CONTRIBUTIONS OF TM5, ECL3 AND TM6 OF HUMAN BCRP TO ITS OLIGOMERIZATION ACTIVITIES AND TRANSPORT FUNCTIONS

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Dedicated to my family, for their endless love, inspiration and support that has guided me throughout my life. To my dearest,

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Abstract

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Contributions of TM5, ECL3 and TM6 of human BCRP to its oligomerization activities and transport functions

Human BCRP is one of the major ATP-binding cassette transporters involved in the development of multidrug resistance in cancer chemotherapy. Overexpression of BCRP in the tumor cell plasma membrane and apical membrane of the gastrointestinal tract leads to decreased intracellular accumulation of various anticancer drugs as well as reduced drug bioavailability. BCRP has been shown to exist on the plasma membrane as higher forms of homo-oligomers. In addition, the oligomerization domain of BCRP has been mapped to the carboxyl-terminal TM5-ECL3-TM6 and this truncated domain, when co-expressed with the full-length BCRP, displays a dominant inhibitory activity on BCRP function. Thus, the oligomerization of BCRP could be a promising target in reversing multidrug resistance mediated by BCRP.

To further dissect the oligomerization domains of human BCRP and test the hypothesis that TM5, ECL3, and TM6 each plays a role in BCRP oligomerization and function, we engineered a series of BCRP domain-swapping constructs with alterations at TM5-ECL3-TM6 and further generated HEK293 cells stably expressing wild-type or each domain-swapping construct of BCRP. Using co-immunoprecipitation and chemical cross-linking, we found that TM5, ECL3,

and TM6 all appear to partially contribute to BCRP oligomerization, which are responsible for the formation of oligomeric BCRP. However, only TM5 appears to be a major contributor to the transport activity and drug resistance mediated by BCRP, while ECL3 or TM6 is insufficient for BCRP functions. Taken together, these findings suggest that homo-oligomeric human BCRP may be formed by the interactions among TM5, ECL3 and TM6, and TM5 is a crucial domain for BCRP functions and BCRP-mediated drug resistance. These findings may further be used to explore targets for therapeutic development to reverse BCRP-mediated drug resistance and increase the bioavailability of anti-cancer drugs for better treatment of multidrug resistant cancers.

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List of Abbreviations

BCRP	Breast cancer resistance protein
MDR	Multidrug resistance
ABC	ATP-binding cassette
Pgp	P-glycoprotein
MRP1	Multidrug resistance associated protein 1
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
VLCFA	Very long chain fatty acid
NBD	Nucleotide binding domain
MSD	Membrane spanning domain
TM	Transmembrane segment
ER	Endoplasmic reticulum
ECL	Extracellular loop
CPT	Camptothecin
TKI	Tyrosine kinase inhibitor
PhA	Pheophorbide a
MTX	Methotrexate
E1S	Estrone 3-sulfate
IAAP	[(125)I]Iodoarylazidoprazosin
NRTI	Nucleoside reverse transcriptase inhibitor
SP	Side population

GI	Gastrointestinal
FACS	Fluorescence-activated cell sorting
RT-PCR	Reverse transcription-polymerase chain reaction
IHC	Immunohistochemistry
AML	Acute myeloid leukemia
ALL	Acute lymphocytic leukemia
ICC	Immunocytochemistry
FCM	Flow cytometry
DFS	Disease-free survival
CGH	Comparative genomic hybridization
FISH	Fluorescence in situ hybridization
dmins	Double minute chromosomes
hsr	Homogeneously staining regions
ER	Estrogen receptor
HIF	Hypoxia-inducible factor
PPARγ	Peroxisome proliferator-activated receptor gamma
PGR	Progesterone receptor
AHR	Aryl hydrocarbon receptor
UTR	Untranslated region
ERE	Estrogen response element
HRE	Hypoxia response element
EMSA	Electrophoretic mobility shift assay
PRE	Progesterone response element

Nrf2	Nuclear factor (erythroid-derived 2)-like 2
DRE	Dioxin-response element
ARE	Antioxidant response element
MRE	MicroRNA response element
ERAD	Endoplasmic reticulum associated degradation
SNP	Single nucleotide polymorphism
FTC	Fumitremorgin C
THC	Tetrahydrocurcumin
DSS	Disuccinimidyl suberate
ECL	Enhanced chemiluminescence
HEK293	Human embryonic kidney 293 cells
HRP	Horseradish peroxidase
GRAVY	Grand average of hydropathicity
FRET	Fluorescence resonance energy transfer
BRET	Bioluminescence resonance energy transfer

I. Introduction

Chemotherapy has been a major form of treatment for various cancers since the 1940s. However, ineffectiveness and failure of the chemotherapy with a single agent was soon observed. This is due to the ability of cancer cells to mutate spontaneously at a rate of approximately 10^{-7} cells per generation, acquiring resistance to the single agent in response to the pressures imposed by the drug treatment (Boesen et al., 1994). In order to resolve this issue, the break through concept of combination therapy was introduced into cancer chemotherapy in the 1960s, which was based on the premise that the emergence of resistant cancer could be prevented with an alternating combination of drugs that have different intracellular targets. Nevertheless, multidrug resistance (MDR), which refers to the ability of organisms and cells to display resistance to a wide range of drugs that are structurally and functionally unrelated, has become a pervasive clinical phenomenon in a majority of cancers ever since the introduction of combination therapy. In spite of the fact that combination therapy has proved its effectiveness in cancers, MDR is now a major obstacle to successful cancer chemotherapy.

Cellular and molecular mechanisms of MDR have been extensively examined and reviewed in detail (Gottesman et al., 1994, Gottesman et al., 2002, Szakacs et al., 2006, Gillet and Gottesman, 2010). Studies with drug-selected model cell lines have demonstrated that overexpression of certain members from the ATP-binding cassette (ABC) transporter superfamily, including P-glycoprotein (Pgp, ABCB1), multidrug resistance associated protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2), is one of the major

mechanisms involved in MDR. The increased expression of these pre-existing ABC transporters on plasma membranes have led to increased efflux and decreased intracellular concentrations of their substrates, many of them being anti-cancer drugs.

The ABC transporters represent the largest family of transmembrane proteins. There are now 48 known human ABC transporters, and they have been divided into seven distinct subfamilies from ABCA through ABCG, based on gene structure similarities and sequence homology (Table 1). Human ABC transporters are exclusively exporters. They utilize the energy from ATP hydrolysis and are predominantly involved in the efflux of essential endogenous compounds, including amino acids, metabolic products, vitamins, lipids and sterols, as well as exogenous drugs and toxins, from the cytoplasm into the extracellular space or the intracellular compartments (endoplasmic reticulum, mitochondria, peroxisome, etc). Therefore, human ABC transporters play essential roles in a majority of physiological, pathological, and pharmacological processes.

Table 1: List of human ABC transporters.

Name	Domain structure	Major functions
	ABCA subfamily	
ABCA1	MSD_1-NBD_1-MSD_2-NBD_2	Cholesterol and phospholipids efflux
ABCA2	MSD_1-NBD_1-MSD_2-NBD_2	Drug resistance
ABCA3	MSD_1-NBD_1-MSD_2-NBD_2	Surfactant secretion
ABCA4	MSD_1-NBD_1-MSD_2-NBD_2	Retina-specific and efflux N-retinylidene-PE
ABCA5	MSD_1-NBD_1-MSD_2-NBD_2	Cholesterol efflux
ABCA6	MSD_1-NBD_1-MSD_2-NBD_2	Macrophage lipid homeostasis?
ABCA7	MSD_1-NBD_1-MSD_2-NBD_2	Lipid homeostasis?
ABCA8	MSD_1-NBD_1-MSD_2-NBD_2	
ABCA9	MSD_1-NBD_1-MSD_2-NBD_2	Monocyte differentiation and macrophage lipid homeostasis?
ABCA10	MSD_1-NBD_1-MSD_2-NBD_2	Macrophage lipid homeostasis?
ABCA12	MSD_1-NBD_1-MSD_2-NBD_2	Keratinocyte differentiation
ABCA13	MSD_1-NBD_1-MSD_2-NBD_2	Susceptibility factor for schizophrenia
	ABCB subfamily	
ABCB1	MSD_1-NBD_1-MSD_2-NBD_2	MDR
ABCB2	MSD-NBD	Peptide efflux
ABCB3	MSD-NBD	Peptide efflux
ABCB4	MSD_1-NBD_1-MSD_2-NBD_2	Phosphatidylcholine (Frank et al.) secretion
ABCB5	MSD_1-NBD_1-MSD_2-NBD_2	Drug resistance
ABCB6	MSD-NBD	Iron efflux
ABCB7	MSD-NBD	Iron/sulfur cluster efflux
ABCB8	MSD-NBD	Drug resistance
ABCB9	MSD-NBD	Peptide efflux
ABCB10	MSD-NBD	Peptide efflux?
ABCB11	MSD_1-NBD_1-MSD_2-NBD_2	Bile salt efflux
	ABCC subfamily	
ABCC1	MSD_0-MSD_1-NBD_1-MSD_2-NBD_2	MDR
ABCC2	MSD_0-MSD_1-NBD_1-MSD_2-NBD_2	Organic anion efflux
ABCC3	MSD_0-MSD_1-NBD_1-MSD_2-NBD_2	Organic anion efflux
ABCC4	MSD_1-NBD_1-MSD_2-NBD_2	Nucleoside efflux
ABCC5	MSD_1-NBD_1-MSD_2-NBD_2	Nucleoside efflux
ABCC6	MSD_0-MSD_1-NBD_1-MSD_2-NBD_2	Organic anion efflux?

ABCC7	MSD_1-NBD_1-MSD_2-NBD_2	Chloride ion channel
ABCC8	MSD_0-MSD_1-NBD_1-MSD_2-NBD_2	K^+ channel regulation
ABCC9	MSD_0-MSD_1-NBD_1-MSD_2-NBD_2	K^+ channel regulation
ABCC10	MSD_0-MSD_1-NBD_1-MSD_2-NBD_2	Organic anion efflux?
ABCC11	MSD_1-NBD_1-MSD_2-NBD_2	Drug resistance
ABCC12	MSD_1-NBD_1-MSD_2-NBD_2	Drug resistance
ABCD subfamily		
ABCD1	MSD-NBD	Very long chain fatty acid (VLCFA) efflux
ABCD2	MSD-NBD	VLCFA and DHA metabolism
ABCD3	MSD-NBD	VLCFA metabolism?
ABCD4	MSD-NBD	VLCFA metabolism?
ABCE subfamily		
ABCE1	NBD_1-NBD_2	Translation termination and ribosome recycling
ABCF subfamily		
ABCF1	NBD_1-NBD_2	
ABCF2	NBD_1-NBD_2	Drug resistance?
ABCF3	NBD_1-NBD_2	
ABCG subfamily		
ABCG1	NBD-MSD	Cholesterol efflux
ABCG2	NBD-MSD	MDR
ABCG4	NBD-MSD	Cholesterol efflux
ABCG5	NBD-MSD	Sterol efflux
ABCG8	NBD-MSD	Sterol efflux

Summarized in (Dean et al., 2001, Cascorbi and Haenisch, 2010)

The human breast cancer resistance protein (BCRP) is one of the human ABC transporters that have been implicated in the MDR in cancer chemotherapy (Szakacs et al., 2006). The BCRP gene was cloned independently from both drug-selected model cell lines and non-drug selected human cDNA library. BCRP cloned by Doyle's group from the human breast cancer MCF-7/AdVp subline was termed BCRP for breast cancer resistance protein (Doyle et al., 1998). Simultaneously, Allikmets' group searched expressed sequence tag databases and described a nearly identical transporter, named ABCP for its high expression in the placenta (Allikmets et al., 1998). Shortly after, the cDNA of this transporter was cloned from a mitoxantrone-selected human colon carcinoma cell line, S1-M1-80, and was designated MXR for mitoxantrone resistance gene (Maliepaard et al., 1999). This transporter was later assigned as ABCG2 by the Human Genome Nomenclature Committee, as a second member of the 'G' subfamily of ABC transporters.

