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Review

Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression

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Abstract

Breast cancer resistance protein (BCRP)/ATP-binding cassette subfamily G member 2 (ABCG2) is an ATP-binding cassette (ABC) transporter identified as a molecular cause of multidrug resistance (MDR) in diverse cancer cells. BCRP physiologically functions as a part of a self-defense mechanism for the organism; it enhances elimination of toxic xenobiotic substances and harmful agents in the gut and biliary tract, as well as through the blood-brain, placental, and possibly blood-testis barriers. BCRP recognizes and transports numerous anticancer drugs including conventional chemotherapeutic and targeted small therapeutic molecules relatively new in clinical use. Thus, BCRP expression in cancer cells directly causes MDR by active efflux of anticancer drugs. Because BCRP is also known to be a stem cell marker, its expression in cancer cells could be a manifestation of metabolic and signaling pathways that confer multiple mechanisms of drug resistance, self-renewal (stemness), and invasiveness (aggressiveness), and thereby impart a poor prognosis. Therefore, blocking BCRP-mediated active efflux may provide a therapeutic benefit for cancers. Delineating the precise molecular mechanisms for *BCRP* gene expression may lead to identification of a novel molecular target to modulate BCRP-mediated MDR. Current evidence suggests that *BCRP* gene transcription is regulated by a number of trans-acting elements including hypoxia inducible factor 1α, estrogen receptor, and peroxisome proliferator-activated receptor. Furthermore, alternative promoter usage, demethylation of the *BCRP* promoter, and histone modification are likely associated with drug-induced BCRP overexpression in cancer cells. Finally, PI3K/AKT signaling may play a critical role in modulating BCRP function under a variety of conditions. These biological events seem involved in a complicated manner. Untangling the events would be an essential first step to developing a method to modulate BCRP function to aid patients with cancer. This review will present a synopsis of the impact of BCRP-mediated MDR in cancer cells, and the molecular mechanisms of acquired MDR currently postulated in a variety of human cancers.

Key words BCRP, ABCG2, multidrug resistance (MDR), transporter, gene expression, tyrosine kinase inhibitors, cancer stem cells

Multidrug resistance (MDR) is a phenomenon in which cancer cells simultaneously become resistant to structurally unrelated chemotherapeutic agents when exposed to a single chemotherapeutic drug. The development of MDR in the course of chemotherapy has been considered as a major obstacle in cancer treatment. For the last three decades, the biological causes underlying MDR have been extensively studied and attributed to diverse molecular mechanisms. Active efflux mediated by drug efflux pumps has been described in a wide variety of cancer cells since *MDR1*, which encodes the membrane transport protein P-glycoprotein (P-gp), was isolated from KB cells selected with vinblastine in 1986[1]. P-gp was the first human ATP-binding cassette (ABC) transporter protein to be identified and is classified as the first member of the B

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subfamily in the ABC transporter superfamily according to the Human Genome Organization. In 1992, a second MDR-causing transporter named multidrug resistance-associated protein 1 (MRP1) was reported [2] and found to cause resistance to xenobiotics and anticancer agents. MRP1 belongs to ABC subfamily C, which consists of 13 members. Although P-gp and MRP1 could impart MDR to cancer cells, neither P-gp nor MRP1 accounted for all of the transport-based drug resistance observed in blast cells from patients with acute leukemia when measured by functional efflux assays[3]. Multidrug-resistant MCF- 7/AdrVp cells lacking expression of P-gp or MRP1 were described following selection of MCF-7 human breast cancer cells with doxorubicin and verapamil [4]. In 1998, Doyle *et al*. [5] isolated a novel ABC transporter from MCF-7/AdrVp cells and showed by transfection of MCF-7 cells that forced overexpression of the transporter reproduced the MDR phenotype of MCF-7/AdrVp cells. The new transporter was named breast cancer resistance protein (BCRP) because it was isolated from multidrug-resistant human breast cancer cells [5]. Two other groups reported finding a similar cDNA at approximately the same time, which were designated as ABCP [6] or MXR [7], respectively. Some review articles describing the function of BCRP in health and disease are available [8-10].

This review discusses recent progress in understanding BCRP as an MDR transporter and focuses on BCRP substrate drugs, particularly novel small molecules developed for targeted therapies, which have impact on cancer treatment. The review also focuses on the molecular regulation of *BCRP* gene expression and summarizes recently proposed mechanisms underlying BCRP overexpression in MDR cancer cells and cancer stem cells.

Functional Configuration of BCRP

According to the Human Gene Nomenclature Committee, BCRP is classified as the second member of the G subfamily of the ABC transporter superfamily (ABCG2). ABC transporters are distinguished by the use of ATP hydrolysis for transporter function and exhibit extensive conservation of the ATP-binding domains throughout evolution across a large number of functionally diverse transmembrane proteins [11]. The typical ABC transporter consists of two highly conserved ATP-binding domains and two transmembrane domains. A smaller group of ABC transporters, including BCRP/ABCG2, are termed half-transporters. BCRP consists of 655 amino acids and possesses six transmembrane helices and one ATP-binding site (Figure 1). Because BCRP is a half-transporter, current evidence

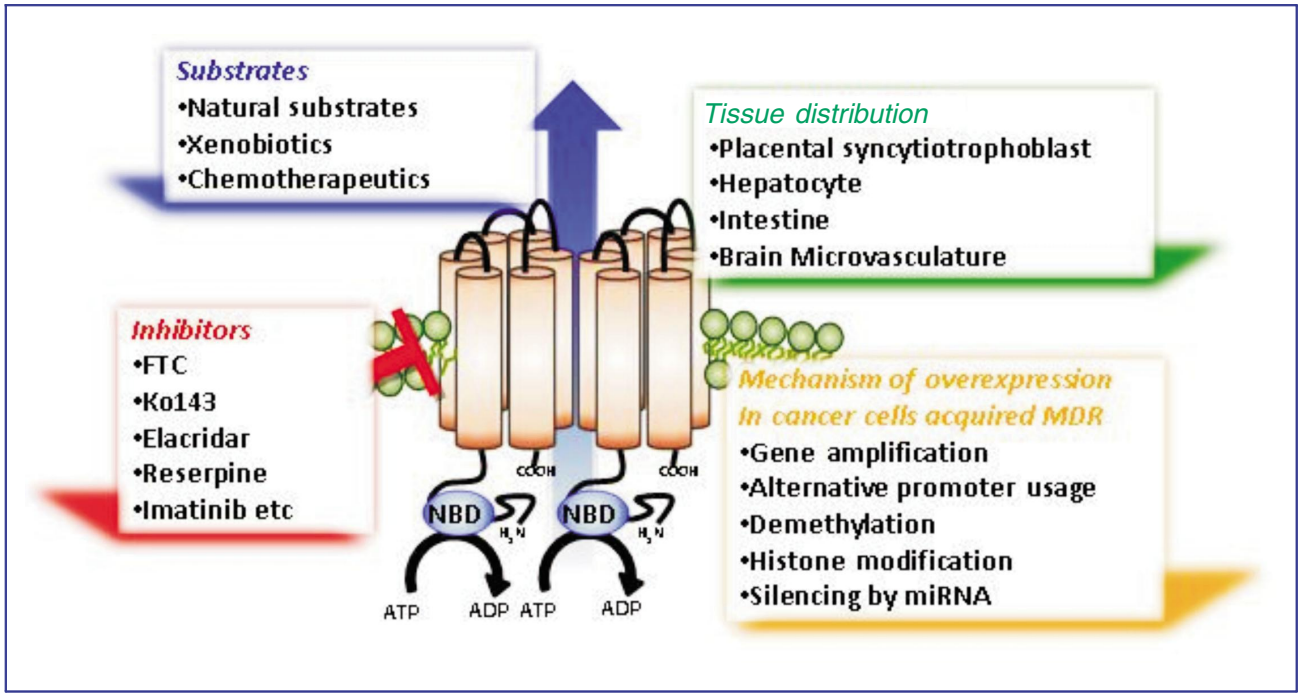

Figure 1. **Summary of BCRP function, tissue distribution, and mechanism of overexpression in drug-resistant cancer cells.** BCRP consists of 6 transmembrane helices and homodimerizes to function at the plasma membranes. It pumps natural substrates, including folate, steroid hormones, and urate; toxic xenobiotics; and anticancer agents, including conventional chemotherapeutics and tyrosine kinase inhibitors. NBD, nucleotide-binding domain to which ATP can bind.

suggests that homodimerization or multimerization is required for transporter activity as illustrated in Figure 1. Our laboratory studied the effect of co-expression of wild-type and dominant-negative BCRP on BCRP-mediated transport in *Xenopus* oocytes[12]. We observed that BCRP-mediated transport of daunorubicin was significantly reduced in a manner dependent on the amount of dominant-negative mutant (S187T) cRNA injected into the oocytes, strongly suggesting that it is essential for BCRP to at least homodimerize to function. Similar observations were made in cultured cells transduced with wild-type and mutant forms of BCRP[13]. Further biochemical analysis using gel-filtration chromatography suggests that BCRP exists as a homotetramer that may act only to regulate the level of functional homodimerized BCRP transporters[14]. Although disulfide bond formation (particularly at cysteine 603) has been postulated to participate in dimer/multimer formation[15,16], *in vivo* studies in intact cells using fluorescence resonance energy transfer techniques recently showed that cysteine 603 is not essential for dimer/oligomer formation[17]. These findings provide a basis for structural and mechanistic analysis of BCRP and related ABC transporters.

Furthermore, to date, mutant forms of BCRP in which amino acid arginine at codon 482 is substituted with threonine or glycine have been reported in various cancer cells when cells were selected with a BCRP substrate chemotherapeutic drug such as doxorubicin[18]. To the best of our knowledge, expression of these mutants has not been reported in clinical specimens[19-21]. Because these mutations alter BCRP substrate specificity, interactions between chemotherapeutic agents and wild-type as well as mutant BCRPs have been extensively studied. These studies are summarized in the "Role of BCRP in MDR" section of this review.

Physiological Function of BCRP

As an efflux transporter for xenobiotics and unwanted toxic compounds, BCRP has been characterized as an important part of self-defense systems in organisms. BCRP substrates are listed in Table 1. This is particularly true at polarized cells in normal tissues, such as placental syncytiotrophoblasts, hepatocytes, and intestinal mucosal cells, where apically expressed BCRP protects organisms by eliminating substances to the maternal circulation, bile ducts, or intestinal lumen, respectively[8]. In brain microvasculature, BCRP is located on the luminal surface of microvessel endothelium[22] and hence, may constitute an important component of the blood-brain barrier. The tissue distribution pattern of BCRP expression reflects its major role in protecting cells from potentially toxic xenobiotics and in assisting the clearance of xenobiotics from the organisms.

Naturally occurring toxic xenobiotics against which BCRP may play a protective role include dietary mutagens and carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons. Area under the plasma concentration-time curve (AUC) was observed to be higher in *Bcrp1*$^{-/-}$ mice compared to wild-type mice following oral or intravenous administration of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine[23] and 2-amino-3-methylimidazo[4,5-f]quinolone[24], which are heterocyclic amines abundantly contained in cooked meat and fish. Polycyclic aromatic hydrocarbons such as benzo[α]pyrene, which endogenously exists as benzo[α]pyrene sulfate[25], and micotoxins including aflatoxin B1[24] are substrates of BCRP. BCRP is also involved in the elimination of photosensitive protoporphyrin IX[26] and hematoporphyrin[27]. Thus, reduced BCRP function may increase the risk for developing protoporphyria and diet-dependent phototoxicity[26,28]. The photosensitizer pheophorbide a, a breakdown product of chlorophyll found in mouse chow, was also reported to be a BCRP-specific substrate[29]. In addition to these substances, BCRP transports folic acid and its polyglutamate conjugates and may play a role in cellular folate homeostasis[30,31].

