated with either the 421CA or the 421AA genotype (142–144). Changes in rosuvastatin PK have been consistently shown to be associated with the 421AA, but not the 421CA genotype (125–128). The other common *ABCG2* SNP, 34G>A, does not significantly affect drug PK.

Table III. Clinical Studies Investigating Impact of the *ABCG2* 421C>A SNP on Pharmacokinetics of Drugs Compared to Subjects Carrying the Wild-Type *ABCB2* Gene

Tested drug	Drug dosing	# of subject ^a	421C>A SNP ^b	Results		Impact ^d	Ref
				AUC Δ^c (%)	C _{max} or C Δ^c (%)		
Diffamotecan	IV single dosing	5 Caucasian	CA	199.35	168.24	Yes	(23)
Teriflunomide	Oral single dosing	8	CA	52.79	28.86	Yes	(118)
Teriflunomide	Oral single dosing	9	CA and AA	82.72	29.85	Yes	(118)
9-aminocamptothecin	Oral multiple dosing	2	CA	258.04		Yes	(119)
Sulfasalazine	Oral single dosing	16 Japanese	CA	92.98	71.43	Yes	(120)
Sulfasalazine	Oral single dosing	9 Japanese	AA	246.2	164.29	Yes	(120)
Sulfasalazine	Oral single dosing	5	CA	137.46	70.79	Yes	(121)
Gefitinib	Oral multiple dosing	7	CA	17.58		Yes	(122)
Sunitinib	Oral retrospective	8	CA	78.57		Yes	(123)
Sunitinib	Oral retrospective	1	AA	221.43		Yes	(123)
Imatinib	Oral multiple dosing	25 Japanese	CA and AA			Yes	(124)
Sunitinib	Oral retrospective	1	AA	221.43		Yes	(123)
Rosuvastatin	Oral single dosing	7 Chinese	CA and AA	78.22	94.12	Yes	(125)
Rosuvastatin	Oral single dosing	6 Chinese	AA	57.96	41.04	Yes	(126)
Rosuvastatin	Oral multiple dosing	39 Chinese	AA		119.5	Yes	(127)
Rosuvastatin	Oral single dosing	4 Caucasian	AA	144.3	131.3	Yes	(128)
Fluvastatin	Oral single dosing	5 Caucasian	AA	72.55	87.4	Yes	(129)
Simvastatin	Oral single dosing	5 Caucasian	AA	110.59	62.5	Yes	(129)
Atorvastatin	Oral single dosing	4 Caucasian	AA	71.54	46.36	Yes	(128)
Irinotecan	IV multiple dosing	23 Asian	CA	28		No	(130)
Irinotecan	IV single dosing	16 Caucasian	CA	-3.37		No	(131)
Irinotecan	IV multiple dosing	42	CA	-0.85		No	(132)
Irinotecan	IV multiple dosing	4	AA	-19.17		No	(132)
Diffamotecan	Oral multiple dosing	5 Caucasian	CA	15.1	2.95	No	(23)
Topotecan	Oral single dosing	2 Caucasian	CA	30.26		No	(133)
Topotecan	IV multiple dosing	2 Caucasian	CA	-0.97		No	(133)
Erlotinib	Oral multiple dosing	13	CA	19.46	12.45	No	(134)
Telatinib	Oral multiple dosing	6 Caucasian	CA	17.09		No	(135)
Docetaxel	IV multiple dosing	21 Asian	CA	10.89	3.81	No	(136)
Docetaxel	IV multiple dosing	5 Asian	AA	5.94	-20.95	No	(136)
Imatinib	Oral multiple dosing	13 Japanese	CA			No	(137)
Imatinib	Oral multiple dosing	32 Korean	CA			No	(138)
Imatinib	Oral multiple dosing	8 Korean	AA			No	(138)
Danuserib	IV single dosing	11	CA			No	(139)
Fluvastatin	Oral single dosing	4 Caucasian	CA	-12.41	-32.28	No	(129)
Fluvastatin	Oral single dosing	7 Chinese	CA	9.02	16.34	No	(140)
Pravastatin	Oral single dosing	4 Caucasian	CA	26.09	53.02	No	(129)
Pravastatin	Oral single dosing	5 Caucasian	AA	-12.32	0.35	No	(129)
Simvastatin	Oral single dosing	4 Caucasian	CA	60.17	30.0	No	(129)
Simvastatin	Oral single dosing	9 Chinese	CA and AA	7.07	3.42	No	(141)
Atorvastatin	Oral single dosing	12 Caucasian	CA	20.38	2.55	No	(128)
Rosuvastatin	Oral single dosing	12 Caucasian	CA	22.31	11.19	No	(128)
Rosuvastatin	Oral single dosing	15 Chinese	CA	7.89	1.27	No	(126)
Rosuvastatin	Oral multiple dosing	108 Chinese	CA			No	(127)
Pitavastatin	Oral single dosing	7 Japanese	CA	19.24	33.65	No	(142)
Pitavastatin	Oral single dosing	21 Chinese	CA	54.02	34.38	No	(143)
Pitavastatin	Oral single dosing	16 Korean	CA	-1.84	-1.73	No	(144)
Pitavastatin	Oral single dosing	3 Japanese	AA	-3.58	34.94	No	(142)
Pitavastatin	Oral retrospective	5 Korean	AA	9.28	-39.19	No	(144)
Nitrofurantoin	Oral single dosing	12 Chinese	CA	9.5	9.83	No	(145)
Nitrofurantoin	Oral single dosing	12 Chinese	AA	4.98	10.06	No	(145)
Sulfasalazine	Oral single dosing	12 Chinese	CA	-47.66	-57.61	No	(146)
Sulfasalazine	Oral single dosing	12 Chinese	AA	95.33	71.07	No	(146)
Telmisartan	Oral single dosing	24	CA	29.37		No	(147)
Telmisartan	Oral single dosing	15 Chinese	CA	2.18	15.4	No	(148)
Telmisartan	Oral single dosing	2	AA	8.82		No	(147)
Telmisartan	Oral single dosing	3 Chinese	AA	36.58	-27.72	No	(148)
Olmesartan	Oral single dosing	25 Korean	CA	3.49	7.02	No	(149)
Olmesartan	Oral single dosing	3 Korean	AA	13.57	21.49	No	(149)
Tacrolimus	Oral multiple dosing	17	CA and AA		37.12	No	(150)
Lamivudine	Oral single dosing	6 Korean	AA	5.45	1.36	No	(151)