Human BCRP is clinically significant in prognosis of both hematopoietic and solid malignancies, in the development of both innate and acquired MDR, and in the regulation of drug bioavailability. Thus, BCRP has been considered as promising in targeted cancer chemotherapy, since inhibition of BCRP increases not only the intracellular level but also the systemic level of anti-cancer BCRP substrates, therefore reversing the MDR mediated by this ABC transporter (Robey et al., 2011).

A. Structure-function relationship of BCRP

As mentioned earlier, all human ABC transporters harbor a distinctive feature of modular architecture, which is composed of at least one hydrophilic cytosolic nucleotide binding domain (NBD) and one hydrophobic membrane-spanning domain (MSD) (Figure 1). Based on the structure and arrangement of NBD and MSD, human ABC transporters can be classified into full transporters, half transporters and non-transporter type ABC proteins (Linton, 2007). Typically, full ABC transporters, such as ABCB1, comprise two homologous halves and are characterized by two MSDs and two NBDs with an arrangement of MSD_1-NBD_1-MSD_2-NBD_2 (Figure 1A). Other types of full ABC transporters, such as ABCC1, have an extra MSD (MSD_0) at the amino terminus with a domain structure of MSD_0-MSD_1-NBD_1-MSD_2-NBD_2 (Figure 1B). On the other hand, some are considered as half transporters because they contain only one MSD and one NBD and are about half the size of a full transporter. These include members of the ABCD subfamily and some of the ABCB subfamily with a domain structure of MSD-NBD (Figure 2C), and members of the ABCG subfamily with a reversed NBD-MSD configuration (Figure 2D). Finally, the ABCE and ABCF subfamilies do not even have MSDs and they are therefore unlikely to have transport ability and are categorized as non-transporter type ABC proteins. The domain structures corresponding to each of all 48 human ABC transporters are listed in Table 1.

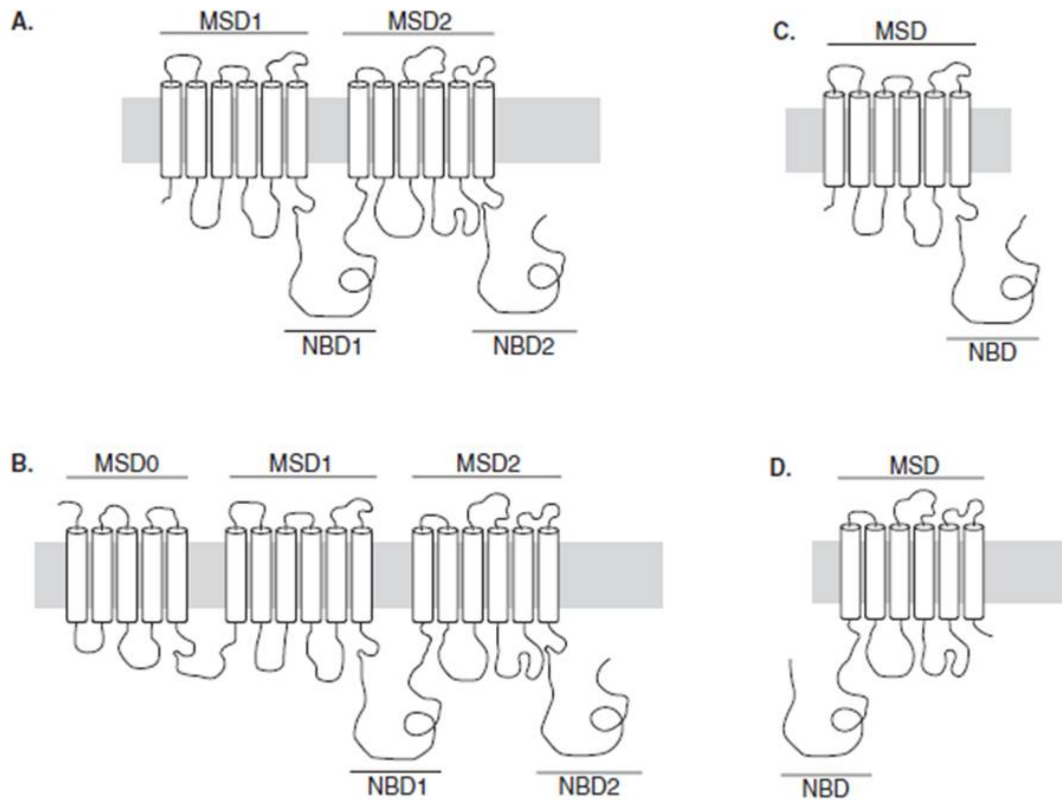

Figure 1: Domain structures of full and half ABC transporters. Schematic domain structures of full (A, B) and half (C, D) ABC transporters are shown with cylinders representing transmembrane segments (TM) linked by loops. The gray boxes represent membrane lipid bilayers.

In terms of function, the MSDs seem to contain substrate-binding sites and usually hold diverse transmembrane segments (typically six putative α-helices per domain), which contribute to substrate specificity. Furthermore, MSDs may form a pore across the membrane through which substrates move (Linton, 2007). On the other hand, the NBDs are highly conserved domains and represent the hallmark feature of the ABC transporter superfamily. In response to ATP hydrolysis following substrate binding, these NBDs located at the cytoplasmic face of the membrane undergo conformational change, which alters both the affinity and orientation of the substrate-binding sites (Linton and Higgins, 2007). This is widely accepted as the 'ATP switch' model powering the function of ABC transporters.

BCRP has been widely considered as a half ABC transporter (Doyle and Ross, 2003), with a reversed domain structure of N-terminal NBD followed by C-terminal MSD (Figure 2). Several characteristic sequence motifs of ABC transporters have been found within the NBD of BCRP (GenBank: AAG52982.1), such as ^{80}GPTGGGKSSL^{89}, a Walker A motif (also called P–loop, GxxGxGKS/T, where x can be any amino acid) and ^{206}ILFLDE^{211}, a Walker B motif (hhhhDE, where h stands for hydrophobic residue) (Walker et al., 1982, Krishnamurthy and Schuetz, 2006). Studies of many ATPases and ABC transporters have shown that the lysine (K) in the Walker A motif interacts with the γ and β phosphate groups of ATP and is essential for ATP hydrolysis, while the aspartate (D) in the Walker B motif coordinates the Mg^{2+} ion of Mg-ATP and is required for nucleotide binding (Carson et al., 1995, Gribble et al., 1997). Indeed, a lysine mutant K86M in the Walker A motif of BCRP, when expressed in Sf9 insect cells, lost both ATP

hydrolytic activity and transport ability for typical BCRP substrates, including mitoxantrone, rhodamine 123 and Hoechst 33342 dye, though the mutant maintained normal protein expression level and ATP binding activity (Ozvegy et al., 2002), suggesting the crucial role of these highly conserved motifs in the catalysis of ATP hydrolysis of BCRP. Henriksen et al. further investigated the K86M BCRP mutant in HEK293 cells and confirmed that this mutation does not affect the protein expression or the dimerization/oligomerization of BCRP (Henriksen et al., 2005b). Instead, the K86M BCRP mutant was inactive by itself and also resulted in a dominant-negative effect on wild-type BCRP function. Moreover, distinct from the normal plasma membrane localization of wild-type BCRP, K86M was localized to the Golgi apparatus followed by retrieval to the endoplasmic reticulum (ER), indicating that NBD might play an important role in the proper surface trafficking of BCRP. On the other hand, mutation of the putative catalytic residue E211 of the Walker B motif resulted in a total loss of ATPase activity and ATP dependent drug transport, suggesting ATP hydrolysis is crucial for BCRP transport activity (Hou et al., 2009). In addition, the D210N mutation results in loss of function with normal protein expression and membrane trafficking (Bhatia et al., 2005).

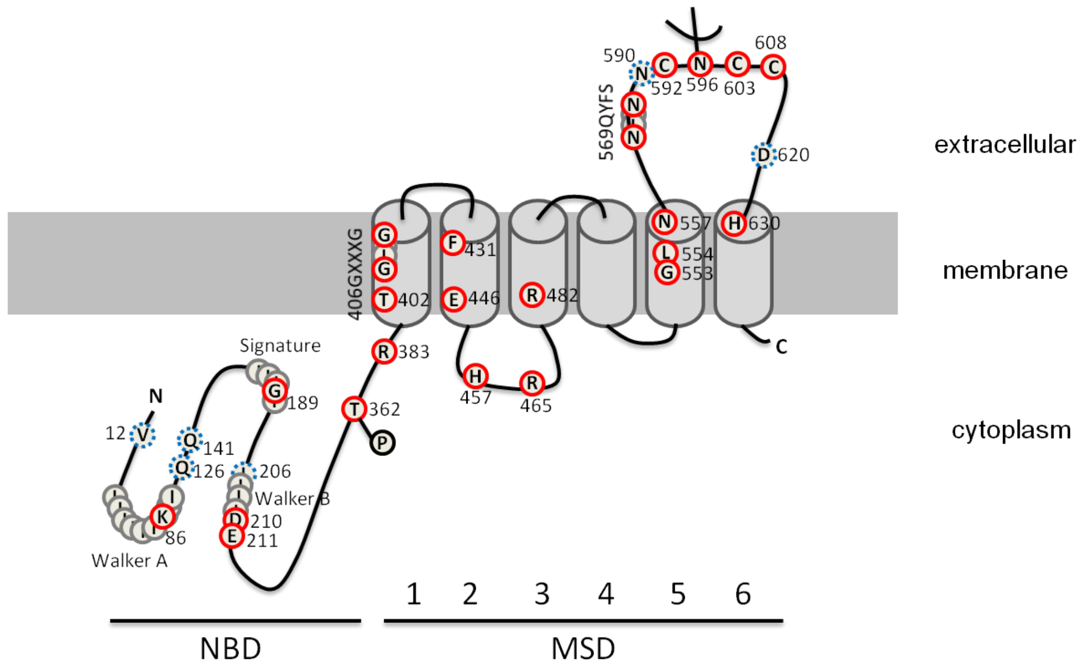

Figure 2: Membrane topology model of BCRP. The depicted model was constructed on the basis of sequence analysis and the available experimental data. Mutations affecting substrate specificity or catalytic activity are indicated with red solid circles. Amino acids as part of the motifs are indicated with gray solid circles. Common SNPs affecting expression or function of BCRP are indicated with blue dashed circles. The gray boxes represent membrane lipid bilayers.

Meanwhile, the most characteristic ABC signature motif (XSXGX) ^{186}VSGGERKRTS^{195}, which might be involved in nucleotide binding and hydrolysis or facilitating interface with the membrane-spanning domain, is located in the nucleotide binding domain of BCRP preceding the Walker B motif (Nakanishi et al., 2003a, Dawson et al., 2007). The significance of the ABC signature motif in BCRP function has been demonstrated (Nakanishi et al., 2003a) in that human BCRP cRNA containing mutations of serine 187 (S187T or S187A) in the ABC signature motif conferred normal protein expression of BCRP in *Xenopus laevis* oocytes, but completely abolished its transport activities.

Recently, two putative steroid-binding sites in human BCRP were identified through homology modeling, including the Walker A motif in the nucleotide binding domain (Mares-Samano et al., 2009) and a steroid-binding element (^{552}SGLLVNL^{558}) in the TM5 of BCRP (Velamakanni et al., 2008). These results might provide more details on the relationship of BCRP structure and substrate binding, as well as development of new therapeutic ligands of BCRP.