BCRP transports conjugates of steroid hormones, such as estrone 3-sulfate (E3S), dehydroepiandrosterone sulfate, and, to lesser extent, estradiol-17β-D-glucuronide[32,33]. Although there is less evidence, 17β-estradiol (E2)[34] and dihydrotestosterone[35] have been indicated as substrates of BCRP. E2 was also shown to effectively inhibit BCRP-mediated transport[34,36]. These findings suggest a role for BCRP in hormone metabolism and regulation. More recently, Dehghan *et al.*[37] identified an association of the non-synonymous coding SNP (Q141K) in exon 5 of the *BCRP* gene with a risk of gout based on a genome-wide association study. This led to the discovery that BCRP plays a role in secretory elimination of uric acid in the proximal tubular cells[38]. Moreover, to date, numerous natural or physiological substrates for BCRP have been identified and have been well reviewed elsewhere[8,39].

BCRP is known to be a marker for pluripotent hematopoietic and tissue stem cells. A characteristic finding on flow cytometric analysis of cells stained with Hoechst 33342 dye is that a side population (SP) of cells possesses low Hoechst 33342 dye accumulation[40], which has been shown to be enriched for stem and progenitor cells[41]. Because the SP was absent in bone marrow from *Bcrp1*$^{-/-}$ mice[42], BCRP has been recognized as a critical determinant of the SP phenotype in not only hematopoietic cells, but also in various normal tissues, including the liver[43], lung[44], heart[45], mammary glands[46], skeletal muscle[47], neurons[48], and corneal stroma[49], and cancer cells[50-57].

Table 1. Drug resistance profile of K562-imatinib cells

Substrate	Transport			Inhibition				
	K_m (μmol/L)	Assay system[a]	Ref	IC_{50} (μmol/L)	K_i (μmol/L)	Substrate used	Assay system[a]	Ref
Natural substrates/dyes								
2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)		MDCK/BCRP1 (TP)	[23]					
2-Amino-3-methylimidazo [4,5-f]quinolone (IQ)		MDCK/BCRP (TP)	[24]					
Benzo[a]pyrene (BP) 3-sulfate		HCT116 NRI (TP)	[25]					
Aflatoxin B1		MDCK/BCRP (TP)	[24]					
Protoporphyrin IX		*Bcrp1*$^{-/-}$ BM, AML/BCRP (AC)	[26]					
Hematoporphyrin	17.8	Sf9 vesicle (AC)	[27]					
Folic acid[b]		HEK/BCRP vesicle (AC)	[31]					
Estrone 3-sulfate	6.8	K562/BCRP vesicle (AC)	[33]					
	16.6	P388/BCRP vesicle (AC)	[32]					
Estradiol-17β-D-glucuronide	44.2	HEK/BCRP vesicle (AC)	[31]					
Dehydroepiandrosterone sulfate (DHEAS)		P388/BCRP vesicle (AC)	[32]	55		E3S	P388/BCRP vesicle (AC)	[32]
17β-Estradiol		*L. lactis*/BCRP (AC)	[34]			H33342	*L. lactis*/BCRP vesicle (AC)	[34]
				4.8		TPT	K562/BCRP (AC)	[36]
Dihydrotestosterone		Mx–RPE (EF)	[35]					
Uric acid		X. Oocyte	[38]					
Fluorescent substrates/probes								
Prazosin-BODIPY		Selected Cancer Cells	[119]					
		HEK/BCRP (AC)	[86]					
Hoechst 33342		A5449/BCRP (SP analysis)	[40]					
LysoTracker® [b]		HEK/BCRP (AC)	[86]					
Rhodamine 123[b]		HEK/BCRP (AC)	[86]					
		X. Oocyte (AC)	[12]					
Pheophorbide a		HEK/BCRP, Selected Cancer Cells (AC)	[29]					
Sulfasalazine	0.7	SB-BCRP-M-VT (AC)	[122]					
D-luciferin		HEK/BCRP (AC)	[125]					
Chemotherapeutics								
Mitoxantrone (MX)	7.0	Sf9 vesicle (ATP)	[85]	61		E3S	P388/BCRP vesicle (AC)	[32]
		X. Oocyte (EF, AC)	[12]					
SN-38	4.0	PC-6/SN2-5H vesicles (AC)	[94]	1.6		E3S	HEK/BCRP vesicle (AC)	[117]
SN-38-glucuronide	26.0	PC-6/SN2 -5H vesicles (AC)	[94]					
9-Aminocamptothecin		HEK/BCRP	[91]					
Indolocarbazole topoisomerase I inhibitors (e.g. NB-506, J-107088)		PC-13/BCRP (AC, EF)	[97]					
Belotecan	>500	MDCKII/BCRP (TP)	[95]					
Doxorubicin (DOX)[b]	5.0	Sf9 vesicles (ATP)	[85]					
Daunorubicin[b]	2.5	Sf9 vesicles (ATP)	[85]					
		HEK/BCRP (EF)	[86]					
		X. Oocyte (AC, EF)	[12]					
Epirubicin[b]			[86]					

(*to be continued*)

Table 1. Drug resistance profile of K562-imatinib cells (*continued*)

Substrate	Transport			Inhibition				
	K_m (μmol/L)	Assay system[a]	Ref	IC_{50} (μmol/L)	K_i (μmol/L)	Substrate used	Assay system[a]	Ref
Chemotherapeutics								
Bisantrene[b]		MCF-7/AdrVp (AC)	[119]					
Methotrexate[c]	681	MCF-7/MX vesicle (AC)	[30]					
	1340	HEK/BCRP vesicle (AC)	[31]					
	1410	HEK/BCRP vesicle (AC)	[131]					
Imatinib	0.15	HiFive vesicles (ATP)	[130]	0.9		H33342		[112]
		HEK/BCRP (AC)	[76]					
Nilotinib	0.008	HiFive vesicles (ATP)	[130]		0.69	MTX	HEK/BCRP vesicle (AC)	[131]
		K562/BCRP	[132]		0.05	H33342	K562/BCRP (AC)	[132]
Dasatinib		Mef/BCRP (AC)	[136]		>2.0	H33342	K562/BCRP (AC)	[132]
CI1033 (Canertinib)		MDA-MB231/BCRP (AC)	[139]					
Gefitinib (Iressa, ZD1839)		HEK293/BCRP R-5 (AC)	[144]	0.4		H33342		[112]
Erlotinib hydrochloride (Tarceva, OSI-774, CP-358774)		HEK293/BCRP R-5 (AC)	[144]	0.13		E3S	K562/BCRP vesicle (AC)	[107]
Lapatinib ditosylate (Tykerb, GW572016)		*Bcrp1*$^{-/-}$ mice (BD)	[152]					
Sunitinib malate (Sutent, SU11248)	0.18	HiFive vesicles (ATP)	[157]					
		MDCK/BCRP (TP)	[159]					
		MDCK/P-gp&BCRP (TP)	[158]					
Sorafenib (Nexavar)		MDCK/BCRP (TP)	[161]					
Axitinib (AG013736)		MCDK/BCRP (TP)	[164]					
Flavopiridol (Alvocidib)		X. Oocytes (AC)	[12]			MX	X. Oocyte (AC)	[12]

[a]Experimental condition used to evaluate BCRP-mediated transport of each compound: BCRP, with forced expression of BCRP; TP, transport assay across monolayer of cells; AC, intracellular accumulation study; ATP, ATPase activity assay; EF, efflux study (retention assay); BD, brain distribution *in vivo* in animals. [b]Reported substrates for mutant *BCRP* (R482T and R482G). [c]Reported substrates for wild-type BCRP (R482). IC_{50}, 50% inhibition concentration; E3S, estrone 3-sulfate; TPT, topotecan; MTX, methotrexate; H33342, Hoechst 33342 dye; DOX, doxorubicin; MX, mitoxantrone; BM, bone marrow.

BCRP Expression in Human Cancers

Acute myelogenous leukemia (AML)

Acute myelogenous leukemia (AML) is a malignant neoplastic disease in which BCRP expression has been the best characterized. Ross *et al.*[58] initially detected *BCRP* mRNA at relatively high levels in approximately one third of 20 AML samples, suggesting that *BCRP* plays a role in resistance to conventional chemotherapies. In the same set of 20 samples, *BCRP* expression seemed to be correlated with MDR1 expression (r = 0.66) although the relationship was not statistically significant. Subsequently, we showed that *BCRP* mRNA expression correlated with *in vitro* resistance to the cyclin-dependent kinase (CDK) inhibitor flavopiridol in blast cells from patients with AML[19]. In a study with paired AML blast cell samples collected before treatment and at the time of relapse or refractoriness, van den Heuvel-Eibrink *et al.*[59] found that *BCRP* mRNA was higher in the relapsed or refractory samples. Benderra *et al.*[60] found that pre-treatment high *BCRP* mRNA level was a prognostic factor for achieving a complete remission (CR) and associated with lower disease-free survival in 149 patients. Furthermore, in a study of 40 patients with newly diagnosed AML, Abbott *et al.*[61] found that *BCRP* mRNA expression was higher in AML samples (78%) than in normal bone marrow; however, only 7% of patients had "functional" levels of *BCRP* mRNA. In that study, there was no correlation between *BCRP* expression and patient outcomes or clinical characteristics, but the data suggested the presence of a small subpopulation of "primitive leukemic stem cells with intrinsic drug efflux capacity" in which *BCRP* mRNA expression was confined. This notion was supported by the findings of van der Kolk *et al.*[62], who detected BCRP protein expression in subpopulations of cells with an immature phenotype ($CD34^+$) but found no increase in

these BCRP$^+$ subpopulati ons at time of relapse in 20 AML patients. Suvannasankha *et al.*[20] also found that *BCRP* mRNA expression correlated poorly to BCRP protein expression but observed that *BCRP* mRNA may be limited to small subpopulations of blast cells pretreatment.

There is compelling evidence, accumulated over the past decade, that BCRP is expressed in premature AML blast cell subpopulations.[9] The highest BCRP expression and function were found in stem-like CD34$^+$/CD38$^-$ cells in both normal and AML marrows [63]. Although blocking BCRP function somewhat reversed drug resistance of CD34$^+$/CD38$^-$ AML blast cells to mitoxantrone, it had little effect on the intracellular accumulation of mitoxantrone. Thus, additional factors are likely involved in the drug resistance exhibited by these cells[63]. An evaluation of the mRNA expression of 47 human ABC transporters in blast cells from 18 AML patients who achieved CR and 13 AML patients who were refractory to induction chemotherapy revealed no difference in ABC transporter expression between the CR and refractory groups; however, the refractory group had significantly higher expression of BCRP and/or MDR1 in CD34$^+$/CD38$^-$ cells[64]. Moreover, a study with 26 bone marrow samples from patients with *de novo* AML done by de Figueriedo-Pontes *et al.*[65] showed that leukemia stem cell population, defined as CD34$^+$/CD38$^-$/CD123$^+$, showed higher BCRP and P-gp expression than other subsets of cell populations, implying that co-expression of both BCRP and P-gp in leukemia stem cells may augment MDR synergistically. Thus, more recent studies of BCRP expression in AML likely confirm that BCRP is often co-expressed with P-gp and connotes a worse prognosis [64-68]. Furthermore, BCRP and P-gp expression appear to be associated with subpopulations of cells with primitive characteristics, such as expression of CD34 but not CD38 [63,65,68,69], although two studies showed no association of BCRP with CD34 expression (but not CD38)[70,71].