^a These are the numbers of subjects carrying the indicated *ABCG2* SNP. The numbers of subjects carrying wild-type *ABCG2* gene vary from studies to studies, but are usually larger than the numbers of subjects carrying the indicated *ABCG2* SNP

^b "CA" or "AA" means that subjects carried the 421C/A heterozygous genotype or the 421A/A homozygous genotype, respectively. "CA and AA" means that subjects were a group of individuals combining both the heterozygous and homozygous genotypes

^c Δ (%) indicates percentage changes in AUC or C_{max}. The AUC data include percentage changes in AUC_(0-t) or AUC_(0-∞)

^d Impact (Yes) indicates that there are statistically significant changes in at least one of the PK parameters: plasma AUC, CL, plasma C_{max}, and plasma C_{min}. Impact (No) indicates that there are no statistically significant changes in any of the PK parameters. For some drugs, PK changes in AUC or C_{max} were not reported and therefore are not shown in this table

Due to its importance in drug disposition, BCRP has been recognized by the FDA to be one of the key drug transporters involved in clinically relevant drug disposition and drug-drug interactions (DDIs) (152). As discussed above, the co-administration of oral GF120918 and topotecan increased the oral bioavailability of topotecan (117). More recently, several BCRP substrate drugs were shown to significantly interact with other drugs that are BCRP inhibitors in humans, implying that BCRP may play a crucial role in such DDIs. These DDIs include the interactions between atorvastatin and tipranavir/ritonavir (153), rosuvastatin and tipranavir/ritonavir (153), rosuvastatin and atazanavir/ritonavir (154), rosuvastatin and lopinavir/ritonavir (155), rosuvastatin and cyclosporine (156), rosuvastatin and eltrombopag (157,158), rosuvastatin and GSK1292263 (159), simvastatin and GSK1292263 (159), sulfasalazine and curcumin (160), and methotrexate and the proton pump inhibitor omeprazole, lansoprazole, or pantoprazole (161,162). Such DDIs all resulted in at least 20% significant increase in plasma AUC, C_{max} , and/or clearance of the BCRP substrate drugs particularly rosuvastatin. Changes in AUC and/or C_{max} of these DDIs are summarized in Table IV. It should be pointed out that the above DDIs could also be caused by inhibiting OATP-mediated uptake of drugs into hepatocytes, thus increasing plasma AUC and/or C_{max} (see below). We also note that, except for methotrexate, the above DDIs were observed almost exclusively with oral administration of BCRP substrates and inhibitors. It is not clear whether the observed DDIs are caused by inhibiting BCRP in the small intestine or in the liver or both. For the DDIs involving statins, inhibiting BCRP in the small intestine leading to greater oral absorption seems to be more likely. This is because, inhibiting BCRP in the liver may increase accumulation in hepatocytes, but does not necessarily affect plasma AUC or C_{max} for the statins which have relatively low membrane permeability and require transporters to cross the cell membrane of hepatocytes. This concept has been illustrated by PBPK modeling using scaling factors obtained by comparing *in vitro* and *in vivo* parameters of pravastatin in rats for hepatic uptake and canalicular efflux (163), and is consistent with the fact that almost all of the clinical studies that show impact of the *ABCG2* 421C>A SNP on drug PK were carried out with oral drug administration (Table III).