Amino acids that are crucial for BCRP function have also been examined extensively. Four amino acids of BCRP, including E446 in TM2, R482 in TM3, N557 in TM5 and H630 in TM6, have demonstrated their contribution to the substrate recognition of BCRP (Miwa et al., 2003). Two other basic residues, H457 and R465, might also be involved in substrate binding of BCRP (Cai et al., 2010). Several amino acids in TM5 are also functionally relevant. L554 is important for transport activity (Kage et al., 2002), and N557 might play an essential role in the proper routing and substrate binding of BCRP (Mohrmann et

al., 2005), while a mutant of the conserved G553 showed reduced protein expression, and abolished N-glysosylation and membrane trafficking of BCRP (Polgar et al., 2006).

Mutation of the conserved R383 located near the cytoplasmic side of TM1 resulted in partial retention in the ER, impaired N-glycosylation, and rapid degradation through the proteasome. Overall, this mutation brought about impairment in BCRP expression and trafficking, indicating that R383 is crucial for the biogenesis of BCRP (Polgar et al., 2009). A highly conserved ^{406}GXXXG^{410} motif, which has been linked with dimerization in other membrane proteins, is also located in the TM1 of BCRP. Mutation of both glycines to alanines maintained a fully functional transporter. However, mutation of glycines to leucines resulted in decreased transport of mitoxantrone, PhA and BODIPY-prazosin, and abolished transport ability for rhodamine 123, without affecting protein expression, membrane trafficking, basal ATPase activity or dimer formation under nonreducing conditions (Polgar et al., 2004). Furthermore, the combined mutations of GXXXG and T402, which is located in TM1 and is adjacent to the GXXXG motif, markedly reduced protein expression, disrupted N-glycosylation and membrane trafficking and promoted proteosome-independent degradation (Polgar et al., 2010).

B. Dimer *vs* oligomer

While the detailed molecular mechanisms of human ABC transporter functions remain elusive, it is widely accepted that a functional ABC transporter requires two MSDs coupled to two NBDs. Co-expression of both N-terminal and

C-terminal halves of ABCB1 in insect cells showed normal drug transport activity comparable to that of intact transporter, while either half expressed individually retained normal trafficking yet lost transport activities (Gao et al., 1996). Similar results observed with ABCC7 (Ostedgaard et al., 1997) and several other prokaryotic ABC transporters (Gauthier et al., 2003) further strengthened this concept. Therefore, the functional unit of full ABC transporters is believed to be a monomer, and half transporters require homo- or hetero-dimerization to be functionally competent.

BCRP is a half transporter and was originally thought to exist and work as a homo-dimer. This hypothesis is supported by a study showing that co-expression of an ATPase-dead BCRP with the wild-type BCRP resulted in reduction of the BCRP transport activity and that BCRP migrated as monomers on SDS-PAGE under reducing conditions but as a dimer complex in the absence of reducing agents (Kage et al., 2002). It was also found that human BCRP expressed in insect cells or bacterial cells retains its function, which argues against the necessity of other mammalian protein partners for BCRP function (Ozvegy et al., 2001). Thus, it was concluded that BCRP probably exists as a homo-dimer linked by disulfide bonds. Attempts have been made to determine the cysteine residues involved in inter-molecular disulfide bond formation, and Cys603 in the extracellular loop 3 (ECL3) of BCRP was identified. Cys603 is not required for protein expression or localization, nor is it essential for ATPase activity or transport function of BCRP (Bhatia et al., 2005, Henriksen et al., 2005a,

Liu et al., 2008), suggesting that the functional BCRP does not necessarily need an intermolecular disulfide bridge for its function and/or oligomerization.

However, emerging new evidence suggests that BCRP may exist as a higher order of oligomer on the plasma membrane. Using chemical cross-linking and non-reducing SDS-PAGE, Litman et al. (Litman et al., 2002) first detected both an apparent BCRP dimer and higher forms of oligomers from whole cell lysate. Similar methods used by Bhatia's group (Bhatia et al., 2005) demonstrated the presence of higher order oligomers of BCRP in both isolated cell membranes and whole cell preparations. Recently, using various biochemical methods such as non-denaturing PAGE, perfluoro-octanoic acid-PAGE (PFO-PAGE), gel filtration chromatography, sucrose gradient sedimentation, chemical cross-linking as well as co-immunoprecipitation, Xu et al. (Xu et al., 2004) demonstrated that the major oligomeric unit of human BCRP in plasma membranes of the drug-resistant cell line MCF7/AdVp3000 is a homo-dodecamer with the minimum stable unit of homo-tetramer. No monomeric or dimeric BCRP was found under non-denaturing conditions, suggesting that the major functional form of BCRP may be a homo-oligomer. Later, the examination of purified human BCRP using cryo-negative staining electron microscopy showed that purified human BCRP may exist as a homo-octomer consisting of four homo-dimeric BCRP complexes (McDevitt et al., 2006). It has also been reported that the purified BCRP in the presence of all solubilized membrane components is a tetrameric complex when expressed in Sf9 cells (Dezi et al., 2010). Although the causes for the discrepancy among the latter three studies are unknown, it is clear that all studies have

demonstrated the presence of higher order oligomeric BCRP complexes. Using deletion mapping and co-immunoprecipitation of differential epitope tagging of BCRP, Xu et al. (Xu et al., 2007) discovered that the oligomerization domain of human BCRP is located in the MSD, consisting of ECL3 with its flanking transmembrane segments (TM5 and TM6). The polypeptide consisting of TM5-ECL3-TM6 not only forms a homo-dodecamer complex itself but also exerts a dominant negative effect on wild-type BCRP drug transport function and drug resistance phenotypes, possibly by forming hetero-complexes with the wild-type BCRP. It is postulated that three different inter-molecular contacts are responsible for the formation of a homo-dodecamer (Xu et al., 2004, Xu et al., 2007), which will be further tested in the current study.

C. Substrates of BCRP and its variants

The substrates of BCRP, identified directly by cellular or vesicular transport assays, or indirectly by substrate-stimulated ATPase activity or cytotoxicity assays, comprise a broad spectrum of anticancer drugs, sulfate and glucuronide conjugates of sterols and xenobiotics, natural compounds and toxins, fluorescence dyes, photosensitizers, antibiotics, etc (a comprehensive list of BCRP substrates is in Table 2).

Among the major groups of BCRP substrates are anticancer drugs, including topoisomerase inhibitors, anthracyclines, camptothecin (CPT) analogs, tyrosine kinase inhibitors (TKI), and antimetabolites, which have identified BCRP as essential for MDR in cancer chemotherapy. BCRP transports many sulfate and glucuronide conjugates of steroids and xenobiotics, which are two common

products of mammalian Phase II metabolism, suggesting that BCRP is involved in drug metabolism. BCRP also mediates the efflux of Pheophorbide a (PhA) (Robey et al., 2004) and many other photosensitizers (Robey et al., 2005), implicating BCRP as a possible cause for photodynamic therapy resistance. BCRP is also involved in the transport of Abeta(1-40) peptides at the blood-brain barrier, and upregulation of BCRP is correlated with Abeta deposition in cerebral vessels, leading to cerebral amyloid angiopathy in many Alzheimer's disease patients (Xiong et al., 2009).

Studies of CPT analogs as BCRP substrates have revealed that the polar groups, especially hydroxyl groups, at the 9 or 10 position of the CPT A ring facilitate interaction with BCRP, and this information could be used to design BCRP insensitive CPT analogues. Also, hydrogen bond formation might be involved in the substrate recognition of BCRP (Rajendra et al., 2003, Ishikawa et al., 2006, Nakagawa et al., 2006). Additional analysis of CPT analogue structures strongly suggests that high-polarity CPT analogues are good BCRP substrates, while the low-polarity counterparts, as poor BCRP substrates, could be considered as BCRP insensitive compounds (Yoshikawa et al., 2004). From this aspect, modification of BCRP substrates could also be considered as a practical approach to overcome the drug resistance mediated by BCRP.

Interestingly, in early studies with the MCF-7/AdVp3000 or mitoxantrone-selected S1-M1-80 cell lines, transport of rhodamine 123 was observed (Litman et al., 2000). However, the transport ability of rhodamine 123 was not seen in several other BCRP overexpressing cell lines (Honjo et al., 2001).

This inconsistency has led to sequencing of the BCRP gene comparing a series of parental and BCRP overexpressing cells, and it was revealed that cells able to transport rhodamine 123 had a glycine (G) or threonine (T) at position 482 in the BCRP protein, while cells expressing wild-type BCRP with an arginine (R) at this site could not transport rhodamine 123. Both mutants and wild-type BCRP were able to efflux mitoxantrone, topotecan, SN-38, Hoechst 33342 (Ozvegy et al., 2002) and BODIPY-prazosin (Robey et al., 2003). However, the 482G/T mutant has a higher affinity for anthracyclines, including doxorubicin, daunorubicin, epirubicin, as well as bisantrene, fluorescence dye rhodamine 123 and lysotracker green (Robey et al., 2003). In contrast, 482R is specific for transport of methotrexate (MTX) (Volk et al., 2000, Volk et al., 2002), MTX diglutamate and triglutamate, as well as folic acid (Chen et al., 2003). These data suggest that amino acid 482 might be a 'hot spot' for substrate specificity of BCRP.

Nevertheless, a study using IAARh123, the photoreactive analogue of rhodamine 123, has surprisingly shown that both 482R and 482T bind directly to IAARh123. 482R could not transport IAARh123, even though it is photolabelled more intensely than 482T, indicating higher binding affinity of 482R to IAARh123 (Alqawi et al., 2004). This piece of data not only provides evidence of the direct binding of substrates to BCRP, but also indicates that an unknown mechanism leads to the difference between 482R and 482T, other than substrate specificity. Indeed, a follow-up study, in which nine 482R mutants were generated and expressed in insect cells, has further demonstrated that amino acid substitution at position 482 induces major changes in not only substrate specificity but also the

transport activity of BCRP (Ozvegy-Laczka et al., 2005). 482R might also play an important role in ATP hydrolysis of BCRP, as demonstrated by basal and stimulated ATPase activity and photoaffinity labeling analyses (Ejendal et al., 2006). Finally, compared to 482R, purified BCRP 482T exhibited an increased ATP hydrolysis rate and affinity for MgATP, as well as decreased sensitivity to vanadate inhibition and preferred binding to ATP, while substrate binding is similar between 482R and 482T (Pozza et al., 2006). Although mutations at position 482 have not been found in any clinical samples (Honjo et al., 2002, Nakanishi et al., 2003b, Zamber et al., 2003), 482 R does provide a superior tool to explore BCRP functions and to assist the modification of BCRP substrates and the development of BCRP inhibitors.

Table 2: Summary of BCRP substrates.