Acute lymphoblastic leukemia(ALL) and chronic myelogenous leukemia (CML)

There is limited information available for BCRP expression in acute lymphoblastic leukemia (ALL). To date, BCRP expression is suggested to be detected at the highest level in B-lineage ALL, although no definite trends were shown between BCRP expression and prognosis of ALL[72,73]. A study of BCRP expression in childhood ALL showed that *BCRP* mRNA was not increased in specimens from children in relapse[73]. No relationship was found between *BCRP* mRNA expression and relapse-free survival in this study. Our study of BCRP function and mRNA and protein expression in blast cells from 30 adult ALL cases by Suvannasankha *et al.*[21] revealed a relatively high frequency of positive staining (37% to 47% of cases) with a variety of anti-BCRP antibodies; however, there was poor concordance of antibody staining, mRNA expression, and functional assays in this study. Positive staining with the BXP21 mouse anti-human monoclonal antibody to BCRP was predictive of a shorter disease-free survival.

In case of chronic myeloid leukemia (CML), previous studies have indicated a high expression of BCRP in premature CML cell populations. Jordanides *et al.*[74] detected aberrant overexpression of BCRP protein in CD34$^+$ cells derived from CML patients. BCRP expression was detected in over 25% of cells from 5 out of 7 individual subjects, and BCRP function was confirmed by active efflux of mitoxantrone out of the cells. Moreover, quantitative gene expression analysis indicated a strong differentiation-associated decrease of *BCRP* mRNA expression in mononuclear cells from patients with CML[75]. In this study, BCRP was more highly expressed in lin$^-$/CD34$^+$/38$^-$ cells derived from CML than in normal bone marrow cells. Although imatinib was shown to be a substrate of BCRP [76,77], BCRP- mediated active efflux of imatinib has not yet been observed in CD34$^+$ CML cells[74,78]. These findings strongly suggest that BCRP expression in CML is confined in primitive cell populations, but the functionality of BCRP in primitive cells is not completely understood.

Solid tumors

BCRP has been reported to be overexpressed in solid tumors. Immunohistochemical studies with BXP-21 antibody demonstrated a high frequency of BCRP immunoreactivity among a panel of 150 untreated human solid tumors comprising 21 tumor types[79]. Specificity of immunohistochemistry was confirmed by the detection of a 72 kDa band representing BCRP on Western blots. According to this article, BCRP expression was widely present in untreated human solid tumors. Kawabata *et al.*[80] used reverse transcription-polymerase chain reaction (RT-PCR) and detected high levels of *BCRP* mRNA expression in 6 of 8 non-small cell lung cancer (NSCLC) cell lines and 5 (22%) of 23 non-small cell lung tumor tissues. In these lung cancer cell lines, topotecan efflux was correlated with the levels of *BCRP* mRNA. In *in vitro* models, BCRP overexpression is commonly observed in cells derived from various types of human solid tumors selected with drugs, such as mitoxantrone[5,7,81,82], topoisomerase inhibitor topotecan[82], flavopiridol[83], and imatinib[84], and seems to be independent of P-gp or MRP expression.

Role of BCRP in MDR

Conventional chemotherapies known as BCRP substrates

BCRP functions as an efflux pump for a wide variety

of xenobiotics inc luding many approved cancer chemotherapies (Table 1). Therefore, emergence of BCRP renders cancer cells resistant to structurally unrelated drugs simultaneously, fulfilling the definition of MDR. BCRP is also known as mitoxantrone resistance protein (MXR) because it was isolated from human colon carcinoma cells (S1M1 80) after *in vitro* selection with mitoxantrone [7]. Mitoxantrone is shown to be a substrate of BCRP in *in vitro* assays[12,85]. Strong cellular resistance to mitoxantrone is attributable to BCRP-mediated efflux, which reduces its intracellular accumulation[5,7,12,81,86]. Following selection with mitoxantrone, BCRP overexpression was observed not only in colon carcinoma cell lines (S1M1 80, HT29RNOV, KM12/MX) but also in a variety of cancer cell lines including breast carcinoma (MCF-7/MX8, MCF-7/Mitox, MDA-MB-231RNOV), gastric carcinoma (EPG85-257RNOV), fibrosarcoma (EPF86-079RNOV), NSCLC (H460/MX), glioblastoma (SF295/MX), and myeloma (8226/MR20)[81,87,88]. Thus, BCRP is a component of the cellular defense mechanism elicited in response to mitoxantrone.

Accumulated evidence on MDR shows that BCRP plays a critical role in the development of resistance to irinotecan-based therapy. Topotecan is a type I topoisomerase inhibitor derived from camptothecin. Selection with topotecan in a human ovarian cancer cell line (IGROV1/T8) [82] and a breast cancer cell line (MCF-7/TPT3000)[89] was found to induce overexpression of BCRP, resulting in acquired resistance to 10-hydroxy-7-ethylcamptothecin (SN-38), the active metabolite of irinotecan; 9-aminocamptothecin; and, to a lesser extent, irinotecan[82,89,90]. Overexpression of both the wild-type and R482T forms of BCRP conferred resistance to 9-aminocamptothecin, which was associated with reduced intracellular drug accumulation, but did not confer resistance to 9-nitrocamptothecin. These observations, in addition to the knowledge that BCRP confers resistance to topotecan and SN-38, suggest that polar residues at the 9 or 10 position of camptothecin may be important to facilitate interactions with BCRP [91]. BCRP was also induced in cancer cells by selection with SN-38[92,93], and further transport study showed that both SN-38 and SN-38-glucuronide are substrates of BCRP [94]. *BCRP* mRNA levels were higher in hepatic metastases obtained from patients after irinotecan-based chemotherapy than in irinotecan-naive metastases, indicating that BCRP was involved in irinotecan resistance *in vivo*[93]. Belotecan, a semi-synthetic topoisomerase I inhibitor, was shown to be transported by BCRP[95]. More recently, novel effective camptothecin analogues of the 7-oxyiminomethyl compound, ST1968 and ST1978, were identified as substrates of BCRP[96].

Although BCRP expression was enhanced in MCF-7 cells after being selected with either doxorubicin (MCF-7/AdrVp cells) or mitoxantrone (MCF-7/MX8 cells), a high level of efflux was noted with rhodamine 123 in MCF-7/AdrVp cells, whereas such efflux was not seen in MCF-7/MX8 cells [87]. This was later explained by the presence of a mutation at codon 482 in the mRNA sequence of *BCRP*, with replacement of the wild-type arginine by threonine or glycine (R482T or R482G)[18,97]. Compared to wild-type *BCRP*, overexpression of either the R482T or R482G *BCRP* mutants conferred greater resistance to anthracyclines, including doxorubicin, daunorubicin and epirubicin, and bisantrene[5,86,98]. Because intracellular accumulation of these substrate drugs was significantly lowered in cells expressing the mutant protein [12,86], mutation at codon 482 has a major impact on substrate specificity of BCRP. Similar findings were made for codon 482 of murine *Bcrp1*/*Abcg2*, except that the mutations were to methionine or serine [99]. Active efflux of hydrophilic antifolates is also affected by the mutations. Wild-type BCRP is capable of transporting methotrexate [30,31,100] and its polyglutamate form [30,31], whereas the mutants likely lack the ability to efflux methotrexate. However, one report described that human embryonic kidney cells stably transfected with R482G-BCRP cDNA displayed resistance to hydrophilic antifolates, including methotrexate, raltitrexed, and GW1843, relative to parental cells [101]. This discrepancy needs to be addressed further. In addition, hydrophobic antifolates may be substrates of both mutants, but not wild-type (R482) BCRP. These mutants have been shown to confer high-level of resistance to lipophilic antifolate inhibitors of dihydrofolate reductase (e.g., trimetrexate and piritrexim) and thymidylate synthase (e.g., AG337 and AG377)[102]. Indeed, the lipophilic antifolate analogue chromophore tetramethylrosamine has been identified as a substrate of both BCRP R482T and R482G but not wild-type BCRP [102]. Codon 482 mutation to methionine in human BCRP has also been reported[103]. To date, however, these mutations have only been found in cultured drug-selected cell lines. Similarly, no BCRP mutations have been observed in clinical samples of blast cells obtained from leukemia patients[19,20,72].

Compared to control transfectants, BCRP-transfected cells have been shown to display only minimal resistance to etoposide and depsipeptide [86] but no resistance to cisplatin, paclitaxel, or vincristine [5]. Recently, the prostate cancer cell line LNCaP was shown to display overexpression of phosphorylated BCRP after *in vitro* selection in the presence of docetaxel, resulting in enhanced decetaxel resistance. This suggests that post-translational modification (PTM) in response to drug may be involved in substrate specificity or functionality of BCRP[104].

Inhibitors for modulation of BCRP function

Because BCRP plays a role in MDR, specifically

modulating BCRP function to sensitize BCRP-overexpressed cancer cells to chemotherapies is of pharmacological interest. To date, several specific inhibitors of BCRP have been reported, and some are currently undergoing clinical trials or are available to treat patients. Among them, fumitremorgin C (FTC) is one of the most specific inhibitors for BCRP and has been frequently used to test BCRP activity in a wide variety of experiments. FTC, a mycotoxin isolated from *Aspergillus* fumigates, was the first reported specific inhibitor for BCRP [105, 106] and is active at micromolar concentrations [31,87,107]. Ko143 is a derivative of FTC and approximately 10 times more potent at BCRP inhibition than FTC[108,109]. GF120918 (also known as Elacridar) was first developed as a P-gp inhibitor and has been shown to block BCRP function. Indeed, GF120918 inhibited BCRP-mediated mitoxantrone transport with an IC_{50} value of 0.31 μmol/L [110]. A pharmacokinetic study demonstrates the effectiveness of GF120918 *in vivo*, showing co-administration of GF120918 can increase the oral bioavailability of topotecan, a BCRP substrate drug, from 30% to 90% [111]. Other BCRP inhibitors include gefitinib (Iressa, ZD1839)[112], flavopiridol [12], 6-prenylchrysin (a hydrophobic flavone) [110], reserpine [26, 113], taxane derivatives (i.e., ortataxel and tRA96023)[114], estrone and antiestrogens (e.g., tamoxifen and TAG-related compounds) [115], imatinib [112,116], certain HIV protease inhibitors[109], and 3-hydroxymethylglutaryl coenzyme A reductase inhibitors [117]. Recently, synthetic compounds related to piperazinopyranones and phenalkylaminobenzopyranones were identified to be strong inhibitors of BCRP comparable to FTC[118]. BCRP inhibitors are summarized in Table 2.