More studies are needed to elucidate the exact mechanisms of the DDIs.

At present, there is not a reliable *in vivo* clinical probe substrate for BCRP. Since many of the statins examined in the DDI studies are also OATP substrates and the inhibitors used can also inhibit OATPs, the exact contribution of BCRP to the DDIs cannot be readily quantified. Because rosuvastatin is the most extensively analyzed drug in numerous clinical studies which consistently pinpoint a role of BCRP in disposition of the drug, it may be an appropriate clinical probe for BCRP. Rosuvastatin is minimally metabolized and is not a P-gp substrate (164). However, since it is also a substrate of OATP1B1, OATP1B3, and OATP2B1 (39), it could be suitable for assessing BCRP activity in organs with low OATP expression such as the small intestine and the blood-brain barrier. Indeed, the strong interaction between oral eltrombopag and rosuvastatin in human subjects has been suggested to be caused by complete inhibition of BCRP in the small intestine by eltrombopag (158). Sulfasalazine has previously been suggested to be used as an *in vivo* probe for assessing the role of BCRP in oral drug bioavailability. This requires further validation because of the report of contradictory results. Another issue for evaluation and reliable prediction of *in vivo* BCRP activity is how to accurately determine the absolute amount of BCRP expressed in tissues and cells and the intracellular drug concentrations directly exposed to BCRP in tissues and cells. For the former, absolute quantification of BCRP by liquid chromatography mass spectrometry may provide a solution (165). For the latter, there has not been an ideal solution yet.

Similarly, although a large number of BCRP inhibitors have been discovered, a highly selective and potent BCRP inhibitor suitable for clinical studies that specifically target BCRP has yet to be established. Most of the drugs that have been shown to interact with BCRP substrate drugs in clinical studies are also inhibitors of other transporters. GF120918 (a dual P-gp/BCRP inhibitor) was used in one clinical study to evaluate the role of BCRP in limiting oral absorption of topotecan (117). This is because topotecan is possibly a good BCRP substrate, but a relatively poor P-gp substrate. However, since GF120918 is not an approved drug, it generally cannot be used in humans.

Table IV. Clinical Drug-Drug Interactions Potentially Involving BCRP

Affected drug	Interacting compound	Drug dosing	AUC Δ (%)	C_{max} Δ (%)	Ref
Rosuvastatin	Atazanavir/Ritonavir	Oral	213	600	(154)
Rosuvastatin	Cyclosporine	Oral	610	960	(156)
Rosuvastatin	Lopinavir/Ritonavir	Oral	110	370	(155)
Rosuvastatin	Tipranavir/Ritonavir	Oral	37	123	(153)
Atorvastatin	Tipranavir/Ritonavir	Oral	836	761	(153)
Rosuvastatin	Eltrombopag	Oral	55	103	(157)
Rosuvastatin	GSK1292263	Oral	39		(159)
Simvastatin	GSK1292263	Oral	34		(159)
Sulfasalazine	Curcumin	Oral	220	180	(160)

Δ (%) indicates percentage changes in AUC or C_{max} . Studies showing interactions of IV methotrexate with proton pump inhibitors did not report changes in AUC or C_{max} , and therefore are not included in this table

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL FUNCTIONS

Due to its wide tissue and cellular distribution, BCRP is believed to have important physiological and pathophysiological functions in tissue and cellular protection and in mediating homeostasis of physiological substrates. Jonker *et al.* (166) fed Bcrp1-knockout and wild-type mice a particular diet enriched in alfalfa-derived chlorophyll and observed phototoxicity in Bcrp1-knockout mice, but not in wild-type mice. It turned out that Bcrp1-knockout mice had much increased absorption and cellular accumulation of the chlorophyll degradation product, pheophorbide A, resulting in severe phototoxic lesions on light-exposed skin (166). Bcrp1 is also expressed on the plasma membrane of mature erythrocytes and was shown to reduce cellular protoporphyrin IX levels (167). Protoporphyrin IX is an important precursor to biologically essential prosthetic groups such as heme, cytochrome c, and chlorophylls. Because elevated cellular accumulation of heme and protoporphyrin IX is associated with formation of membrane lipid-damaging reactive oxygen species, it is speculated that BCRP plays an important role in protecting cells from oxidative damage by decreasing cellular accumulation of porphyrins (167).