Substrates	Reference
Topoisomerase inhibitors	
Mitoxantrone (topoisomerase II inhibitor)	(Doyle and Ross, 2003)
Bisantrene (topoisomerase II inhibitor)	(Litman et al., 2000)
Etoposide (topoisomeriase II inihibitor)	(Yuan et al., 2009)
Becatecarin (topoisomerase II inhibitor)	(Robey et al., 2009)
NB-506, J-107088 (topoisomerase I inhibitors)	(Komatani et al., 2001)
Anthracyclines (Topoisomerase II inhibitors)	
Daunorubicin	(Ozvegy et al., 2001)
Doxorubicin (Adriamycin)	(Ozvegy et al., 2001)
Epirubicin	(Robey et al., 2003)
Pirarubicin	(Yuan et al., 2009)
Camptothecin analogs (Topoisomerase I inhibitors)	
Topotecan	(Maliepaard et al., 1999)
SN-38	(Maliepaard et al., 1999)
CPT-11	(Maliepaard et al., 2001b)
9-aminocamptothecin	(Maliepaard et al., 2001b)
NX211	(Maliepaard et al., 2001b)
DX-8951f	(van Hattum et al., 2002)
Homocamptothecins	(Bates et al., 2004)
BN80915 (diflomotecan)	(Bates et al., 2004)
Gimatecan	(Marchetti et al., 2007)
Belotecan	(Li et al., 2008)
Tyrosine kinase inhibitors	
Gefitinib	(Elkind et al., 2005)
Dasatinib	(Hiwase et al., 2008)
Erlotinib	(Marchetti et al., 2008)
Vandetanib	(Azzariti et al., 2010)
Nilotinib	(Hegedus et al., 2009)
Sorafenib	(Agarwal et al., 2011)
Tandutinib	(Yang et al., 2010)
CI1033 (Pan-HER TKI)	(Erlichman et al., 2001)
CP-724,714 (HER2 TKI)	(Feng et al., 2009)
Symadex (fms-like tyrosine kinase 3 inhibitor)	(Bram et al., 2009)
Antimetabolites	
MTX, MTX diglutamate, MTX triglutamate (antifolate)	(Chen et al., 2003)
GW1843, Tomudex (antifolates)	(Shafran et al., 2005)
Trimetrexatte, piritrexim, metoprine, pyrimethamine (lipophilic antifolates)*	(Bram et al., 2006)
5-fluorouracil (pyrimidine analog)	(Yuan et al., 2009)

CdAMP (nucleotide), cladribine (nucleoside)	(de Wolf et al., 2008)
Other anticancer drugs	
Flavopiridol (cyclin-dependent kinase inhibitor)	(Nakanishi et al., 2003b)
JNJ-7706621 (CDK and aurora kinases inhibitor)	(Seamon et al., 2006)
Bicalutamide (non-steroidal anti-androgen)	(Colabufo et al., 2008)
NSC73306	(Wu et al., 2007)
Phenethyl isothiocyanate (PEITC)	(Ji and Morris, 2004)
TH-337 (indazole-based tubulin inhibitors)	(Meng et al., 2008)
Sulfate and glucuronide conjugates of xenobiotics	
Estrone 3-sulfate (E1S)	(Suzuki et al., 2003)
17beta-estradiol sulfate	(Imai et al., 2003)
DHEAS	(Suzuki et al., 2003)
4[35S]-methylumbelliferone sulfate	(Suzuki et al., 2003)
E3040 sulfate	(Suzuki et al., 2003)
Troglitazone sulfate	(Enokizono et al., 2007)
3-O-sulfate conjugate of 17alpha-ethinylestradiol	(Han et al., 2010)
SN-38-glucuronide	(Nakatomi et al., 2001)
[3H]17beta-estradiol-17beta-D-glucuronide	(Suzuki et al., 2003)
[14C]4-methylumbelliferone glucuronide	(Suzuki et al., 2003)
BP-3-sulfate and BP-3-glucuronide	(Ebert et al., 2005)
Phenolic MPA glucuronide	(Miura et al., 2008)
Photosensitizers	
Pheophorbide a	(Robey et al., 2004)
Pyropheophorbide a methyl ester	(Robey et al., 2005)
Chlorine E6	(Robey et al., 2005)
5-aminolevulinic acid	(Robey et al., 2005)
Phytoporphyrin	(Robey et al., 2006)
HPPH	(Zheng et al., 2009)
Natural compounds and toxins	
Folic acid	(Chen et al., 2003)
Urate	(Woodward et al., 2009)
Genistein	(Imai et al., 2004)
Riboflavin (vitamin B2)	(van Herwaarden et al., 2007)
Vitamin K3, plumbagin	(Shukla et al., 2007)
Glutathione (GSH)	(Brechbuhl et al., 2009)
Sphingosine 1-phosphate	(Takabe et al., 2010)
PhIP (carcinogen)	(Pavek et al., 2005)
PPIX (heme precursor)	(Zhou et al., 2005a)
Fluorescent dyes	
Rhodamine 123	(Litman et al., 2000)
IAARh123	(Alqawi et al., 2004)

Hoechst 33342	(Ozvegy et al., 2002)
Lysotracker green	(Robey et al., 2003)
BODIPY-prazosin	(Cygalova et al., 2009)
D-luciferin (firefly luciferase substrate)	(Zhang et al., 2009)
Cholyl-L-lysyl-fluorescein (fluorescent bile salt derivative)	(de Waart et al., 2010)
BODIPY-FL-dihydropyridine	(Shukla et al., 2006)
Others	
[(125)I]Iodoarylazidoprazosin (IAAP), [(3)H]azidopine	(Shukla et al., 2006)
Sulfasalazine (anti-inflammatory)	(van der Heijden et al., 2004)
Erythromycin (macrolide antibiotic)	(Janvilisri et al., 2005)
Ciprofloxacin, ofloxacin, norfloxacin,enrofloxacin, grepafloxacin, ulifloxacin (fluoroquinolone antibiotics)	(Merino et al., 2006, Pulido et al., 2006, Ando et al., 2007)
Nitrofurantoin (urinary tract antibiotic)	(Merino et al., 2005b)
Moxidectin (parasiticide)	(Perez et al., 2009)
Albendazole suloxide and oxfendazole (anthelmintics)	(Merino et al., 2005a)
Ganciclovir (antiviral drug)	(Hu and Liu, 2010)
Zidovudine (NRTI)	(Wang et al., 2003)
Lamivudine (NRTI)	(Kim et al., 2007)
Leflunomide and A771726 (antirheumatic drugs)	(Kis et al., 2009)
Diclofenac (analgesic and anti-inflammatory drug)	(Lagas et al., 2009)
Cimetidine (histamine H2-receptor antagonist)	(Pavek et al., 2005)
ME3277 (hydrophilic glycoprotein IIb/IIIa antagonist)	(Kondo et al., 2005)
Pitavastatin (HMG-CoA reductase inhibitor)	(Hirano et al., 2005)
Rosuvastatin (HMG-CoA reductase inhibitor)	(Huang et al., 2006)
Dipyridamole (thromboxane synthase inhibitor)	(Zhang et al., 2005)
Glyburide (hypoglycemic agent)	(Gedeon et al., 2008)
Nicardipine, nifedipine, nitrendipine (Ca^{2+} channel blocker)	(Shukla et al., 2006)
Olmesartan medoxomil (angiotensin II AT1-R antagonist)	(Yamada et al., 2007)
Befloxatone (selective monoamine oxidase inhibitor)	(Tournier et al., 2011)
Prazosin (alpha-1-adrenergic receptor antagonist)	(Litman et al., 2000)
Riluzole (Na^+ channel blocker)	(Milane et al., 2009)
Amyloid-beta	(Tai et al., 2009)
Zoledronic acid (osteotropic compound)	(Kars et al., 2007)
Hesperetin conjugates (flavonoid)	(Brand et al., 2008)
Kaempferol (flavonoid)	(An et al., 2011)

D. Physiological significance of BCRP

The substrate spectrum and the tissue distribution of BCRP have implicated a key role of this transporter in the protection of the human body against xenobiotics. In human tissues, BCRP is prominently expressed in placental syncytiotrophoblasts, in the epithelium of the small intestine and colon, in the liver canalicular membrane, and in ducts and lobules of the breast (Maliepaard et al., 2001a). BCRP expression has also been detected in the luminal membrane of epithelial cells in normal gallbladders (Aust et al., 2004), in alveolar pneumocytes, sebaceous glands, interstitial cells of the testes, prostate epithelium, endocervical cells of the uterus, squamous epithelium of the cervix, islet and acinar cells of the pancreas, the zona reticularis layer of the adrenal gland, kidney cortical tubules, and hepatocytes (Fetsch et al., 2006, Huls et al., 2008). BCRP is also present in venous and capillary endothelium. Furthermore, BCRP is predominantly localized on the plasma membrane of certain cell types of the above tissues (Rocchi et al., 2000), many of which harbor a secretory or barrier function, and this specific distribution profile is closely related to the physiological role of human BCRP. More importantly, BCRP is highly expressed in various stem cells and serves as an important determinant of the side population (SP) phenotype (Zhou et al., 2001).

1. BCRP as first line defense in the GI tract

Human BCRP is physiologically expressed in the apical membrane of epithelial cells in the gastrointestinal (GI) tract, with maximal expression in the duodenum and a gradual decrease along the GI tract to the rectum (Gutmann et

al., 2005). BCRP is also constitutively expressed in the liver canalicular membranes, which supports a possible protective role of BCRP against xenobiotic absorption and towards toxic metabolite excretion. It has been shown that administration of GF120918, a dual inhibitor of ABCB1 and BCRP, resulted in a significant increase in the bioavailability and systemic concentration of topotecan after oral administration (Kruijtzer et al., 2002). Moreover, it is also reported that GF120918 could markedly reduce the biliary and renal excretion of topotecan after intravenous administration. Considering that topotecan has higher affinity for BCRP than with ABCB1, inhibition of BCRP by GF120918 is likely the major mechanism responsible for the increased intestinal absorption and decreased biliary and renal excretion.

In addition, data collected from Bcrp-null mice help to appreciate this first line defense role of BCRP. Bcrp-null mice are more susceptible to phototoxic skin lesions, which are caused by accumulation of pheophorbide a, a chlorophyll degradation product found in food and supplements. This has implicated that BCRP is crucial in protecting the human body from the development of protoporphyria and food-related phototoxicity (Jonker et al., 2002). Meanwhile, BCRP is responsible for the efflux of sulfate and glucuronide conjugates of xenobiotics and hormones, which are mostly products of phase II metabolism, suggesting that BCRP has a major role in extruding toxic metabolites, mostly through the biliary pathway (Dietrich et al., 2003).

2. BCRP in the blood-brain barrier

BCRP is constitutively expressed at the blood-brain barrier, mainly at the luminal cell surface of microvessel endothelium, and serves as a crucial barrier to drug access into the brain (Cooray et al., 2002, Zhang et al., 2003, Aronica et al., 2005). Indeed, BCRP works with ABCB1 in the blood-brain barrier and is responsible for restricting numerous xenobiotics from entering the brain. The positive impact is that BCRP protects the brain from the toxicity of xenobiotics, while the negative impact is that BCRP impedes therapeutic agents from reaching their intracerebral targets.

3. BCRP in the placenta

BCRP expression is highest on the plasma membrane of the chorionic villi in the placenta (Litman et al., 2002). This cellular localization indicates that BCRP plays a major role in protecting the fetus against toxic compounds ingested by the mother. In Bcrp-null mice, the fetal exposure to topotecan and other dietary toxins increased significantly (Jonker et al., 2000), further confirming the protecting role of BCRP in the placenta.

4. BCRP in stem cells

A fascinating property of hematopoietic stem cells is their ability to actively extrude Hoechst 33342, a fluorescent dye. The low Hoechst 33342 staining cells isolated by subsequent fluorescence-activated cell sorting (FACS) are termed as 'side population' (SP) (Goodell et al., 1996), which have been shown to possess stem cell-like characteristics in a variety of tissues (Zhou et al., 2001, Bunting, 2002, Lechner et al., 2002, Summer et al., 2003). BCRP is expressed at a higher

level in SP cells, compared to non-SP cells, and has been identified as the Hoechst 33342 efflux pump in SP (Kim et al., 2002, Scharenberg et al., 2002). Moreover, ectopic expression of BCRP conferred a SP phenotype in HEK293 cells, indicating that BCRP might serve as an attractive candidate marker for stem cells (Scharenberg et al., 2002). On the other hand, BCRP is expressed differentiational during hematopoiesis, with the highest levels in the primitive bone marrow stem cell populations, followed by a sharp reduction in response to stem cell differentiation, suggesting possible dual roles of BCRP in maintaining human pluripotent stem cells in an undifferentiated state, and in protecting these stem cells from xenobiotics or other toxins *in vivo*. (Zhou et al., 2001, Bunting, 2002, Scharenberg et al., 2002).

Studies with Bcrp-null mice further confirmed that BCRP is necessary for the SP phenotype, since loss of Bcrp expression resulted in a drastic decrease in SP cells in the bone marrow and skeletal muscle. Notably, it has also been shown that the hematopoietic cells of Bcrp-null mice became more sensitive to the cytotoxicity of mitoxantrone, confirming the physiological protection function of Bcrp in hematopoietic cells (Zhou et al., 2002).