Specific probe substrates to characterize BCRP function

To date, several probe substrates for BCRP have

Table 2. Inhibitors for BCRP

Inhibitor	IC_{50} (μmol/L)	K_i (μmol/L)	Substrate used	Assay system[a]	Ref
FTC		1.0	MX	S1M1 80 vesicle (ATP)	[87]
		0.30	MTX	HEK/BCRP vesicle (AC)	[31]
	0.25	0.55	E3S	K562/BCRP vesicle (AC)	[107]
		0.47	PhA	MDCK/BCRP (TP)	[109]
Ko143	0.01		PhA	MDCK/BCRP	[109]
Elacridar (GF120918)	0.31		MX	Sf9 vesicles	[110]
6-Prenylchrysin	0.29		MX	Sf9 vesicles	[110]
Reserpine	<10		H33342	Saos2/BCRP (AC)	[113]
Ortataxel	<10		MX	8226/MR20	[114]
tRA96023	<10		MX	8226/MR20	[114]
Tamoxifen and antiestrogens (TAG compounds)			TPT	K562/BCRP (AC)	[115]
Phenalkylamine derivatives (5b, 5c)	~ 1		MX	HCT116/R (AC)	[118]
Piperazine derivatives (4c)	~10		MX	HCT116/R (AC)	[118]
Daunomycin	59		E3S	P388/BCRP vesicle (AC9	[32]
Anti-HIV therapeutics					
Amprenavir	181		PhA	MDCK/BCRP (TP)	[109]
Atazanavir	69.1		PhA	MDCK/BCRP (TP)	[109]
Lopinavir	7.66		PhA	MDCK/BCRP (TP)	[109]
Nelfinavir	13.5		PhA	MDCK/BCRP (TP)	[109]
Saquinavir	27.4		PhA	MDCK/BCRP (TP)	[109]
Delavirdine	18.7		PhA	MDCK/BCRP (TP)	[109]
Efavirenz	20.6		PhA	MDCK/BCRP (TP)	[109]
HMG-CoA reductase inhibitors					
Atorvastatin		14.3	E3S	HEK/BCRP vesicle (AC)	[117]
Cerivastatin		18.1	E3S	HEK/BCRP vesicle (AC)	[117]
Fluvastatin		5.43	E3S	HEK/BCRP vesicle (AC)	[117]
Pitavastatin		2.92	E3S	HEK/BCRP vesicle (AC)	[117]
Rosuvastatin		15.4	E3S	HEK/BCRP vesicle (AC)	[117]
Simvastatin acid		18.0	E3S	HEK/BCRP vesicle (AC)	[117]

Footnotes as in Table 1.

been reported. These agents are useful for evaluating the effect of drugs on BCRP-mediated transport. The fluorescence-labeled compound, BODIPY-prazosin, is one of the most frequently used probe substrates for BCRP [87,119]. LysoTracker Green® and rhodamine 123 were described as good substrates for both codon 482 mutants of BCRP but not for wild-type BCRP [12,87]. Mitoxantrone is often used as a fluorescent substrate in flow cytometry-based functional assays[7,86], but it is also a weak substrate for P-gp. Robey *et al.*[29] demonstrated that pheophorbide a is a fluorescent substrate specific to wild-type and mutant BCRP, and that using a flow cytometric assay with this compound is convenient to determine expression of functional BCRP. Earlier pharmacokinetic studies showed that the transport of sulfasalazine across a monolayer of Caco-2 human colon carcinoma cells from the basolateral to apical side was significantly reduced by FTC but not by cyclosporine A, a typical inhibitor for P-gp, indicating that sulfasalazine is a substrate of BCRP[120]. In *Bcrp1*$^{-/-}$ mice, the AUC of sulfasalazine was over 100-fold greater than that in wild-type mice [121]. Affinity of sulfasalazine to BCRP was estimated at approximately 70 μmol/L by *in vitro* membrane vesicle study [122]. Furthermore, clinical studies revealed that measured plasma concentrations *in vivo* correlate with single nucleotide polymorphisms in the *BCRP* gene (c.421C>A)[123]. Selection of T-lymphocytes by sulfasalazine caused BCRP overexpression[124]. These findings suggest that sulfasalazine can be used as a probe substrate to evaluate BCRP function. More recently, intracellular accumulation of D-luciferin was shown to be modulated by FTC [125]. D-luciferin bioluminescent output *in vivo* was substantially influenced by BCRP function within the regions of interest. Furthermore, FTC had no effect on the bioluminescent output in cells overexpressing human *P-gp*, *MRP1*, or *MRP2* genes; therefore D-luciferin-based bioluminescent imaging has been suggested as a new high-throughput method for identifying modulators of BCRP function[126].

BCRP and Molecular Targeted Therapy

Tyrosine kinase inhibitors (TKIs) for BCR-ABL

Previously, Özvegy-Laczka *et al.* [112] described a clear interaction between tyrosine kinase inhibitors, including imatinib mesylate (originally known as Gleevec, STI571) and BCRP, which modulate BCRP transport and stimulate BCRP-specific ATPase activity. There are conflicting reports on whether imatinib is a substrate transported by BCRP. Imatinib is shown to be a substrate for BCRP in human embryonic kidney HEK293 cells transfected with *BCRP* [76], whereas it is not in Soas2 cells with forced expression of BCRP [116] or primitive CML CD34$^+$ cells aberrantly overexpressing BCRP [74]. The bioavailability, pharmacokinetics, and disposition of imatinib are shown to be influenced by BCRP, suggesting that BCRP functions as an imatinib transporter *in vivo*[127]. Subsequently, chronic exposure of Caco-2 human colon carcinoma cells to imatinib is found to result in induction of BCRP expression[84], representing a novel potential mechanism of acquired drug resistance in cancer patients treated with imatinib over a long time. Although BCRP did not confer a survival advantage to imatinib-treated Saos2 cells compared to mock-transfected ones[116], this could be because Saos2 are not growth- or survival-dependent upon the intracellular targets of imatinib, including BCR-ABL, c-KIT, or platelet-derived growth factor receptor (PDGFR). We examined the effects of BCRP expression on cellular resistance to imatinib in human CML-derived K562 cells, whose growth is dependent on BCR-ABL, and demonstrated that forced expression of BCRP in these cells caused resistance to imatinib cytotoxicity that was overcome by the BCRP-specific inhibitor FTC[128]. Interestingly, this effect was attenuated by imatinib-mediated inhibition of BCR-ABL, which in turn down-regulated BCRP expression post- transcriptionally via the PI3K/AKT pathway. This imatinib-mediated down-regulation of BCRP was further confirmed by Dohse *et al.*[129]; however, detailed mechanisms should be further examined.

Nilotinib (Tasigna, AMN107) is a second-generation BCR-ABL TKI used to surmount resistance or intolerance to imatinib in patients with Ph(+) CML. Photoaffinity labeling assays [130] and kinetic analyses [131] have shown that nilotinib competitively inhibits BCRP-mediated drug efflux. The Ki value of nilotinib for BCRP-mediated methotrexate transport was 0.69 μmol/L, suggesting that nilotinib is a highly potent inhibitor of BCRP [131]. Nilotinib was also shown to be a high-affinity substrate of BCRP and P-gp[132]. Because BCRP is highly expressed on CML CD34$^+$ cells [74], several studies have been conducted to determine whether the inhibition of BCRP activity potentiates the effect of nilotinib on apoptosis, BCR-ABL inhibition, or CML CD34$^+$ cell proliferation. So far, it is unlikely that BCRP and P-gp will have any effect on the clinical response to this drug [133]. BODIPY-labeled nilotinib was also found to be a substrate for P-gp and BCRP and has been proposed as a potentially useful probe for functional analysis of both transporters in cancer cells and other preclinical studies[134].

Dasatinib (Sprycel, BMS-354825) is another second-generation dual kinase inhibitor of ABL and SRC. In *in vitro* assays, dasatinib is approximately 300 times more active than imatinib and effective against most imatinib-resistant BCR-ABL active-site mutants, except the T315I mutation [135]. Ko143 significantly increased

dasatinib uptake in BCRP-overexpressing cell lines and reduced the IC_{50} of dasatinib[136]. *In vivo* brain distribution studies showed that brain-to-plasma concentration ratio of dasatinib was significantly higher in *Mdr1a/b*$^{-/-}$*Bcrp1*$^{-/-}$ than in *Mdr1a/b*$^{-/-}$ mice and increased in wild-type mice with co-administration with Ko143[137]. These findings are a clear demonstration that dasatinib is a potent substrate for BCRP.

Bosutinib (SKI-606), a third-generation TKI inhibitor, is useful in patients whose leukemia is resistant to both first- and second-generation TKI inhibitors. Unlike imatinib, bosutinib inhibits both SRC and ABL[138]. Current evidence regarding the interaction of bosutinib with ABC transporters is limited. In *in vitro* growth assays, neither P-gp nor BCRP induced resistance to bosutinib, although their transport activities were inhibited by bosutinib at relatively high concentrations (> 2 μmol/L)[132].

TKIs for epidermal growth factor receptor (EGFR, HER1) and HER2

CI1033 (also named canertinib) was the first EGFR TKI that was shown to be a substrate of BCRP. Intracellular accumulation of CI1033 was diminished in BCRP-expressing T98G glioblastoma and HCT8 colon cancer cells[139]. This was further confirmed by a trans-cellular transport study using MDCK/BCRP cells[140].

Gefitinib (Iressa, ZD1839) is an orally active, small-molecule inhibitor of EGFR and a first-generation TKI. Gefitinib, a strong inhibitor of BCRP, modulated ATPase activity of BCRP in a plasma membrane vesicle study[112] and reversed BCRP-mediated resistance to SN38[141,142] and topotecan[143]. Gefitinib accumulation was lowered in HEK293 cells transfected with BCRP, suggesting that gefitinib is a substrate of BCRP[144]. Another study showed that BCRP conferred resistance to gefitinib[141,145] and that chronic exposure of colorectal carcinoma cells to gefitinib induced BCRP expression[146]. Hence, BCRP has been postulated to play a critical role in cellular resistance to and pharmacokinetic disposition of gefitinib. The systemic exposure of gefitinib was greater in patients heterozygous at the BCRP c.421C>A locus than in patients with wild-type BCRP[144]. In an animal pharmacokinetic study, brain accumulation of gefitinib increased approximately 70-fold in *Mdr1a/b*$^{-/-}$*Bcrp1*$^{-/-}$ mice compared to wild-type mice, suggesting that the distribution of gefitinib to the brain is highly restricted by both P-gp and BCRP[147]. Because concomitant administration of gefitinib with topotecan could enhance topotecan penetration to the central nervous system, co-administration of gefitinib with a BCRP substrate drug may provide a potential benefit for patients with brain tumors.

Erlotinib hydrochloride (Tarceva, OSI-774, CP-358774), another orally-active EGFR TKI, is similar to gefitinib in structure. Erlotinib reverses P-gp- and BCRP-mediated MDR in cancer cells through direct inhibition of their drug efflux function[148]. Erlotinib competitively inhibited BCRP-mediated estrone 3-sulfate (E3S) transport with an IC_{50} value of 0.13 μmol/L, which is comparable to FTC, suggesting that erlotinib is one of the most powerful inhibitors for BCRP among the TKIs[107]. An *in vitro* accumulation study in HEK293 cells transfected with BCRP showed that erlotinib is a substrate for BCRP[144]. Furthermore, *in vivo* systemic exposure of orally administered erlotinib was significantly increased in *Mdr1a/b*$^{-/-}$*/Bcrp1*$^{-/-}$ mice compared with wild-type mice, and brain distribution of erlotinib was observed to be restricted by both BCRP and P-gp[149]. According to the possible clinical consequences of interactions between P-gp/BCRP-inhibiting substrates and erlotinib, erlotinib pharmacokinetics *in vivo* may be affected by drug-drug interaction on P-gp or BCRP, which needs to be addressed in patients.

Lapatinib ditosylate (Tykerb, GW572016) is an orally active, dual tyrosine kinase inhibitor that interrupts both EGFR and HER2 signaling and is used primarily to treat breast cancer[150]. Lapatinib sensitized MDR cells by inhibiting BCRP transport activity directly[151]. The brain-to-plasma ratio of lapatinib was over 10-fold higher in *Mdr1a/b*$^{-/-}$*/Bcrp1*$^{-/-}$ mice compared to *Mdr1a/b*$^{-/-}$ mice, suggesting that lapatinib is a substrate for BCRP[152]. Synergistic interactions were seen with lapatinib and SN38, and this effect was attributed to increased intracellular accumulation of SN38 as a result of the inhibitory effect of lapatinib on BCRP-mediated SN38 efflux[153].

Vandetanib (Zactima, ZD6474) is a multitargeted TKI that affects EGFR and VEGFR2, and is RET kinase. Chronic exposure of HT-29 colorectal cancer cells to vandetanib reportedly induced BCRP expression[146]; however, BCRP overexpression did not confer resistance to vandetanib in drug-resistant S1M1 80 cells[154]. Vandetanib treatment significantly increased the intracellular accumulation of BCRP substrate drugs in a dose-dependent manner[154], suggesting that vandetanib functions as an MDR sensitizer.