Zhou *et al.* (57) showed that the “side-population” (SP) cells in mouse blood that are enriched for stem cells expressed the highest level of Bcrp1 mRNA compared with other cell populations. This is the first demonstration that Bcrp1 is highly expressed in hematopoietic stem cells. Subsequently, BCRP expression was observed in stem cells from a variety of human tissues including blood (168), pancreas islets (169), and liver (170). Thus, BCRP is considered a stem cell marker. It was natural to speculate that BCRP provides cellular protection for stem cells. Indeed, Krishnamurthy *et al.* (171) have demonstrated that BCRP protects hematopoietic stem cells under hypoxic conditions by preventing the accumulation of heme that causes mitochondrial death and that BCRP expression is upregulated in stem cells under hypoxic conditions. BCRP is highly expressed in cancer stem cells. Cancer stem cells are inherently present in the tumor cell populations. Due to high expression of BCRP (and possibly other ABC transporters such as P-gp as well), cancer stem cells are resistant to chemotherapeutics. After chemotherapy, these cells survive and ultimately differentiate to mature tumor cells (13). This is a new theory about the development of multidrug resistance in cancers which will possibly impact the practice of chemotherapy by selectively targeting cancer stem cells.

BCRP is most abundantly expressed in the placenta among normal human tissues and has been proposed to protect the placental trophoblasts from oxidative damage (172). Intrauterine growth restriction (IUGR) is a condition that is known to be associated with excessive oxidative stress (173). BCRP was found to have significantly reduced expression in the placenta from IUGR pregnancies compared to normal pregnancies, suggesting that the capacity of BCRP in protecting placental cells is possibly diminished in subjects with IUGR (172).

BCRP has recently been implicated for the development of gout (174). The common *ABCG2* 421C>A SNP has been shown to be significantly associated with elevated serum uric levels and the onset of gout. *In vitro* transport studies confirmed that uric acid is a BCRP substrate, and cells expressing the Q141K

variant resulting from the *ABCG2* 421C>A SNP had lower uric acid efflux activity than cells expressing wild-type BCRP. Subjects carrying the *ABCG2* 421C>A SNP are likely at an increased risk for developing gout due to reduced activity of BCRP for renal elimination of uric acid.

BCRP has also been implicated in the development of Alzheimer's disease (AD). AD is possibly caused by abnormal accumulation of amyloid-beta (A β) peptides in the brain, leading to neurotoxicity. It has been shown that A β peptides are likely BCRP substrate, and that brain accumulation of A β ₁₋₄₀ in Bcrp1^{-/-} mice is much higher than that in wild-type mice (175). Furthermore, BCRP expression in AD brain with cerebral amyloid angiopathy is induced (175). BCRP may therefore play a role in protecting the brain from developing the AD.

Lastly, it has recently been shown that *ABCG2* null alleles define a new blood type, the Jr(a-) blood type, and red blood cells from the individuals with the Jr(a-) blood type do not express BCRP (176). However, the Jr(a-) individuals with *ABCG2* null alleles appear phenotypically normal (176). The role of BCRP in determining the Jr(a-) blood type and pharmacological implications of this observation are currently not known. It would be important to know if and how drug disposition is altered in this unique natural BCRP knockout population.

REGULATION OF BCRP EXPRESSION

BCRP expression can be regulated at the transcriptional level. In humans, the promoter region of the *ABCG2* gene is designated E1A and E1B/C. The predominant BCRP promoter is E1B/C which was initially characterized by Bailey-Dell *et al.* (177). This promoter is TATA-less, contains several SP1 sites, and is downstream of a putative CpG island (177). To date, the *cis* regulatory elements identified in the BCRP promoter include an estrogen response element (ERE), a progesterone response element (PRE), a hypoxia response element (HRE), an antioxidant response element (ARE), an aryl hydrocarbon response element (AhRE), and the active nuclear factor κ B subunit (NF κ B) response element (2). Thus, the *ABCG2* gene is upregulated under hypoxic conditions via the hypoxia-inducible factor 1 α (HIF-1 α) (171), by estradiol through estrogen receptor α (ER α) (178), by progesterone via progesterone receptor B (PRB) (179), and by aryl hydrocarbon receptor agonists through the aryl hydrocarbon receptor (AhR) (180). BCRP expression has also been shown to be induced via the peroxisome proliferators-activated receptor gamma (PPAR γ) (181) or downregulated by dexamethasone possibly via glucocorticoid receptor (GR) (182). Nevertheless, regulation of the *ABCG2* gene and/or BCRP protein expression seems quite complicated. There are controversial reports particularly for regulation of the *ABCG2* gene and/or BCRP protein expression by steroid hormones. There are studies showing upregulation of the *ABCG2* gene and induction of BCRP protein by estradiol (178,183,184); however, other studies showed downregulation of BCRP by estradiol possibly via posttranscriptional regulation (185–187). Likewise, we reported induction of BCRP by progesterone in human placental BeWo cells (179,186), but