E. Pathophysiological significance of BCRP

Although human BCRP is widely expressed in normal tissues, overexpression of BCRP has been found frequently in various drug-selected cancer cell lines, and contributes to the clinical MDR of hematopoietic malignancies and solid tumors. Increased BCRP expression has also been linked to cancer stem cells.

1. BCRP in MDR

As mentioned earlier, BCRP was originally cloned from an Adriamycin selected breast cancer cell line MCF-7/AdVp, which exhibited resistance to a range of cytotoxic agents, including mitoxantrone, doxobucibin and daunorubicin, but had no increased expression of ABCB1 (Doyle et al., 1998). Overexpression of BCRP has been found in and correlated to the MDR phenotypes of numerous drug-selected cancer cell lines derived from various tumor types, including topotecan-selected ovarian tumor cell line T8 (Maliepaard et al., 1999), mitoxantrone-selected colon cancer cell line S1-M1-80 (Litman et al., 2000) and HT29 (Perego et al., 2001), SN-38-selected human small cell lung cancer (SCLC) cells PC-6/SN2-5 (Kawabata et al., 2001), mitoxantrone-selected human gastric carcinoma cell line EPG85-257RNOV (Stein et al., 2002), gefitinib-resistant non-small cell lung cancer (NSCLC) cells (Nagashima et al., 2006), epirubicin-resistant human hepatocyte carcinoma cells HLE-EPI (Kamiyama et al., 2006), and topotecan and doxorubicin selected human multiple myeloma cells (Turner et al., 2006). However, the BCRP-mediated drug resistance profiles found in these cell lines vary, which might be attributed to different cell origin or the involvement of other resistance factors, including other MDR ABC transporters, drug metabolizing enzymes, DNA repair, and apoptosis (Jaeger, 2009). Therefore, a complex model is required for better evaluation of MDR in cell lines.

A recent approach by Szakacs et al. (Szakacs et al., 2004) has profiled mRNA expressions of the 48 known human ABC transporters and their correlation with the growth inhibition of 1,429 anticancer drug candidates in 60 diverse

cancer cell lines (the NCI-60). The results could more accurately predict which transporters are more likely related to resistance to a certain drug tested. This group further designed a microarray platform, the ABC-ToxChip, to evaluate the transcriptional profiles of a comprehensive set of genes involved in MDR between parental and drug-selected cell lines (Annereau et al., 2004). The sensitivity and accuracy of this method has been confirmed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC), suggesting that this microarray could serve as a helpful tool to clarify ABC transporters and other mechanisms responsible for MDR.

2. BCRP in hematopoietic malignancies

Although cellular models are powerful tools to examine the MDR phenotypes mediated by BCRP, the clinical relevance of BCRP in human cancers was mainly established in clinical samples. There has been considerable evidence demonstrating overexpression of BCRP in many different hematopoietic malignancies since its discovery. Early studies by Ross et al. have indicated relatively high levels of BCRP expression in 33% of acute myeloid leukemia (AML) blast cells (Ross et al., 2000). However, the contribution of BCRP for drug resistance or drug response in AML patients is so far inconclusive (Table 3). It was reported that BCRP expression could predict a decrease in complete remission as well as 4-year overall survival in AML patients with daunorubicin or mitoxantrone treatment (Benderra et al., 2004). Also, another study in AML patients of older age has demonstrated the relationship between higher Pgp/BCRP co-expression with lower complete response rate and overall survival

rate (van den Heuvel-Eibrink et al., 2007). In addition, Benderra et al. have shown that AML patients expressing one or none of functional ABCB1, ABCC3 or BCRP have a better prognosis than those patients expressing two or all of the above transporters (Benderra et al., 2005). These data suggest that modulation of all susceptible ABC transporters may provide therapeutic synergy and promote a better prognosis.

The role of BCRP expression in acute lymphocytic leukemia (ALL) has also been implicated but stays inconclusive. Suvannasankha's group has reported a correlation between BCRP expression and prognosis in adult ALL patients (Suvannasankha et al., 2004) while several other groups did not find such a relationship between BCRP expression and response in childhood ALL (Sauerbrey et al., 2002). One explanation for the discordance might be the different experimental methods used, different antidies used or different criteria for samples (adult or childhood) used (Table 3). It might also be necessary to expand the sample size. There is still a lot of work to be done in order to clarify the role of BCRP in drug resistance in hematopoietic malignancies.

Table 3: BCRP expression in human hematopoietic malignancies.

Type	Correlation	Methods	Size	Reference
AML	Yes	RT-PCR	20	(Ross et al., 2000)
AML	No (mitoxantrone, topotecan or doxorubicin based therapy)	ICC (BXP34)	20	(Sargent et al., 2001)
AML	Yes (daunorubicin based therapy)	ICC (BXP34)	20	(Sargent et al., 2001)
AML	No	FCM (BXP34 & BXP21)	20	(van der Kolk et al., 2002)
AML	Yes (relapse/refractory)	RT-PCR	20	(van den Heuvel-Eibrink et al., 2002)
AML (child)	Yes (prognosis, relapse)	RT-PCR	59	(Steinbach et al., 2002)
AML (adult)	no	RT-PCR	40	(Abbott et al., 2002)
AML	no	RT-PCR	51	(Galimberti et al., 2004)
AML adult)	Yes (prognosis on daunorubicin and mitoxantrone therapy)	RT-PCR	149	(Benderra et al., 2004)
AML (adult)	Yes (complete remission, DFS, overall survival)	FCM (BXP21)	85	(Benderra et al., 2005)
AML	Yes (relapse and DFS)	FCM (BXP34)	73	(Damiani et al., 2006)
AML	Yes (complete response rate)	RT-PCR	154	(van den Heuvel-Eibrink et al., 2007)
AML	Yes (DFS, relapse, overall survival to fludarabine-based therapy)	RT-PCR	138	(Damiani et al., 2010)
ALL (child)	No (prognosis)	RT-PCR	67	(Sauerbrey et al., 2002)
ALL	Yes	FCM (BXP34)	46	(Plasschaert et al., 2003)
ALL (adult)	Yes (DFS)	ICC (BXP21)	30	(Suvannasankha et al., 2004)

3. BCRP in solid tumors

Recently, correlations between BCRP expression with solid tumors have been reported (Table 4). In Diestra's study, a total of 150 untreated tumor samples from various origins were examined, and increased expression of BCRP was found in tumors from the digestive tract, endometrium, lung, as well as melanoma (Diestra et al., 2002). In breast cancer patients, BCRP expression is also found to correlate with response to anthracycline-based chemotherapy (Burger et al., 2003). Increased BCRP expression is also related to loss of differentiation and shorter survival in oral squamous cell carcinomas (Friedrich et al., 2004). In contrast, various other studies indicate that there was no relationship between BCRP expression and response in breast cancer (Kanzaki et al., 2001), advanced bladder tumors (Diestra et al., 2003) or testicular germ cell cancers (Zurita et al., 2003), which strongly calls for validated assays and longitudinal studies to definitively assess the contribution of BCRP to drug resistance in solid tumors. Interestingly, Gupta et al. reported reduction of BCRP mRNA and protein in colorectal and cervical cancer tissues (Gupta et al., 2006). This observation implies that BCRP may play a role in tumorigenesis in several cancer types, where reduction of BCRP permits the accumulation of genotoxins or other carcinogenic cytokines.

Table 4: BCRP expression in human solid tumors.

Type	Correlation	Methods	Size	Reference
Breast carcinoma	No (doxorubicin-based treatment)	RT-PCR	43	(Kanzaki et al., 2001)
Breast carcinoma	No (anthracycline-based therapy)	IHC (BXP21 & BXP34)	52	(Faneyte et al., 2002)
Breast cancer	Yes (anthracycline-based therapy)	RT-PCR	59	(Burger et al., 2003)
Digestive tract tumors	Yes	IHC (BXP21)	32	(Diestra et al., 2002)
Colorectal and cervical cancer	Yes (downregulation)	IHC	154	(Gupta et al., 2006)
Endometrial carcinoma	Yes	IHC (BXP21)	5	(Diestra et al., 2002)
Lung tumors	Yes	IHC (BXP21)	10	(Diestra et al., 2002)
NSCLC	Yes (PFS and overall survival to platinum-based therapy)	IHC (BXP21)	72	(Yoh et al., 2004)
NSCLC	Yes (short survival to cisplatin-based therapy)	IHC (BXP21)	156	(Ota et al., 2009)
SCLC	Yes (response and PFS to platinum-based therapy)	IHC (BXP21)	130	(Kim et al., 2009b)
Melanoma	yes	IHC (BXP21)	5	(Diestra et al., 2002)
Melanoma	no	RT-PCR	18	(Deichmann et al., 2005)
Retinoblastoma	Yes (invasion)	IHC (5D3)	39	(Mohan et al., 2006)
Retinoblastoma	No	IHC (BXP21)	18	(Wilson et al., 2006)
Esophageal carcinoma	Yes	RT-PCR	100	(Tsunoda et al., 2006)
T/NK-cell lymphoma	Yes	IHC	45	(Saglam et al., 2008)
Diffuse large B-cell lymphoma	Yes (prognosis)	IHC (BXP21)	67	(Kim et al., 2009a)

F. Regulation of BCRP expression

Increased expression of BCRP is frequently seen in both drug resistant cancer cell lines and clinical tumor tissues. Numerous studies have demonstrated that the regulation of BCRP expression occurs at several levels, including gene amplification, transcriptional, posttranscriptional and epigenetic modifications.

1. Gene amplification of BCRP

The amplification of the BCRP gene was first reported in a variety of drug resistant human cancer cell lines selected by mitoxantrone (Ross et al., 1999). Knutsen et al. verified this phenomenon and also demonstrated chromosome translocation in two mitoxantrone-resistant sublines of human breast cancer cell line, MCF-7 AdVp3000 and MCF-7 MX, by both comparative genomic hybridization (CGH) and Southern blot (Knutsen et al., 2000). The mitoxantrone-sensitive parental cell line MCF-7, on the other hand, had no copy number amplification or chromosome translocation of the BCRP gene. In addition, it has also been shown that the resistance level to SN-38 in colorectal cancer cells was positively correlated to BCRP gene amplification, which resulted in a marked decrease in the intracellular concentration of SN-38 (Candeil et al., 2004).

Later, Knutsen's group extended the study to investigate the mechanisms of BCRP gene amplification (Rao et al., 2005). SF295 glioblastoma cells were exposed to an increasing concentration of mitoxantrone and four sublines with increasing resistance to mitoxantone, named as SF295 MX50, MX100, MX250, and MX500, were generated. Southern blot analysis confirmed gene amplification in all mitoxantrone-resistant SF295 sublines. Furthermore, fluorescence in situ

hybridization (FISH) demonstrated that with low levels of drug selection (MX50 and MX100), amplification of the BCRP gene was via double minute chromosomes (dmins). With higher levels of drug selection (MX250 and MX500), BCRP gene amplification was conferred more by homogeneously staining regions (hsr) than by double minute chromosomes. These results indicate that BCRP gene amplification under drug selection probably occurred initially as double minute chromosomes and switched to chromosomal reintegration of the amplicon at multiple chromosomes to generate a more stable genotype.

2. Transcriptional regulation of BCRP

The human BCRP gene is located on chromosome 4q22 and spans more than 66 kbp (Knutsen et al., 2000). It contains 16 exons and 15 introns, which encode a membrane protein of 655 amino acids (Kanzaki et al., 2002). The first exon contains most of the 5'-untranslated region (5'-UTR) of the BCRP gene, while the translational start site is located in exon 2. The BCRP gene has a TATA-less promoter with its basal promoter activity conferred by the sequence 312 bp directly upstream from the transcriptional start site. A CCAAT box is present in -274 bp and removal of this CCAAT box reduces the transcription acitivity of the BCRP gene. There are five putative Sp1 sites downstream from a putative CpG island, which is a common feature of promoters lacking a TATA box. It is found that the homeobox gene MSX2 recruits SP1 to the Sp1 binding sites within BCRP promoter and upregulates BCRP gene transcription in pancreatic cancer cells (Hamada et al., 2011).