TKIs for Class III kinases (c-KIT and PDGFR)

Sunitinib malate (Sutent, SU11248) is a multi-targeted small-molecule TKI used to treat renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumors[155]. Sunitinib was observed to inhibit BCRP transport activity and stimulate its ATPase activity in a concentration-dependent manner[156,157]. Direct binding of sunitinib to BCRP was demonstrated by photoaffinity labeling and antibody binding assays[157]. A recent *in vitro* transport study showed that sunitinib is a substrate for both BCRP and P-gp[158]. An *in vivo* pharmacokinetic analysis revealed a 23-fold increase in brain

accumulation of sunitinib in *Mdr1a/b*$^{-/-}$/*Bcrp1*$^{-/-}$ mice, whereas there was only a 2.3-fold increase in *Mdr1a/b*$^{-/-}$ mice and no increase in *Bcrp1*$^{-/-}$ mice, suggesting that sunitinib is cooperatively restricted by both P-gp and Bcrp1 at the blood-brain barrier[159].

Sorafenib tosilate (Nexavar) is another multitargeted TKI used to treat hepatocellular carcinoma (HCC) and RCC[160]. Like sunitinib, sorafenib was shown to potently inhibit BCRP-mediated drug efflux[156]. A recent transcellular study showed that sorafenib is efficiently transported by BCRP rather than P-gp[161]. In an *in vivo* pharmacokinetic analysis, brain accumulation of oral sorafenib increased in *Bcrp1*$^{-/-}$ mice compared with wild-type mice, suggesting that its distribution to the brain is primarily restricted by BCRP[161-163].

TKIs for VEGFR

Axitinib (AG013736) is a small-molecule indazole derivative and newly developed effective TKI, which inhibits vascular endothelial growth factor receptor (VEGFR)-1, -2, and -3 as well as PDGFR and cKIT. A transport study using monolayers of MDCK II cells expressing BCRP showed that axitinib is a moderate substrate of BCRP[164]. The combination of axitinib and SN38 was found to exert a strong synergistic effect on antiproliferative and proapoptotic activities in endothelial and other cancer cells. This effect could be due to increased intracellular accumulation of SN38 by axitinib-mediated inhibition of BCRP and ATP7A expression, which is the result of extracellular signal-regulated kinase (ERK)1/2 deactivation and AKT phosphorylation in human pancreatic cancer cell lines[165]. However, such a synergistic interaction was not observed with SN38 and a similar TKI, pazopanib (Votrient), suggesting that an interaction of pazopanib with BCRP is unlikely[153].

Apatinib (YN968D1), another promising inhibitor of receptor tyrosine kinases including VEGFR2, is currently in phase III clinical trials in China to determine its efficacy in treating gastric cancer and NSCLC[166]. Apatinib reversed P-gp- and BCRP-mediated MDR by inhibiting their transport function, but not by suppressing the AKT or ERK1/2 pathway. Use of these TKIs with other conventional chemotherapeutics may provide a clinical benefit for patients to circumvent MDR to other conventional antitumer drugs.

Cyclin-dependent kinase (CDK) inhibitors

Flavopiridol (Alvocidib, HMR1275, also known as L86-8275) is a synthetic N-methylpiperidinyl chlorophenyl flavone and the first CDK3 inhibitor that is currently in clinical trials[167,168]. In one study, flavopiridol induced overexpression of ABCG2 but not P-gp or MRP1 in MCF-7 cells[83]. Furthermore, resistance to flavopiridol was observed in selected BCRP-overexpressing cell lines but not in P-gp- or MRP1-overexpressing cell lines[83]. Flavopiridol was shown as a substrate for wild-type and mutant BCRP (482T) in *Xenopus* oocytes injected with *BCRP* cRNA[12]. We also found that *BCRP* mRNA expression correlated proportionally with cell viability in the presence of 250 nmol/L flavopiridol (r = 0.86, P = 0.003) and negatively with apoptosis induced by flavopiridol in blast cell specimens derived from patients with AML, suggesting that BCRP plays a role in leukemia cellular resistance to flavopiridol[19].

Another conventional CDK inhibitor that was described to interact with BCRP is purvalanol A[169]. An *et al.*[169] showed that purvalanol A can effectively block the SRC signaling and cell cycle progression. These investigators studied the effect of a series of CDK inhibitors on BCRP-mediated transport by photosensitivity assay. Among compounds tested, purvalanol A inhibited BCRP-mediated hematoporphyrin most potently with an IC_{50} value of 3.5 μmol/L. WHP-180 is also a CDK inhibitor that was shown to inhibit BCRP function, but the rest of the agents tested, including bohemine, seliciclib (roscovitine), and olomoucine, showed only minimal effect on BCRP-mediated transport. According to the findings, planar structure of these CDK inhibitors may be an important factor for interactions with the active site of BCRP.

Interaction of BCRP with new CDK inhibitors under development has been reported. JNJ-7706621 (3,5-diamino-1,2,4-triazole) is an effective inhibitor for both CDK and aurora kinases[170]. Selection of human cervical carcinoma HeLa cells with JNJ-7706621 caused overexpression of BCRP, resulting in MDR in these cells (HeLa-6621)[171]. In the same study, the AUC of oral JNJ-7706621 was approximately 3-fold higher in *Bcrp1*$^{-/-}$ mice compared with wild-type mice, implying that this agent is transported by BCRP.

Molecular Mechanisms for BCRP Gene Expression

Transcriptional regulation of BCRP under physiological conditions

The human BCRP gene is localized to chromosome 4 (4q22), spans over 66 kb, and consists of 16 exons and 15 introns. The exon-intron organization of BCRP gene was originally described by Bailey-Dell *et al.*[172]. The translation start site is located in exon 2 (Ex2), and the translation termination site is located in Ex16. A putative transcription start site (TSS, +1), whose nucleotide position was defined as previously described (GenBank AF15130.1), was found 529 bp upstream of the Ex1 and Ex2 junction and an active proximal

promoter region was identified at nucleotides -300 to -50 relative to the TSS in cell lines of human choriocarcinoma (JAR, JEG-3, BeWo cells) and breast cancer (MCF-7)[172]. In the same study, the promoter region was characterized as TATA-less and as having a CAAT box and several Sp1 sites downstream from a putative CpG island (Figure 2). This is similar to the promoters reported for the *MDR1* (*ABCB1*) and *MRP1* (*ABCC1*) genes, which lack TATA boxes and have multiple Sp1 sites[173,174].

To date, several cis-acting elements have been reported in the proximal promoter region of BCRP by many groups, as summarized in Figure 2. Krishnamurthy *et al.*[26] found that *BCRP* gene transcription is activated by binding of the hypoxia-inducible factor 1α (HIF1α)/ ARNT heterodimer to a hypoxia response element (HRE) under low oxygen conditions. Because BCRP interacts with heme and other porphyrins, its regulation by hypoxia suggests that BCRP plays a role in protecting cells/tissue from protoporphyrin accumulation under hypoxia. Moreover, hormonal regulation of *BCRP* transcription has been reported during pregnancy in regard to its protective role for the fetus at the placental barrier[175-177]. Indeed, E2-mediated up-regulation of *BCRP* mRNA expression was shown in estrogen receptor (ER)-positive breast cancer T47D cells[178] and primary placental trophoblast cells[179]. Ee *et al.*[178] found that this stimulatory effect is mediated through the classical pathway that involves binding of E2/ER complexes on the estrogen response element in the *BCRP* promoter region. Subsequent studies observed estrogen-mediated transcriptional and post-transcriptional down-regulation of *BCRP* in BeWo choriocarcinoma cells[177] and in breast cancer cells[180], respectively. However, Wang *et al.*[175] reported that BCRP mRNA and protein expression was further increased by progesterone (P4) in combination with E2 in BeWo cells, suggesting that E2 and P4 are synergistically involved in up-regulation of BCRP expression. Although the precise mechanism is under investigation, these investigators demonstrated that P4-mediated up-regulation of BCRP could be triggered by direct binding of progesterone receptor B to a progesterone response element identified in the *BCRP* promoter[181]. Core sequences of that PRE were located at the same position as the ER element core sequence[178,181,182]. Therefore, *BCRP* transcription may be regulated by both ERs and PRs in a complex manner.

Other nuclear receptors are likely involved in transcriptional regulation of *BCRP*. BCRP is found to be transcriptionally up-regulated via activation of peroxisome proliferator-activated receptor (PPAR) γ in human myeloid dendritic cells[183]. A 150-bp long, conserved

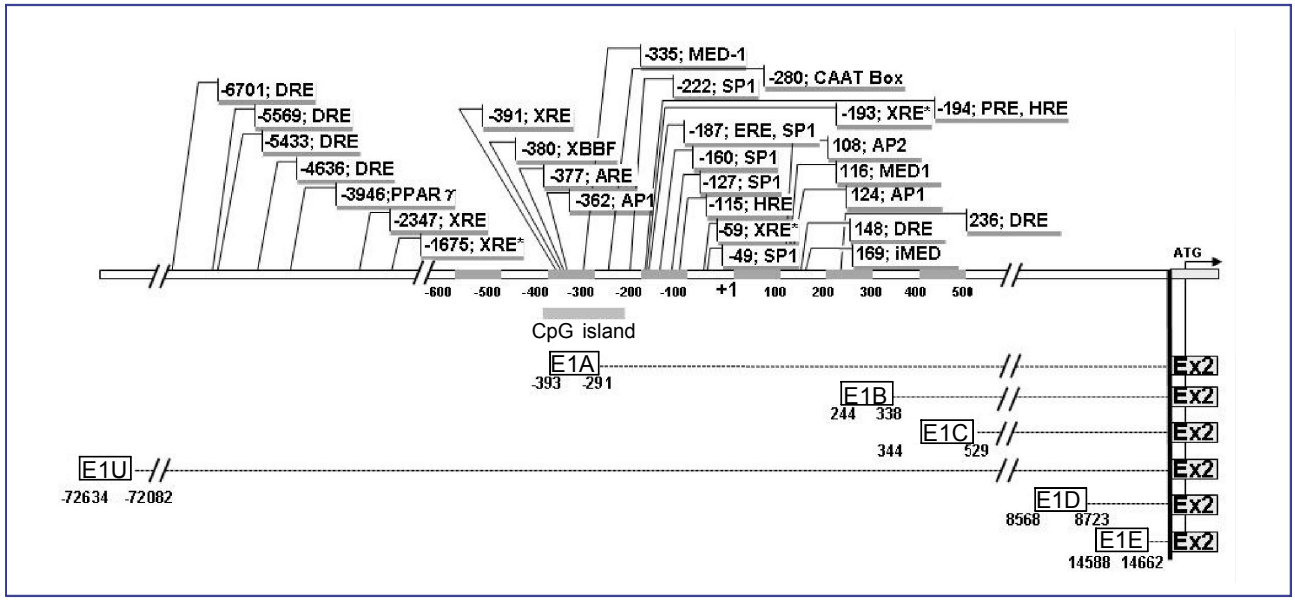

Figure 2. **Cis-regulatory elements in BCRP promoter and splice variants of BCRP transcripts.** Identified cis-acting elements are shown in the promoter region of *BCRP* gene with identified splice variants of BCRP mRNA. A putative transcription start site (TSS, +1), defined as previously described (GenBank AF15130.1), was found 529 bp upstream of the Ex1 and 2 junction, and 18899 bp upstream of Ex2. An active proximal promoter region was identified at nucleotides –300 to –50 relative to the TSS. The same Ex2 acceptor is used for all 5′-UTR exons (E1U, E1A, E1B, E1C, E1E, and E1D). Variable TSSs are found for E1U, E1B, and E1C. Figure is not completely to scale and all nucleotide positions are shown relative to the TSS (+1). XRE sites with superscript "*" are also identified as core sequences for DRE. PPAR, peroxisome proliferator-activated receptor; XRE, xenobiotic response element; DRE, dioxin response element; XBBF, X-box binding factor; ARE, antioxidant response element; HRE, hypoxia response element; PRE, progesterone response element; MED, multiple start site element downstream; iMED, inverted MED.

enhancer region containing three functional PPAR response elements was identified upstream of human *BCRP*, and binding of the PPARγ/retinoid X receptor (RXR) heterodimer to this region was confirmed. Moreover, *BCRP* was transcriptionally up- regulated by *PPAR*α-specific agonists (e.g. Wy14643 or GW7674) in the intestines and liver of wild-type mice but not in PPARα-null mice. Hence, *BCRP* transcription has been suggested to be regulated in a PPARα-dependent manner[184].