others showed downregulation of BCRP by progesterone in human breast cancer cells (188). Reasons of such contradicting data are not known, but may be related to cell- or organ-specific regulation (e.g., organ-specific promoters) or whether transcriptional or posttranscriptional regulation plays a predominant role in experimental systems used in these studies or if there are cooperative interactions between hormones and other regulatory factors. Binding of ER has been shown to enhance transcriptional regulation of the *ABCG2* gene by cytokines through p65 or NF κ B (189).

Epigenetic regulation of the *ABCG2* gene has also been reported. In drug-resistant cancer cell lines, elevated BCRP levels were reported to be associated with hypomethylation or unmethylation of the CpG island (190,191) and with histone hyperacetylation of the *ABCG2* promoter (191). BCRP expression can be downregulated by microRNAs by binding to the 3' UTR of the *ABCG2* gene and negative modulation of transcript stability and protein translation (192). Several such microRNAs including hsa-miR-519c, hsa-miR520h, and hsa-miR328 have been identified (193–195).

Posttranslational regulation can affect translocation and expression of BCRP on cell surface. As stated earlier, estradiol was shown to downregulate BCRP expression in ER α -positive cancer cells by decreasing BCRP protein synthesis and maturation (185) or in brain capillaries through a nongenomic pathway (187). Pim-1 kinase phosphorylates BCRP and promotes its dimerization and plasma membrane trafficking (196). BCRP protein expression can also be decreased by long-term exposure (>24 h) of cells to certain compounds (197). For a more extensive and comprehensive review on regulation of BCRP, please refer to the excellent review by Natarajan *et al.* (2).

CONCLUSION

In the past several years, we have seen significant progress in understanding the role of BCRP in drug transport. Our knowledge about drugs and xenobiotics as BCRP substrates and/or inhibitors has increased dramatically. However, the mechanism by which BCRP acts to transport drugs or xenobiotics and is inhibited is still poorly understood at the molecular level. This awaits further biochemical, biophysical, and computational studies on BCRP, including mutational analyses to enhance the scope of our understanding of amino acids involved in drug interaction and transport selectivity, determination of high-resolution 3D structures, identification of drug binding sites, and computational prediction of BCRP substrates and inhibitors. Such studies will provide the molecular basis for developing new ways to circumvent drug resistance in cancers as well as predict and modulate drug disposition such as increasing brain drug penetration.

There is mounting evidence now to support the notion that BCRP plays an important role in drug disposition. Hence, to predict the impact of BCRP on drug pharmacokinetics and drug-drug interactions in humans, it is essential to understand how BCRP expression is regulated by xenobiotics or physiological and pathological conditions, and how to extrapolate BCRP activity from *in vitro* or animal data to *in vivo* data in humans. In this regard, it is critical to identify an appropriate *in vivo* clinical probe substrate and inhibitor

for BCRP and develop methods to accurately quantify the absolute amount of BCRP expressed in tissues and cells as well as the intracellular concentrations of drugs and xenobiotics exposed to BCRP in tissues and cells.

Owing to the importance of BCRP in drug disposition and in developing gout, caution should be taken when a BCRP substrate drug with narrow therapeutic window or a potent BCRP inhibitor is to be administered to patients carrying the *ABCG2* 421C>A SNP so that undesired toxicities or side effects of medications may be avoided.

ACKNOWLEDGMENTS

This work is supported in part by the NIH Grant DA032507. We gratefully thank Dr. Isabelle Ragueneau-Majlessi and Sophie Argon for search of the UW Metabolism and Transport Drug Interaction Database (DIDB) and the UW ePKGene Database for the impact of BCRP and *ABCG2* SNPs on drug PK. We greatly acknowledge Dr. Zsolt Bikadi for preparing Fig. 2. Due to a limitation in the number of references imposed by the journal, many excellent studies cannot be cited in this review article. We appreciate the contributions of all of the authors to this important field of research.

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