To date, six major transcription regulation pathways, including alternative promoters, estrogen receptor alpha (ERα), hypoxia-inducible factor 1 (HIF-1), peroxisome proliferator-activated receptor gamma (PPARγ), progesterone receptor (PGR) and aryl hydrocarbon receptor (AHR), have been identified to regulate BCRP mRNA expression.

The 5'-UTR of BCRP mRNA in cell lines highly expressing BCRP have been examined and compared to normal tissues. At least three forms of the untranslated exon 1 (E1a, E1b, E1c) with a common exon 2 were revealed, suggesting the existence of alternative promoters of BCRP. These exon 1 variants present tissue-specific expression, while E1c seems to be translated more efficiently than E1a (Nakanishi et al., 2006).

Sequence analysis of the 5'-flanking region of the BCRP gene has led to the discovery of a putative estrogen response element (ERE) between the -188 to -172 bp of the BCRP promoter (Ee et al., 2004b). Deletion and site-directed mutagenic analysis confirmed the existence of ERE in the region between -243 and -115 bp. It has been further demonstrated that 17β-estradiol (E2) could promote the mRNA expression of BCRP, through activation of estrogen receptor alpha (ERα), which directly binds to the ERE located in the BCRP promoter. Using a genome-wide analysis of promoter elements for transcription factor binding sites, Kamalakaran et al. validated that BCRP promoter does carry a functional ERE and that the BCRP gene is estrogen responsive (Kamalakaran et al., 2005). However, the regulation of BCRP by estrogen or its conjugates is not

limited to transcriptional level through ERE, but also related to posttranslational regulation via several signaling pathways.

Studies by Krishnamurthy et al. have demonstrated that hypoxia also increases the mRNA expression of BCRP in three different human cell lines (Krishnamurthy et al., 2004). Further computer algorithm analysis of the 5'-flanking sequence of human BCRP revealed three putative hypoxia response elements (HREs), all located upstream of the transcription start site. Using site-directed mutagenesis and electrophoretic mobility shift assays (EMSA), it was demonstrated that HIF-1 complex specifically binds to the BCRP promoter through the only functional HRE (-116 bp) and promotes the transcription of the BCRP gene. This upregulation of the BCRP gene by activated HIF-1 under hypoxia condition might be responsible for one of the mechanisms in some tumors that facilitate drug resistance. Moreover, IL-6 or ER stress inducer could synergistically increase BCRP expression through the site overlapping XBP-1 and HIF-1 binding sites on the BCRP promoter, indicating HRE might be involved in the effect of ER stress on BCRP expression (Nakamichi et al., 2009).

Besides ERE and HRE, nuclear receptor responsive elements are usually seen in ABC transporter promoters responsible for transcriptional regulation (Repa et al., 2000, Chawla et al., 2001). Szatmari et al. first reported upregulation of BCRP mRNA in human myeloid lineage, monocyte-derived dendritic cells upon PPARγ agonist rosiglitazone treatment (Szatmari et al., 2006). This effect was completely abolished by PPARγ antagonist or PPARγ siRNA, indicating that it is indeed PPARγ receptor-specific. To elucidate the mechanisms of the above

phenomenon, the promoter sequence of the BCRP gene was analyzed, and three potential PPAR response elements were found in a conserved region around 150 bp of length (-3946 to -3796 bp). EMSA analysis further demonstrated that all three putative elements were able to bind PPARγ-RXR heterodimers specifically, suggesting that this genomic region likely plays an important role in the PPARγ dependent transcriptional regulation of BCRP expression.

Nonetheless, a novel progesterone response element (PRE) has been identified between -243 to -115 bp of the BCRP promoter region (Vore and Leggas, 2008, Wang et al., 2008). Progesterone significantly increased BCRP mRNA level in PGR-B-transfected but not PGR-A-transfected cells. Although EMSA confirmed the direct binding of PRE with either PGR-B or PGR-A, mutations in PRE only decreased the progesterone-response in PGR-B-transfected but not PGR-A-transfected cells, and further deletion of the PRE largely abrogated the progesterone effect. Interestingly, cotransfection of PGR-A and PGR-B significantly decreased the progesterone-response compared with PGR-B transfection alone, indicating progesterone induced BCRP mRNA expression through PGR-B, while PGR-A inhibits the effect of PGR-B via an undefined mechanism.

Last but not least, Ebert et al. showed that either AHR or the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) agonists could upregulate BCRP mRNA in human colon cancer cells (Ebert et al., 2005, 2007). Mechanism studies have identified an active, proximal dioxin-response element (DRE) at -194 to -190 bp upstream of the transcription start site of the human BCRP gene, which is

responsible for the direct binding of AHR and the subsequent induction of BCRP mRNA (Tan et al., 2010). Moreover, an antioxidant response element (ARE) at -431 to -420 bp of BCRP 5'-UTR is responsible for Nrf2-mediated BCRP expression through interaction with Nrf2 (Singh et al., 2010). It is noteworthy that the functional DRE is in close proximity to ERE, HRE, as well as PRE, which might suggest that the proximal regions of these motifs are essential for the recruitment of transcriptional activation machinery onto the BCRP gene and promote transcription. However, whether these elements work in concert or compete with each other is largely unknown, and further investigation might provide valuable information for the characterization of transcriptional regulation of BCRP and other ABC transporters.

3. Posttranscriptional regulation of BCRP

Human BCRP is extensively N-glycosylated. Sequence analysis revealed three potential N-linked glycosylation sites at positions 418, 557 and 596. Through site-directed mutagenesis, Asp596 (N596) was demonstrated to be the only N-glycosylation site in BCRP (Figure 1). Nonetheless, disruption of N-glycosylation modification in BCRP only brought about subtle impairments in plasma membrane trafficking and rhodamine 123 transport activity, which indicates N-glycosylation on N596 is not essential for the protein expression, membrane trafficking, ATPase activities or transport functions (Diop and Hrycyna, 2005). Mohrmann et al. verified that N596 is the only N-glycosylation site of BCRP and that N-glycosylation at 596 is not important for plasma membrane routing (Mohrmann et al., 2005).

Initially, phosphorylation of BCRP is not detected from the ovarian carcinoma cell line T8, and it seems that phosphorylation is not required for BCRP functions since substrate transport in T8 cells is effective (Mohrmann et al., 2005). However, Xie et al. used a yeast two-hybrid screening assay and identified BCRP as one of the interacting partners of Pim-1L, a serine/threonine kinase (Xie et al., 2008). Pim-1L colocalizes with BCRP on the plasma membrane and induces the phosphorylation of BCRP at T362 (Figure 1) in prostate cancer cells. Knocking down Pim-1L or T362A mutant resulted in decreased drug resistance, impaired plasma membrane localization and reduced BCRP oligomer formation, indicating that phosphorylation of BCRP at T362 is crucial for its expression and function.

BCRP is also under posttranscriptional regulation by microRNAs, a class of noncoding RNA genes. In the CD34(+)CD38(-) hematopoietic stem cells isolated from human umbilical cord blood, hsa-miR-520h inhibits mRNA expression of BCRP and possibly promotes the differentiation of hematopoietic stem cells (Liao et al., 2008), while in pancreatic cancer cells, hsa-miR-520h downregulates both mRNA and protein levels of BCRP, resulting in inhibition of cell migration and invasion (Wang et al., 2010). Interestingly, microRNA binds to a proximal miRNA response element (MRE) within the 3'-UTR of BCRP in various cancer cell lines and suppresses its expression (Li et al., 2011). However, drug resistant cells lost this putative MRE, therefore evading mRNA degradation and protein translation repression of BCRP mediated by microRNAs, leading to overexpression of BCRP (To et al., 2009). Another microRNA, miR-328, also targets 3'-UTR of the BCRP

gene and decreases BCRP mRNA and protein levels through mRNA cleavage (Pan et al., 2009).

4. Epigenetic regulation of BCRP

Promoter methylation has also been shown to regulate BCRP expression in both human multiple myeloma cells and patients' plasma cells, with demethylation of BCRP promoter contributing to increased mRNA and protein expression (Turner et al., 2006). The promoter methylation of BCRP was also examined in renal carcinoma cell lines. Increased BCRP expression was seen after demethylation treatment, indicating that methylation resulted in the silencing of the BCRP gene. Chromatin immunoprecipitation assay showed that methylated promoter of BCRP interacted with methyl CpG binding domain proteins MBD2 and MeCP2, which further recruited histone deacetylase 1 and a corepressor, resulting in interruption of BCRP transcription (To et al., 2006). Moreover, inhibition of DNA methylation in PC-6 lung cancer cells greatly increased both mRNA and protein expression of BCRP, and the promoter methylation of BCRP is inversely correlated with BCRP expression in both SCLC and NSCLC cells (Nakano et al., 2008), indicating that promoter demethylation of BCRP could be a common regulatory mechanism for BCRP upregulation in cancer cells.

In addition, BCRP promoter is also regulated by histone modification. Following drug selection in several cancer cell lines, increased histone H3 acetylation and reduced class I histone deacetylases associated with BCRP promoter were observed (To et al., 2008). Increased BCRP expression requires three prerequisites, removal of the repressive histone marker (trimethylated

histone H3 lysine), recruitment of RNA polymerase II, and recruitment of a chromatin-remodeling factor to the BCRP promoter. These studies further address the complexity of BCRP gene regulation.

5. Regulation of BCRP degradation

Wild-type BCRP is degraded in lysomes. However, misfolded BCRP proteins, such as Cys592 and Cys608 mutants, which lack the intramolecular disulfide bond and lose proper membrane trafficking, has been shown to be removed from the ER by retrotranslocation to the cytosol compartment, ubiquitinated by ubiquitin ligase and degraded in proteasomes (Wakabayashi et al., 2007, Wakabayashi-Nakao et al., 2009). In addition, Asp596 mutant, which lost N-glycosylation important for stabilizing de novo synthesized BCRP, also underwent ubiquitin-mediated proteasomal degradation (Nakagawa et al., 2009). In a recent study, it has been further revealed that Derlin-1, a part of the complex that mediates ER associated degradation (ERAD), promotes the degradation of wild-type BCRP through suppression of ER to Golgi transport, therefore providing a novel regulatory mechanism of BCRP degradation (Sugiyama et al., 2011).

6. Pharmacogenetics of BCRP

Pharmacogenetic variations of human BCRP have brought another level of regulation for the expression or function of BCRP, with the most common single nucleotide polymorphisms (SNPs) depicted in Figure 1.

Imai's group took the first step by isolating BCRP cDNA from 11 cancer cell lines for sequencing. Three variants, including G34A (V12M), and C421A (Q141K) SNPs and a splicing variant 944-949 deletion (delta315-6) were

identified (Imai et al., 2002). In addition, a C376T mutant, which leads to premature termination, was present in 3 out of 124 individuals, which resulted in loss of expression of BCRP protein (Imai et al., 2002). Zamber et al. moved the work forward through a large scale study to investigate the frequencies of all known coding region SNPs using DNA samples from 222 human subjects, representing eleven distinct ethnic groups (Zamber et al., 2003). Together, nine SNPs were identified, including four nonsynonymous, G34A, C421A, A616C (I206L), and A1768T (N590Y), as well as three synonymous and two intronic SNPs. Of all the SNPs discovered in this study, G34A and C421A are the most common.