Previously, aryl hydrocarbon receptor (AHR) agonists, such as polycyclic aromatics (e.g. BP, indolo [3,2-b]carbazole and benzo[k]fluoranthene) and phytochemicals (e.g. quercetin, chrysin, and flavone), were shown to induce BCRP expression in Caco-2 cells, indicating an involvement of AHR in *BCRP* transcription[25,185]. *BCRP* mRNA expression was recently found to be enhanced by binding of the AHR/ARNT heterodimer in proximal and distal regions upstream of the *BCRP* TSS that contain AHR elements [AHREs, also known as xenobiotic response elements (XREs)][186,187].

Nrf2, a nuclear factor-erythroid 2-related transcription factor, plays a critical role in transcriptional up-regulation of many metabolizing enzymes and transporters that rescue cells from oxidative stress and/or electrophilic stress mediated by extracellular stimuli such as xenobiotics[188]. Nrf2 was found to function as a transcription factor for *BCRP* gene expression in hepatocytes: silencing *Nrf2* gene expression abolished induction of *BCRP* mRNA expression mediated by the Nrf2 inducer, tert-butylhydroquinone[189]. Detailed promoter analysis using luciferase reporter assays revealed an antioxidant response element (ARE) critical for the Nrf2-mediated expression in lung cancer cells[190]. These findings provide a rationale for up-regulation of functional BCRP by xenobiotics.

Furthermore, growth factors are also likely involved in *BCRP* gene transcription. Epidermal growth factor (EGF)-mediated activation of *BCRP* mRNA expression has been observed in ovarian[191] and breast[175] cancer cell lines. In breast cancer cells, this effect could involve ERK1/2 and c-jun N-terminal kinase, suggesting that *BCRP* mRNA expression may be under control of the MEK-ERK signaling pathway[175]. This notion is supported by the evidence that activation of EGF receptors (e.g. EGFR and HER2) increased the side population (SP) fraction in head and neck squamous cell carcinoma[192] and breast cancer cells[53]. However, the downstream transcription factors and *trans*-acting effectors have not been identified for EGF-mediated signaling pathways. Further studies are warranted to understand the mechanism underlying BCRP up-regulation by growth factors.

Alternative promoter utilization of BCRP

In general, alternative splice variants of 5′-untranslated leader exons are associated with alternative promoter usage. Such usage can result in diversity of gene expression in a variety of ways, including tissue- or cell type-specific gene expression and changes in the efficiency with which the mRNA with different leader exons is translated. We discovered at least three 5′-UTR splice variants of Ex1 (designated as E1A, E1B, and E1C) that are alternatively spliced to the common Ex2, compliant with the GT-AG rule. E1A, E1B, and E1C start at nucleotides -393, +244, and +344 and end at nucleotides -291, +338, and +529, respectively (Figure 2)[77]. These alternative splice variants are expressed in a tissue-specific manner among human normal tissues, suggesting transcriptional regulation by tissue-specific alternative promoter usage. In addition to the splice variants, our GenBank search predicted two more mRNA variants: one with an Ex1 located approximately 73 kb upstream from the TSS (designated E1U) and one that lacks Ex1 (that starts from Ex2, designated as E1-). This was further confirmed by Poonkuzhali *et al.*[193], who performed an extensive review of the expressed sequence tag (EST) database and genome assembly using Aceview (http://ncbi.nih.gov/IEB/Research/Acembly) and the University of California Santa Cruz genome browser (http://genome.ucsc.edu/). At least three different alternative first exons were found for BCRP: Ex1a (corresponding to E1C[77]), Ex1b (E1A), and Ex1c (E1U)[193]. Interestingly, liver samples that generated a *BCRP* transcript using Ex1b had significantly lower *BCRP* mRNA levels. Zong *et al.*[194] reported three isoforms of mouse *Bcrp1* mRNA that differ in their 5′-UTR (also denoted as E1a, E1b, and E1c) like human *BCRP*. Importantly, they found that the expression of mouse *Bcrp1* during hematopoiesis is transcriptionally regulated by alternative use of multiple leader exons and promoters in a developmental stage-specific manner, indicating a promising mechanism for the lineage-specific expression of BCRP in hematopoiesis in both humans and mice.

To date, a set of *cis*- and *trans*-acting control elements distinct for each alternative promoter has not yet been identified in humans or mice. Such information may help to explain how BCRP is expressed in a tissue-specific manner. Nevertheless, some light has been shed on this subject recently. Natarajan *et al.*[195] found that *Bcrp1* E1b mRNA spice variant expression, which is predominant in mouse intestine, is regulated by binding of phosphorylated cAMP-response element binding protein (p-CREB) to its *cis* site on the mouse E1b promoter region. Possibly, p-CREB may be involved in transcriptional regulation of the human *BCRP* mRNA variant expressed in the small intestine, E1C. This is currently under investigation in our laboratory.

BCRP alternative promoter usage was recently studied in bone marrow samples derived from pediatric

patients with M7 AM L by Campbell *et al*.[196]. They identified, similar to our prediction from a GenBank search, the leader exon E1U to be located approximately 73 kb upstream from TSS and reported small exons (designated $E1U_2$-$E1U_5$) between E1U and E1A (Figure 2). They also found two other Ex1s (designated E1D and E1E) located between E1C and Ex2 and reported that these Ex1s were expressed less frequently in bone marrow samples. Although the function of the extensive variability in the *BCRP* 5′-UTR is not yet fully understood, further investigations may clarify the role of alternative promoters in the tissue- and cell type-specific regulation of BCRP expression.

Post-translational regulation of BCRP function

Post-translational modifications (PTMs) of BCRP, including N-glycosylation and phosphorylation, have been reported in normal and cancer cells. The first described BCRP PTM was N-glycosylation of asparagine 596[197]. Although this modification did not affect BCRP function, it may be involved in expression of functional BCRP. N-glycosylation status was shown to be important for apical sorting in polarized cells such as hepatocytes[198]. We found that the proteasome is involved, at least in part, in the post-translational down-regulation of BCRP[128]. Nakagawa *et al*.[199,200] showed that N-glyco-

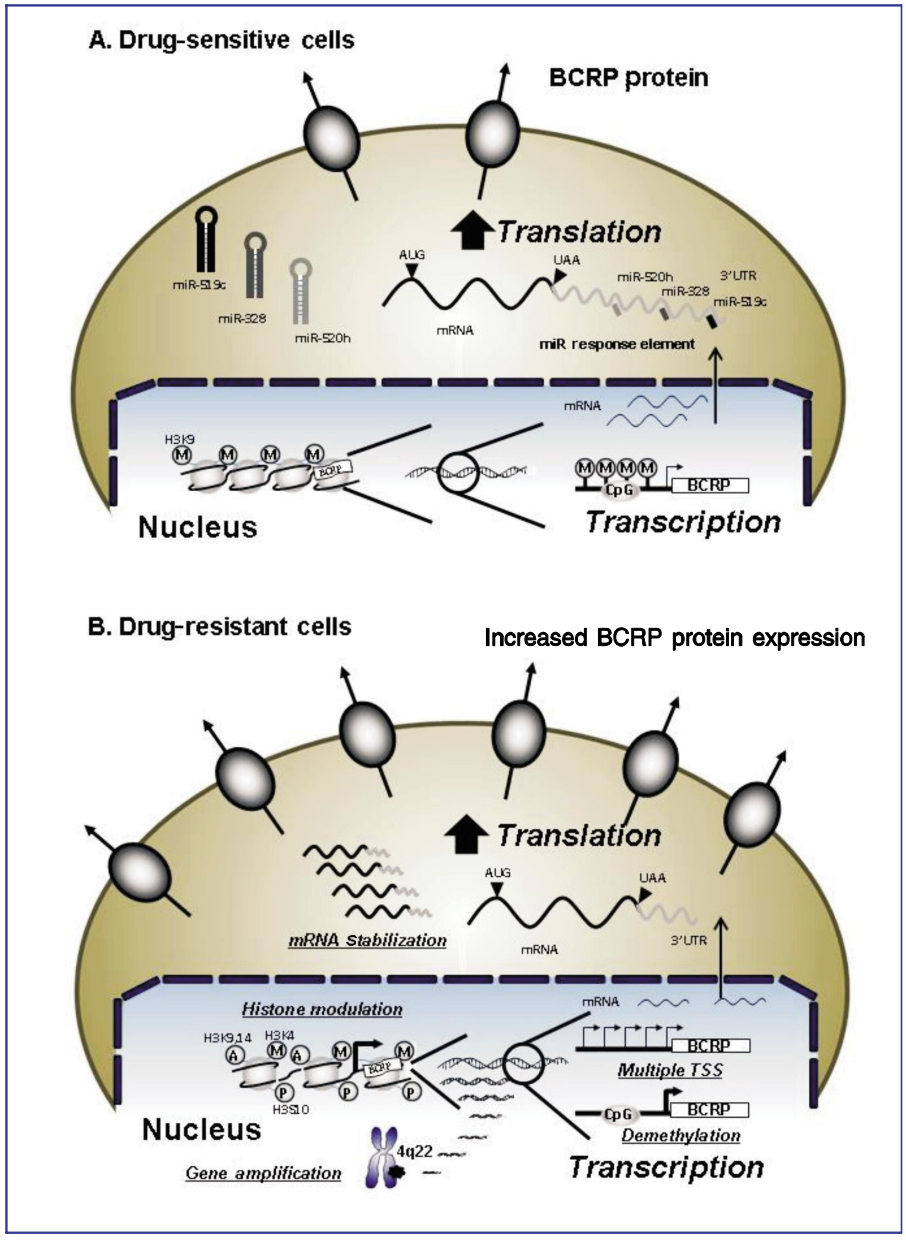

Figure 3. **Putative mechanisms for *BCRP* overexpression in drug-resistant cells.** A, in drug-sensitive cells, BCRP transcription is regulated by histone 3 trimethylated at lysine 9 (H3K9me3) and the proximal promoter region is reported to be methylated in cells prior to drug selection or treatment. Synthesized mRNA is negatively regulated by possible candidate miRs, including *miR-519c*, *miR-328*, and *miR-520h*, which are purported to bind miR response elements in 3′-UTR of *BCRP* mRNA. Thus, BCRP expression is transcriptionally and post-transcriptionally regulated. B, in drug-resistant cells, several possible mechanisms are hypothesized based on current evidence. *BCRP* gene amplification is observed in some drug-selected cancer cells. Transcription factors become more accessible because of histone 3 modulations, including acetylation at lysine 9 and 14 (H3K9ac, H3K14ac), methylation at lysine 4 (H3K4me), and phosphorylation at serine 10 (H3S10p). Multiple TSSs are likely used for induction of *BCRP*. Demethylation of CpG islands around promoter regions may contribute to overexpression of *BCRP* in response to drugs. Once *BCRP* mRNA is synthesized, its 3′-UTR becomes truncated, resulting in deletion of miR response elements, which in turn increases BCRP mRNA stability and levels. M: methylation; A: acetylation; P: phosphorylation.

sylation status may be important for BCRP post-translational down-regulation induced by ubiquitin-mediated, ER-associated degradation during the trafficking process. Once BCRP is shuttled to the plasma membrane, it is known to be degraded by the endosome-lysosome pathway after remaining in the plasma membrane domain for a certain period [201,202]. Translocation of BCRP is also regulated by PTMs. Xie *et al*. [104] found that BCRP is phosphorylated by the serine/threonine kinase Pim-1 (Pim-1L, 44kDa) at threonine 362, which is essential for its translocation to the plasma membrane, in human prostate cancer LNCaP cells. LNCaP cells transfected with *Pim-1L* cDNA became resistant to docetaxel, which was thought not to be substrate for BCRP. These findings suggest that phosphorylation at T362 is critical for translocation and substrate specificity of BCRP. Furthermore, disrupting the Pim-1 signaling to reverse BCRP-mediated MDR may be a potential therapeutic approach[104].

Post-translational regulation of BCRP has been extensively studied in relation to the PI3K/AKT signaling axis in a variety of cells. The first report showed that impaired PI3K/AKT signaling (in *Akt1*$^{-/-}$ mice) caused loss of the SP fraction in the bone marrow, as well as localization of BCRP in the endoplasmic reticulum and absence of BCRP in the active plasma membrane location [203]. To date, similar observations have been made in tumors including glioma [204] and HCC [205]. The PI3K/AKT signaling pathway may be involved in previously reported E2-induced post-transcriptional down-regulation in breast and choriocarcinoma cell lines [177,180]. Although the precise mechanism remains unclear, Hartz *et al*. [206] showed that BCRP transport function was promptly reduced in isolated brain capillaries treated with E2. This was further explained by internalization of BCRP protein after short-term exposure to E2 [207]. Under long-term exposure conditions, E2 signals through ERβ to activate the PTEN/PI3K/AKT/GSK axis, resulting in BCRP degradation through a process in which ubiquitination and proteasomal proteolysis may be involved.

In neurospheres produced from gliomas, loss of PTEN increased the SP fraction, and this increase was abolished with a PI3K inhibitor, LY294002. These results suggest that the PTEN/PI3K/AKT pathway is critical for BCRP function[204,208]. In BCR-ABL-dependent CML cells, we noted that inhibition of BCR-ABL by imatinib blocks the PI3K/AKT pathway, leading to post-transcriptional down-regulation of BCRP surface expression, which could attenuate the BCRP-mediated resistance to TKIs and confound experiments characterizing the interaction of TKIs with BCRP [128]. Similar observations were made with nilotinib and dasatinib in BCR-ABL-dependent model systems showing a concomitant down-regulation of phosphorylated CrkI, which undergoes phosphorylation by BCR-ABL as a substrate [129]. However, similar post-transcriptional down-regulation was also observed following imatinib treatment in head and neck squamous cell carcinoma in which BCR-ABL is not expressed [209]. This effect may be mediated by blocking tyrosine kinases other than BCR-ABL, such as c-KIT or SRC; hence, the precise mechanism needs to be addressed further.

Folate deprivation was found to induce BCRP expression associated with mitoxantrone resistance in Caco-2 cells. Interestingly, BCRP expression was associated with a cytoplasmic compartment in these cells, possibly resulting in intracellular drug sequestration as a mechanism of resistance, rather than the classical mechanism of efflux of drugs from cells[210].

Regulation of BCRP expression in side population and other stem cells

Since the first report of a distinct SP in neuroblastoma cells [50], SP cells have been reported in various human cancer cell lines, including gastrointestinal [51], lung [52], breast [53,54], hepatic [55], esophageal [56], and pancreatic [57] cancer cell lines. SP cells have been shown to be highly tumorigenic in immunodeficient mice and to possess a potent ability for colony formation and proliferation. Accordingly, SP cells are enriched for cancer stem cells (CSCs). The low Hoechst dye accumulation that is characteristic of the SP fraction is mainly mediated by expression of functional BCRP [40,53]; therefore, SP cells have been thought to be highly resistant to conventional chemotherapeutic agents. Modulation of BCRP function in the CSC population may provide a promising way to overcome MDR by efficiently eradicating CSCs. To date, molecular mechanisms underlying BCRP expression in CSCs are not fully understood. Krishnamurthy *et al*. [26] showed that, under hypoxia, progenitor cells from *Bcrp1*$^{-/-}$ mice had reduced ability to form colonies as compared with those from *Bcrp1*$^{+/+}$ mice. Inhibiting heme biosynthesis rescued *Bcrp1*$^{-/-}$ progenitor cells. Thus, BCRP has been postulated to play a critical role in protecting progenitors/stem cells from intracellular accumulation of heme-related molecules (e.g. porphyrin) to enable cell survival under conditions of hypoxia. In this regard, BCRP transcription may be regulated in stem cells via binding of the HIF1/ARNT heterodimer to the HRE in the promoter region of *BCRP* upon hypoxic demand.

The PI3K/AKT pathway may be essential for expression of functional BCRP in stem cells. Recently, we found that HER2 expression was significantly correlated with the presence of an SP in luminal types of breast cancer cell lines and primary cells from breast cancer patients [53]. The occurrence of SP and *in vivo* tumorigenicity of SP cells decreased when cells were treated with HER2 signaling inhibitor AG825 or trastuzumab, which reduced phosphorylation of HER3

and AKT. These results suggest that PI3K/AKT signaling triggered by HER2/HER3 heterodimers contributes to expression of functional BCRP in SP cells. Accordingly, HER2-induced enhancement of SP cells may be a new rationale for the aggressive phenotype of HER2$^+$ breast tumors. How BCRP functionality is augmented by the HER2 signaling is poorly understood, and further study is warranted to clarify this molecular mechanism.

The pluripotent *Oct4* gene may play a role in maintenance of cancer stem cell-like properties[211]. Oct4 induces transcription of T-cell leukemia/lymphoma 1A (TCL1A), resulting in activation of AKT in embryonic stem cells[212]. Wang *et al*.[213] found that overexpression of Oct4 resulted in induction of BCRP expression in chemoresistant HCC cell lines and tumor samples from patients with HCC, suggesting that BCRP is under control of the Oct4/TCL1/AKT signaling pathway in HCCs. Because the AKT signaling may modulate BCRP function through regulating its intracellular localization[203,208] and post-transcriptionally down-regulating BCRP expression in BCR-ABL$^+$ CML cells[128], these findings suggest there is likely a link between upstream effectors of AKT and BCRP in stem cell populations. However, how the AKT signaling regulates BCRP function through translocation, up-regulation of BCRP expression, or other mechanisms still remains unknown.

Sal-like protein 4 (SALL4), a zinc finger transcription factor essential for histogenesis, was recently shown to activate BCRP expression indirectly, suggesting that SALL4 regulates the function of BCRP in SP cells[214]. The homeobox gene *MSX2* is an inducer of epithelial-mesenchymal transition. *In vitro* promoter analysis suggests that MSX2 requires the SP1-binding site in the *BCRP* promoter region so that MX2 and SP1 may cooperatively regulate *BCRP* transcription in pancreatic cancer stem cells[215]. *BCRP* expression can be transcriptionally silenced by TGF-β through direct binding of its downstream targets, Smad2/3, to the *BCRP* promoter/enhancer, resulting in a decrease in the number of SP cells. This observation suggests a role for TGF-β in negative regulation to maintain the cancer-initiating cells within gastric cancer[216]. In addition to such complex transcriptional regulation, post-transcriptional regulation of *BCRP* mediated by microRNAs (miRs) has been reported in some cancer cells. In the stem-like (BCRP$^+$) cell population fractionated from RB143 human retinoblastoma cells, *miR-328*, *miR-519c*, and *miR-520h* levels are found to be lowered 9-, 15-, and 3-fold, respectively, suggesting that the high level of BCRP expression is regulated by these miRs[217]. Other mechanisms may relate to the methylation status of the CpG island region of the *BCRP* promoter. In human prostate cancer, BCRP is reportedly up-regulated in response to the DNA- demethylating reagent 5-aza-dC in cancer-initiating cells marked by CD117$^+$/BCRP$^+$, suggesting that *BCRP* transcription is negatively regulated by hypermethylation of CpG sites in the promoter[218].

Putative Mechanisms for Over-expression of BCRP in Response to Drug Treatments

Previously reported mechanisms for differential expression of BCRP in cells selected with chemotherapeutic agents compared to parental, drug-sensitive cells include gene amplification, histone modification, and miR-mediated regulation. Recent progresses are illustrated in Figure 3.

Gene amplification

Comparative genomic hybridization and cDNA hybridization studies demonstrated a high level of *BCRP* amplification in cancer cell lines treated with mitoxantrone (MCF-7/MX) and, to a less extent, with doxorubicin in the presence of verapamil (MCF-7/AdrVp3000)[219]. These cell lines, produced by high selective pressure, highly overexpress BCRP. However, no amplification was observed in another cancer cell line selected with mitoxantrone (S1M1 80)[219]. This result was further confirmed by Volk *et al*.[88], who showed that not all cancer cell lines selected with mitoxantrone had *BCRP* gene amplification. We examined amplification of *BCRP* in MDR cancer cells, MCF-7 breast cancer cell sublines (MCF/MX and MCF/AdrVp), and human ovarian cancer IGROV1 sublines (IGROV1/MX and IGROV1/T8) by quantitative PCR. *BCRP* gene copy number was 15-fold greater in MCF-7/MX (selected with mitoxantrone) than in parental cells or MCF-7/AdrVp cells (selected with doxorubicin with verapamil)[77]. No *BCRP* amplification occurred in IGROV1/MX3 (selected with mitoxantrone) or IGROV1/T8 (selected with topotecan) cells relative to their respective parental cells. These findings suggest that gene amplification may be induced in response to drugs; however, gene amplification does not necessarily occur in all drug-selected MDR cancer cells. The reason for this differential effect is poorly understood.

Multiple transcription start sites (TSSs) and alternative promoter usage

In MCF-7 and IGROV1 cells, each splice variant (E1A, E1B, or E1C) generally has a distinct major TSS, whereas the TSS for these Ex1 variants in drug-resistant MCF-7/AdrVp and IGROV1/T8 cells are more heterogeneous. This is particularly true for the E1C variant, such that no single TSS can be assigned for a

given exonal variant[77]. Moreover, our study showed that the proportion of E1C *BCRP* transcripts increased to up to 47% of the total *BCRP* mRNA transcripts in MCF-7/AdrVp cells, whereas this transcript variant was only 23% of the *BCRP* transcripts in parental MCF-7 cells. In contrast, proportion of the E1A transcripts to total transcripts was much less (approximately 6%) than that of E1C in MCF-7/AdrVp cells; however, the E1A transcript was predominant over E1C in MCF-7 cells. Similar observations were made in sublines of drug-selected IGROV1 cells, implying that alternative promoters using different TSSs are used to induce *BCRP* transcription. Heterogeneity of the TSS for Ex1 observed in drug-resistant MCF-7/AdrVp cells reflects the previous report by Ince *et al*.[220] that multiple TSSs in *Mdr1* were often observed in actinomycin D-selected sublines of Chinese hamster lung DC-3F cells, which overexpress *Mdr1* mRNA without a concomitant increase in *Mdr1* gene amplification. The same investigators later described that this multiple TSSs could be associated with the response element, termed as MED-1 (multiple start site element downstream), in the *Mdr1* promoter region for RNA polymerase II, which can initiate transcription at distinctive sites. In human *MDR1* gene, response element termed inverted MED (iMED) was indicated to act as a *cis*-activator for the *MDR1* gene[221,222]. Indeed, MED-1 and iMED elements are located in the *BCRP* promoter region as we reported[77] (Figure 2). Further study of the role that these response elements play in *BCRP* expression is reasonable to identifying *trans*-acting factors for each promoter region.