G34A and C421A mutants, when expressed in polarized LLC-PK1 cells, both displayed significantly increased drug accumulation and decreased drug efflux compared to wild-type BCRP. However, the molecular mechanisms of this observed drug resistance for G34A and C421A are totally different. Confocal microscopy showed that the dysfunction of G34A could be attributed to disturbed apical plasma membrane localization, while the dysfunction of C421A mutant is due to decreased ATPase activity (Mizuarai et al., 2004, Morisaki et al., 2005). The functional analysis of these naturally occurring SNPs might facilitate our understanding of the structure-function relationship of BCRP. C421A mutant also showed great reduction in protein expression and drug resistance compared to wild-type *in vitro*, although mRNA levels were similar (Imai et al., 2002). However, *in vivo* study failed to identify the difference in intestinal BCRP mRNA or protein levels between C421 homozygouts and C421A heterozygouts (Zamber et al.,

2003), suggesting that other to-be-defined variations *in vivo* might contribute to the expression of BCRP as well.

G. Modulation of BCRP functions

In cancer chemotherapy, the ultimate goal targeting BCRP is to inhibit BCRP-mediated extrusion of these anticancer drugs to increase the effectiveness of treatment. Considerable efforts have been expanded to find chemosensitizers that will inhibit the function of BCRP and thereby reverse MDR.

1. Modulation of BCRP by small molecular inhibitors

The first inhibitor of BCRP, fumitremorgin C (FTC), was reported before BCRP had been discovered and was shown to inhibit resistance in the mitoxantrone selected S1-M1-3.2 colon cancer cell line (Rabindran et al., 1998). FTC was also shown to specifically inhibit BCRP mediated transport of chemotherapeutic agents in stably transfected cell lines (Rabindran et al., 2000). Unfortunately, clinical use of FTC was prevented by its neurotoxicity, leading to the development of a new tetracyclic analogue of FTC, Ko143 (Allen et al., 2002). Ko143 appeared to be a specific and potent inhibitor of both human BCRP and murine Bcrp. Most importantly, Ko143 was nontoxic *in vitro* at therapeutic concentrations and *in vivo* in mice either through oral or intraperitoneal administration (Allen et al., 2002). Subsequently, other FTC-type inhibitors, including the indolyl diketopiperazines (van Loevezijn et al., 2001) and tryprostatin A (Woehlecke et al., 2003) were screened and assessed.

On the other hand, numerous ABCB1 inhibitors were examined for their ability to inhibit BCRP. For example, a potent ABCB1 inhibitor elacridar

(GF120918) was also a BCRP inhibitor (de Bruin et al., 1999, Maliepaard et al., 2001b). Reserpine has also been shown to inhibit BCRP-mediated Hoechst 33342 transport (Zhou et al., 2001, Wierdl et al., 2003), and cyclosporin A has been demonstrated to inhibit ATPase activity of BCRP (Ozvegy et al., 2001, Qadir et al., 2005). Other specific ABCB1 inhibitors that have demonstrated potent BCPR inhibition include tariquidar (XR9576) (Robey et al., 2004), PSC-833 (Garcia-Escarp et al., 2004), a series of newly synthesized 1,4-dihydropyridines and pyridines, such as dihydropyridines, niguldipine, nicardipine and nitrendipine (Zhou et al., 2005b), and chromanone derivatives, such as piperazinobenzopyranones and phenalkylaminobenzopyranones (Boumendjel et al., 2005).

Recently, several TKIs have been shown to act as BCRP inhibitors. First, CI1033 was shown to reverse BCRP mediated resistance to SN-38 and topotecan (Erlichman et al., 2001). Gefitinib (Iressa; ZD1839) has also been shown to inhibit BCRP mediated drug resistance, as do imatinib mesylate (Gleevec, STI571), EKI-785, nilotinib, erlotinib, lapatinib and sunitinib at low concentrations (Houghton et al., 2004, Ozvegy-Laczka et al., 2004, Nakamura et al., 2005, Brendel et al., 2007, Shi et al., 2007, Dai et al., 2008, Dai et al., 2009). Since BCRP has been shown either to directly transport or to confer resistance to CI1033, gefitinib, and imatinib (Burger et al., 2004, Elkind et al., 2005), it is likely that these TKIs act as competitive inhibitors.

Flavonoids, a class of polyphenolic compounds widely present in foods and herbal products, are also found to be another class of BCRP inhibitors.

Silymarin, hesperetin, quercetin, and daidzein, as well as the stilbene resveratrol, were shown to increase intracellular accumulation of mitoxantrone and BODIPY-prazosin in BCRP overexpressing cells (Cooray et al., 2004). Similarly, chrysin and biochanin A have also been shown to be potent inhibitors of BCRP (Zhang et al., 2004b). It has also been reported that genestein, naringenin, acacetin, kaempferol and glycosylated flavonoids reversed resistance to SN-38 and mitoxantrone in BCRP overexpressing K562 cells (Imai et al., 2004). It has also been postulated that flavonoids inhibit BCRP via interaction with its NBD (Morris and Zhang, 2006). Treatment with multiple flavonoids has revealed an additive effect in BCRP inhibition, implying the approach of 'flavonoid cocktails' might achieve ideal effects in reversing MDR of BCRP (Zhang et al., 2004a).

Several other potent BCRP inhibitors include novobiocin, an aminocoumarin antibiotic that could decrease resistance to topotecan, SN-38 and mitoxantrone at low concentrations through competitive inhibition (Shiozawa et al., 2004). UCN-01, a cyclin-dependent kinase inhibitor, could also inhibit BCRP-mediated xenobiotics transport (Robey et al., 2004). Some human immunodeficiency virus protease inhibitors (HPIs), including ritonavir, saquinavir, nelfinavir, and lopinavir could effectively inhibit the transport activity of wildtype BCRP (482R), with less effect on its mutants (482T/G). None of the HPIs tested were BCRP substrates, indicating BCRP expression should be taken into account for drug-drug interaction when HPIs are coadministered with BCRP substrates (Gupta et al., 2004, Weiss et al., 2007).

Moreover, curcumin represents a current trend of screening natural compounds for cancer chemotherapy. Curcumin could inhibit the transport and resistance of mitoxantrone or PhA in BCRP overexpressing cells, without affecting ATP binding or expression of BCRP (Chearwae et al., 2006). Tetrahydrocurcumin (THC), a major metabolite of curcumin, also exhibited potent inhibition of BCRP, ABCB1 and ABCC1 (Limtrakul et al., 2007). More importantly, THC inhibited the binding of IAAP with BCRP, implying that THC inhibits BCRP function through direct interaction with the drug binding site of BCRP, instead of an off-target effect.

Our group has also discovered a novel inhibitor of BCRP, PZ-39. It has been shown that PZ-39 directly inhibits the transport activity of BCRP acutely and accelerates the lysosome degradation pathway of BCRP chronically. It is so far a potent and specific BCRP inhibitor, which has no effect on ABCB1 or ABCC1 (Peng et al., 2009). Inhibitor induced BCRP degradation provides a novel mechanism of BCRP inhibition and might become an effective way of reversing BCRP-mediated MDR (Peng et al., 2010).

Recently, the development of broad-spectrum MDR modulators has also demonstrated their value in reversing MDR mediated by several ABC transporters. The semisynthetic taxane analogue ortataxel inhibits drug efflux mediated by ABCB1, ABCC1 and BCRP. However, ortataxel is not optimal as a clinical MDR reversing agent because of its cytotoxicity, imposed by the C-13 side chain of the taxane molecule, which inhibits microtubule depolymerization. Therefore, noncytotoxic taxane-based reversal agents (tRA) were designed by eliminating

the C-13 side chain, and the resulting tRA 98006 (Brooks et al., 2003) strongly modulates daunorubicin and mitoxantrone efflux and sensitizes the cells overexpressing the three MDR transporters to their cytotoxicities. Interestingly, another tRA 96023 modulates only ABCB1 and 482R BCRP, but not ABCC1 (Minderman et al., 2004a). tRAs might be promising to be developed as broad-spectrum MDR modulators in the future. In addition, the pipecolinate derivative VX-710 (biricodar; Incel) has also shown inhibitory effects toward ABCB1, ABCC1 and 482R BCRP (Minderman et al., 2004b), while a novel class of tetrahydroisoquinolin-ethyl-phenylamine (WK-X-34 and XR9577) brought superior effectiveness in ABCB1 and BCRP inhibition (Jekerle et al., 2006, Jekerle et al., 2007), suggesting their potentials as clinical broad-spectrum MDR modulators.

2. Modulation of BCRP by gene therapy

Besides direct inhibition of ABC transporter functions, suppression of the expression of the BCRP gene through hammerhead ribozyme-based or antisense oligonucleotide-based treatment has been demonstrated to be a powerful therapeutic strategy to overcome drug resistance mediated by ABC transporters (Kobayashi et al., 1994, Stuart et al., 2000). Hammerhead ribozyme destroys target genes directly via endoribonucleolytic activities, and it can be designed to cleave a specific mRNA molecule with a certain motif, thus might also be applied for downregulation of BCRP gene, therefore restoring drug sensitivity of cancer cells overexpressing BCRP. Kowalski's group designed six hammerhead ribozymes directed against the BCRP encoding gene. One such anti-BCRP

ribozyme, RzB1, showed high endoribonucleolytic cleavage activity at physiologicl pH and temperature in a cell free system (Kowalski et al., 2001). After being further introduced into cell culture system, RzB1 successfully decreased both mRNA and protein levels of BCRP and reversed the drug-resistant phenotype, not only in atypical MDR human gastric carcinoma cell lines with moderate BCRP expression (Kowalski et al., 2002), but also in MDR MCF7/MX cells with a very high expression level of BCRP (Kowalski et al., 2004). Moreover, a newly engineered multitarget multiribozyme potently reverses BCRP-mediated MDR and inhibits ABCB1 and ABCC2 at the same time (Kowalski et al., 2005). This approach provides yet another novel tool for gene therapies against broad-spectrum MDR transporters.

Additionally, antisense oligonucleotide has also been applied to modulate BCRP gene expression. Transfer of BCRP antisense oligonucleotide into topotecan-resistant ovarian cancer cells brought about a 60% reduction of BCRP mRNA and a substantial decrease of resistance index to topotecan, indicating antisense oligonucleotide could partially reverse BCRP-mediated drug resistance (Jia et al., 2003).

Recently, a new approach using RNA interference has been applied for the specific knockdown of BCRP. siRNA significantly resulted in the downregulation of both exogenous and endogenous BCRP, therefore increasing the accumulation of topotecan (Ee et al., 2004a). However, Li et al. reported that siRNAs only partially reversed the MDR mediated by BCRP, indicating design of BCRP-specific siRNA or transfection efficiency might affect the application of this

approach *in vitro* (Li et al., 2005). Nevertheless, Priebsch's group applied both specific anti-BCRP siRNAs and shRNAs in human gastric carcinoma cells with an atypical MDR phenotype and achieved complete reversal of MDR mediated by BCRP (Priebsch et al., 2006). These data re-assured the feasibility of siRNA- and shRNA-mediated gene therapy in reversing BCRP-dependent MDR.

H. Specific aims of the present work

BCRP is one of the major ABC transporters involved in the development of MDR in cancer chemotherapy. Overexpression of BCRP in tumor cell plasma membranes leads to decreased intracellular accumulation of various anticancer drugs. BCRP has also been suggested to be important in regulating drug bioavailability. Thus, inhibition of BCRP function or expression will increase not only the intracellular level of anti-cancer drugs but also the systemic drug levels, therefore, reversing multidrug resistance. Compared to other typical ABC transporters, BCRP is considered to be a half transporter and it has been shown in several studies that the major form of BCRP on the plasma membrane is homo-oligomer and its oligomerization domain has been mapped to the domain consisting of C-terminal TM5-ECL3-TM6. Furthermore, this oligomerization domain hetero-oligomerizes with full-length BCRP and significantly inhibits its drug efflux activity. Therefore, the oligomerization of BCRP could be a novel and promising target in reversing MDR mediated by BCRP specifically.