Demethylation

Methylation of the 5′-carbon of cytosine in the CpG island of a gene promoter is a well-known epigenetic modification that silences gene expression. Methylation has been reported in the CpG island within the proximal *BCRP* promoter region in RCC cell lines such as UOK121 and UOK143. Furthermore, *BCRP* expression was up-regulated when these cells were treated with the DNA-demethylating agent, 5-aza-2′-deoxycitidine (5-aza-dC)[223]. Other investigators found that DNA demethylation is a molecular mechanism by which *BCRP* is overexpressed in response to drug exposure. Turner *et al*.[224] found that *BCRP* transcription is up-regulated because of demethylation of the CpG island in the promoter in human multiple myeloma 8226 cells selected with mitoxantrone (8226/MR). *BCRP* promoter methylation appeared to be present not only in CCRF-CEM and Jurkat leukemia cells and primary T-ALL lymphoblasts obtained from ALL patients, but also in IGROV1 ovarian carcinoma cells[225]. In solid tumors, we examined the methylation status of the *BCRP* promoter in drug-resistant MCF-7/AdrVp breast cancer cells and IGROV1/MX3 and IGROV1/T8 ovarian cancer cells and found no demethylation compared to parental cells[77]. However, Bram *et al*.[225] described prominent *BCRP* promoter demethylation in IGROV1/MX3 and IGROV1/T8 cells compared with their parental IGROV1 cells. Because no *BCRP* gene amplification was found in these cells, demethylation of the promoter region could be the cause for *BCRP* up-regulation in response to drugs.

Histone modifications

Recently, it has become increasingly evident that histone modulation affects BCRP expression in response to drug exposure. Histone deacetylase inhibitors (HDACi), including vorinostat and romidepsin, are in clinical use[226]. Indeed, BCRP activation has been observed in malignant peripheral blood mononuclear cells and S1 colorectal cancer cells treated with romidepsin, which increases acetylated histone H3[227]. Another HDACi phenylbutyrate[228] induced BCRP expression in S1 and KG-1a leukemia cells. Histone hyperacetylation resulting from a single-step selection with anticancer agents (e.g. doxorubicin) was associated with overexpression of BCRP in cancer cell lines MCF-7, IGROV1, and S1[229]. Furthermore, Hauswald *et al*.[230] demonstrated that BCRP and P-gp could be induced in mononuclear leukemic cells derived from a patient with relapsed AML when treated with HDACi (e.g. phenylbutyrate), with histone being hyperacetylated in the promoter region of *BCRP*. These findings show that exposure of cancer cells to HDACi induces a drug resistance phenotype that might negatively affect treatment effectiveness. To *et al*.[226] hypothesized an association of the *BCRP* promoter with histone H3 trimethylated at Lys 9 (H3K9me3) keeps BCRP expression low in drug-sensitive cells. According to the hypothetical model, once the cells are treated with drugs, H3 modifications, including acetylation at Lys 9 and 14 (H3K9ac, H3K14ac), trimethylation at Lys 4 (H3K4me3), and phosphorylation at Ser 10 (H3S10ph), induce recruitment of a chromatin remodeling factor (Brg-1) and RNA polymerase IIs, resulting in withdrawal of class I HDACs from the *BCRP* promoter. This modification functions as a switch to open the chromatin configuration to enhance *BCRP* transcription. Thus, H3 modulation seems to be a relevant rationale for manipulating BCRP expression; however, this needs to be examined further because HDACi such as romidepsin has not induced *BCRP* mRNA expression in all cell lines studied so far[226].

Regulation of *BCRP* gene expression by microRNA (miR)

To date, several miR response elements that might

target *BCRP* mRNA 3′-UTR have been identified using multiple algorithms[231,232]. Among them, *miR-519c* may be involved in BCRP overexpression in drug-resistant cancer cells. To *et al.*[232] found that *BCRP* mRNA with a truncated 3′-UTR is expressed in drug-resistant S1M1 80 cells but not in their parental S1 cells. Because the shorter 3′-UTR lacked the miR response element target site(s) for *miR-519c*, *BCRP* mRNA became stabilized, resulting in BCRP overexpression in the drug-resistant cells. Shortening of the *BCRP* mRNA 3′-UTR has been reported in various BCRP-overexpressing resistant cell lines, including MCF-7/FLV1000, SF295/MX2000, H460/MX20, and A549/Beca250[233]. Thus, instability of BCRP mRNA 3′-UTR in response to drugs may be one of the mechanisms for induction of BCRP expression in selected cancer cells. *miR-328* is also involved in post-transcriptional regulation of *BCRP* because *miR-328* negatively regulates both mRNA and protein expression of BCRP and is underexpressed in MDR cancer cells such as MCF-7/MX100 cells[231]. Another miR postulated to affect BCRP expression is *miR-520h*. Although BCRP expression was unchanged in MCF-7/MX100 cells with forced expression of *miR-520h* [217], *miR-520h* is likely involved in BCRP expression during differentiation of hematopoietic stem cells. *miR-520h* is enriched in hematopoietic stem cells but underexpressed in $CD34^+$ cells, thereby maintaining BCRP expression in the stem cell population[234]. This was also noted in pancreatic cancer PANC-2 cells because introduction of *miR-520h* resulted in cell migration and invasion as well as reduction of the side population[235].

BCRP Polymorphisms and Cancer Treatment Outcomes

Many single nucleotide polymorphisms (SNPs) have been observed in the *BCRP* gene[236]. Honjo *et al.*[18] first described 7 SNPs in the intron sequences, 3 non-coding SNPs in the UTR, 2 synonymous SNPs, and 3 non-synonymous SNPs [c.34G>A (V12M, Ex2; rs2231137), c.421C>A (Q141K, Ex5; rs2231142), and c.1858G>A (D620N, Ex16; rs34783571)]. Transport activity of BCRP variant proteins V12M and Q141K have been studied the most frequently and have been found to decrease BCRP function significantly when transfected into cultured cells[237-239]. Reduction of the activity of the V12M variant resulted from its decreased expression at the apical membranes[238]. Tamura *et al.*[240,241] reanalyzed the transport activity of BCRP protein when 7 known BCRP variants due to SNPs, including V12M, Q141K, F208S (c.623T>C, Ex6; rs1061018), and S248P (c.742T>C, Ex7; rs3116448), were expressed in Flp-In-293 cells. The results indicated that relative to wild-type BCRP-expressing cells, the IC_{50} value of SN38 against Q141K-expressing Flp-In-293 cells was reduced approximately 50%, but it was not reduced in V12M-expressing cells. Interestingly, the IC_{50} values of SN38, mitoxantrone, doxorubicin, daunorubicin, and etoposide for Flp-In-293 cells expressing other variants were significantly lower than those for cells expressing wild-type BCRP. Notably, F208S and S441N were not expressed in the cells, suggesting that the rest of the variants (S248P, F431L, and F489L) may also impart impaired function of BCRP [241]. Another study showed that the P269S (c.805C>T, Ex7; rs34678167) and Q126Stop (c.376C>T, Ex4; rs72552713) alleles were found among Korean subjects, and that the transport activity of the P296S variant decreased based on methotrexate uptake by vesicles expressing this variant[242]. Not all *BCRP* SNPs are associated with diminished transporter activity, however. The I206L (c.616A>C, Ex6; rs12721643) variant was found to have high transporter activity but low protein expression when transfected into HEK cells, whereas the N590Y (c.1768A>T, Ex15; rs34264773) and D620N had higher expression but lower activity[243].

The effects of promoter and non-coding region SNPs on BCRP expression in the liver, intestines and lymphoblasts were recently investigated by Poonkuzhali *et al.*[193]. Forty-one SNPs were found in the promoter region and 49 in the introns. Promoter and intron 1 alleles were found to be associated with altered *BCRP* mRNA expression. Furthermore, a decrease in BCRP expression may be caused by -30477C>G (rs2127861), -15622C>T (in upstream region), and 1143G>A (in intron 1; rs2622604), and an increase in BCRP expression may be caused by -15994C>T (rs7699188), -15846A>C (in upstream region), and 12283T>C and 16702C>T (in intron 1; rs2046134). Interestingly, these investigators showed that individuals with the -15994C>T polymorphism had significantly higher clearance of oral imatinib.

Previous pharmacokinetic studies have shown that people with *BCRP* polymorphisms can experience significant alterations in the absorption, distribution, metabolism, or elimination of BCRP substrate drugs. The oral bioavailability of topotecan increased significantly in patients heterozygous for the c.421C>A allele [244]. Another work showed that cancer patients heterozygous for the c.421C>A (Q141K) allele had approximately 3-fold higher plasma levels of intravenous diflomotecan (a 10,11-difluoro-homocamptothecin), a new promising topoisomerase I inhibitor with enhanced plasma stability and superior preclinical anti-tumor activity, than those harboring the wild-type allele, implying that diflomotecan can be transported by BCRP[245]. The heterozygous c.421C>A allele also did not appear to affect the pharmacokinetics of irinotecan or its metabolite SN38 [246] despite that BCRP transports SN38 and its glucuronide [90,94]. Similarly, the c.34G>A or c.421C>A

polymorphisms of *BCRP* were not associated with alterations in the pharmacokinetics of irinotecan, SN38, or SN38 glucuronide, nor were they associated with the tumor response rate or toxicity in Korean patients with advanced NSCLC treated with irinotecan [247]. Recent progress in research of both functional SNPs and BCRP inhibitory agents that modulate the *in vivo* pharmacokinetics and pharmacodynamics of BCRP substrate drugs are reviewed elsewhere[248].

In a study of 200 Japanese patients with non-papillary RCC and age- and sex-matched controls, Korenaga *et al.* [249] investigated the c.421C>A (Q141K) allele and found that individuals with the wild-type C/C genotype had a higher risk for developing non-papillary RCC . These authors concluded that *BCRP* may be a candidate RCC susceptibility gene. Increased susceptibility and shorter survival of patients with diffuse large B-cell lymphoma was observed in subjects with the c.34G>A or c.421C>A alleles[250]. A recent study by Hahn *et al.* [251] showed that patients with the c.421C>A (C/A) allele and hormone-refractory prostate cancer had significantly longer survival beyond 15 months compared to those with the wild-type (C/C) genotype when treated with docetaxel-based combination chemotherapy . Similarly, Müller *et al.* [252] found shorter overall survival after treatment with platinum-based regimens in a large series of patients with small cell lung cancer and NSCLC who carried the 421A allele of *BCRP*. Because platinum-based drugs are not identified as substrates for BCRP, the precise reason for this phenotype is not clear. Further study is needed to address this issue.

Conclusions

BCRP plays a significant role in pharmacokinetics and contributes to MDR in cancer. Therefore, to overcome MDR, it is important to understand how this transporter exerts its function and how its expression is regulated, as this information may provide clues for developing compounds that modulate BCRP activity. As we reviewed here, many mechanisms are involved in BCRP expression, suggesting that a complex overall mechanism underlies expression among a variety of tissues. BCRP function is likely regulated by the PI3K/AKT signaling pathway in a transcriptional and post-translational manner; however, the precise molecular mechanism used by this signaling cascade has not yet been determined. Because this signaling pathway is also critical for cancer cell survival and proliferation, further investigations may clarify common molecular targets that modulate BCRP expression and activity as well as cell proliferation and growth. Cancer stem cells have been recently the subject of increased scrutiny, and BCRP expression in such cell populations is garnering attention because of its potential to confer drug resistance. Current evidence does not explain sufficiently how BCRP is regulated in undifferentiated cells. Therefore, it is worthwhile to address this issue in terms of circumventing transporter-mediated MDR acquired by cancer cells by modulating activity of MDR transporters such as BCRP to sensitize cancer stem cells to conventional cancer chemotherapeutic agents and to efficiently eradicate them from tumor mass, thereby providing a clinical benefit for patients.

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