The hypothesis of my project is that TM5, ECL3 or TM6 each plays a role in BCRP oligomerization and that this oligomerization formation can be used as a target for developing chemosensitizing agents to overcome BCRP-mediated MDR

in cancer chemotherapy. To test the above hypotheses, two specific aims are accomplished. The first aim is to determine if ECL3 is solely responsible for BCRP oligomerization. Using co-immunoprecipitation and chemical cross-linking analyses, I have found that ECL3 is essential for the oligomerization of BCRP, while neither the three cysteines nor the ^{569}QYFS motif within ECL3 is important for the oligomer formation. On the other hand, both TM5 and TM6 contribute to oligomer formation, but to a lesser extent when compared to full-length BCRP. These data suggest that ECL3, TM5 and TM6 all play a partial role in the oligmerization of BCRP and that each domain might contain at least one interacting site responsible for the formation of oligomeric BCRP. The second aim is to further determine the role of ECL3, TM5 or TM6 in the function of BCRP. Our results demonstrated that domain-swapping of TM5/TM6 by TM1/TM2 markedly reduced BCRP functions, indicating ECL3 alone is insufficient to retain functionality. However, TM5 is a major contributor of BCRP function, while TM6 alone is not sufficient to retain the transport activity or resistance phenotype of BCRP.

The outcome from this study should lead to a better understanding of how TM5, ECL3 and TM6 contribute to the oligomerization and function of BCRP and thus extend our knowledge of the structure-function relationship of BCRP and help to develop better treatment regimens that may be used to specifically reverse the MDR mediated by BCRP.

II. Materials and Methods

A. Materials

All primers for engineering BCRP constructs with PCR (polymerase chain reaction) were obtained from Invitrogen by Life Technologies, Inc. and pfu DNA polymerases were purchased from Stratagene by Agilent Techonologies. All DNA plasmid preparation kits were from QIAGEN. Cell culture medium DMEM, antibiotics, and trypsin were from Cellgro by Mediatech, Inc. and Lonza, respectively. DNA and protein molecular weight markers were obtained from Fermentas Life Sciences. The chemical cross-linking reagent disuccinimidyl suberate (DSS) was from Pierce by Thermo Fisher Scientific. ECL (Enhanced chemiluminescence) Western Blot detection reagents were obtained from Amersham Pharmacia Biotech by GE Healthcare. All other reagents of analytic grade or higher were purchased from Sigma-Aldrich.

B. Transient and stable cell transfection

HEK293 cells (human embryonic kidney 293 cells) with stable expression of HA-tagged BCRP were grown at 37°C with 5% CO_2 in DMEM, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, with the presence of 0.3 mg/ml G418. For transient transfection, pcDNA 3.1(+) plasmid containing different Myc-tagged BCRP constructs or vector control were transfected into HEK293 cells or HEK293 cells with stable expression of HA-tagged BCRP at 90% confluency using LipofectAMINE according to the manufacturer's instructions. The cells were harvested for cell lysate preparations 24 hours after transient transfection.

HEK293 cells were grown at 37°C with 5% CO_2 in DMEM, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For stable transfection, pcDNA 3.1(+) plasmid containing different Myc-tagged BCRP constructs or vector control were first transfected into HEK293 cells using LipofectAMINE according to the manufacturer's instructions. 48 hours following transfection, different dilutions of the transfected cells were selected with 0.8 mg/ml of G418 for 3 weeks. The stable clones with similar expression levels of all Myc-tagged BCRP constructs were verified by Western Blot and maintained in 0.3 mg/ml G418.

C. Cell lysate preparation

For cell lysate preparation, cells were harvested and lysed in ice-cold lysis buffer (150 mM NaCl, 25 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, with freshly added 2 mM PMSF and 1 mM DTT before use). The cell lysates were passed through 26 gauge needles at least 20 times followed by incubation of 30 minutes on ice. The lysates were then centrifuged at 12,000 × g for 15 minutes. Protein concentrations were determined by Bio-Rad protein assay dye kit, and the absorbance was measured by SmartSpec 3000 Spectrophotometer (Bio-Rad Laboratories, Inc.).

D. Co-immunoprecipation and Western Blot

For co-immunoprecipation, 500 μg fresh cell lysates were mixed with 2 μg normal mouse IgG, diluted to 1 ml with ice-cold lysis buffer used for cell lysate preparation and incubated for 2 hours at 4°C. The suspensions were precleared with 40 μl protein G-PLUS agarose beads (Santa Cruz Biotechnology, Inc.) for 2

hours at 4°C. After brief centrifugation at 500 × g for 1 minute, the supernatants were transferred to new microcentrifuge tubes and precipitated with monoclonal mouse anti-Myc (Cell Signaling Technology, 1:500 dilution) or anti-HA antibodies (Covance, 1:500 dilution) for 4 hours at 4°C. After centrifugation at 10,000 × g for 15 minutes at 4°C, the immune complexes in supernatants were transferred to fresh microcentrifuge tubes and were allowed to bind to 40 μl protein G-PLUS agarose beads overnight at 4°C with constant rotation. Agarose beads were washed 5 times with 1 ml lysis buffer, and the immunoprecipitates were resuspended in 40 μl 2× SDS-PAGE sample buffer.

For Western Blot analysis, cell lysates or immunoprecipitates were denatured in 2 × SDS-PAGE sample buffer for 30 minutes at room temperature. The samples were subjected to SDS-PAGE on 10% or 12% Tris-glycine SDS-polyacrylamide gel. The separated proteins were transferred onto a polyvinylidene fluoride membrane (Osmonics, Inc) and blocked in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for 1 hour at room temperature. The membranes were then incubated with the primary antibodies for 1 hour at room temperature. The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated Rat anti-mouse Kappa IgG (SouthernBiotech) using ECL detection reagents.

E. Plasma membrane preparation

Plasma membranes were prepared as described previously with minor modifications (Yang et al., 2002). Briefly, the cells were washed with ice-cold PBS and resuspended in hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10

mM Tris, pH 7.4, 2 mM PMSF) at 10^6 cells/ml followed by homogenization and centrifugation at 1,000 × *g* for 10 min. The plasma membrane fractions were prepared by layering the 1,000 × *g* supernatant on top of a 35% sucrose cushion containing 10 mM Tris, pH 7.4 and 1 mM EDTA, followed by centrifugation at 38,000 rpm for 1 h. The membranes at the interface between the supernatant and the sucrose cushion were collected, mixed with STBS buffer (250 mM sucrose, 150 mM NaCl and 10 mM Tris, pH 7.5) and pelleted by centrifugation at 29,000 rpm for 1 h. The final membrane pellets were resuspended in STBS buffer and stored at -80°C.

F. Chemical cross-linking

Chemical cross-linking was performed with DSS. Briefly, confluent cells in 150 mm dishes were washed three times with KCl/Hepes buffer (90 mM KCl, 50 mM Hepes, pH 7.5) and incubated with 2 mM DSS in the KCl/Hepes buffer for 45 minutes at room temperature. Tris-HCl (pH 7.4) was added to a final concentration of 2 mM to quench the reaction. The cells were then collected for plasma membrane preparation as described above. 5 µg of plasma membranes treated with DSS dissolved in DMSO or DMSO only were treated with an equal volume of 2 × SDS extraction buffer for 30 minutes at room temperature followed by centrifugation at 11,000 × g for 10 minutes. The supernatants were then loaded onto precast 4-15% gradient Tris/glycine polyacrylamide gels (Bio-Rad Laboratories, Inc.), and electrophoresis was run at 140 volts with SDS running buffer. Proteins were transferred to polyvinylidene fluoride membranes followed by detection with Western Blot.

G. MTT assay

A total of 3,000 HEK293 stable cells were plated in 96-well flat bottom plates for 24 hours and then exposed to Adriamycin (0, 1.6, 6.4, 25.6, 102.4, 410, 1640 ng/mL), or mitoxantrone (0, 4, 16, 64, 256, 1024, 4096 nM). After 72 hours, 20 µL of 5 mg/mL MTT solution in PBS were added to each well. After brief centrifugation and removal of the medium, 100 µl of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a MRX absorbance reader (Dynex Technologies, Inc.). Triplicate wells were assayed for each condition, and S.D. was determined.

H. Drug accumulation assay

The drug accumulation assay was performed as previously described (Peng et al., 2009) with minor modifications. 10^6 cells were trypsinized, resuspended in 1 mL DMEM with 20 µM mitoxantrone, and incubated at 37°C for 30 minutes. Cells were then collected by centrifugation at 500 × g and washed three times with ice-cold DMEM. The cells were then resuspended in 1 mL DMEM and subjected to analysis by flow cytometry using BD FACSCalibur APC Analyzer. The data were analyzed using Cell Quest Pro (BD Biosciences).

III. Experimental Results

A. ECL3 is responsible for oligomerization in truncated BCRP construct

In a previous study, it has been demonstrated that the oligomerization site of BCRP is located in its C-terminus consisting of TM5-ECL3-TM6 (Xu et al., 2007). To further map the oligomerization site, we dissected this region into three domains containing TM5, ECL3 and TM6, respectively. Through sequence alignment, we have found that ECL3 is highly conserved among human, rat and mouse (Figure 3). Moreover, Cys603, Cys592 and Cys608, which are responsible for the formation of intermolecular and intramolecular disulfide bond in BCRP (Wakabayashi et al., 2007), are located in ECL3. A QXXS motif (^{569}QYFS), which has been shown to facilitate the transmembrane domain interactions in membrane proteins (Sal-Man et al., 2005), was also found in the ECL3 of BCRP and is conserved among human, rat and mouse (Figure 3). Therefore, I first tested if ECL3 is responsible for BCRP oligomerization. Since it was previously found that ectopically expressed Myc-BCRP^{TM12} construct consisting of the N-terminal NBD through TM2 (Figure 4A-2) retained plasma membrane trafficking yet did not confer oligomerization activity, I engineered a construct by swapping the ECL1 in Myc-BCRP^{TM12} to ECL3, resulting in the Myc-tagged construct of Myc-BCRP$^{TM12–ECL3}$ (Figure 4A-3), to investigate the sole role of ECL3 in the oligomerization of BCRP. This construct, together with Myc-BCRP^{TM56} (Figure 4A-1) as a positive control and Myc-BCRP^{TM12} as a negative control (Xu et al., 2007), was transiently transfected into HEK293 stable cells that stably expressing

HA-tagged full-length BCRP (Figure 4, HA-BCRP^{FLWT}), to determine if Myc-BCRP$^{TM12–ECL3}$ interacts with the full-length BCRP.

Twenty-four hours after transfection, cell lysates were collected and tested for co-expression of the full-length and truncated BCRP constructs via Western Blot using either anti-HA or anti-Myc antibodies. All constructs were well expressed in HEK293 stable cells (Figure 4B). These lysates were then subjected to co-immunoprecipitation using anti-Myc antibodies (Figure 4C) followed by Western Blot analyses probed with anti-HA antibodies. As shown in Figure 4C, Myc-BCRP$^{TM12–ECL3}$ co-immunoprecipitates HA-BCRP^{FLWT} (lane 3). A detailed analysis of the amount of coprecipitated BCRP constructs after normalization to their expression levels shows that the relative level of coprecipitation of Myc-BCRP$^{TM12–ECL3}$ is reduced compared to Myc-BCRP^{TM56} (Figure 4D). Therefore, the replacement of ECL1 by ECL3 (Myc-BCRP$^{TM12–ECL3}$) enabled its interaction with HA-BCRP^{FLWT}, to a lesser extent when compared to Myc-BCRP^{TM56}, suggesting that ECL3 alone plays a partial role in the oligomerziation of BCRP.

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Rat             MSSSNDHVLVPMSQRNKNGLPGMSSRGARTLAEGDVLSFHHITYRVKVKSGFL-VRKTAE 59
Mouse           MSSSNDHVLVPMSQRNNNGLPRMNSRAVRTLAEGDVLSFHHITYRVKVKSGFL-VRKTVE 59
Human           MSSSNVEVFIPVSQGNTNGFPATASNDLKAFTEGAVLSFHNICYRVKLKSGFLPCRKPVE 60
                ***** .*::*:** *.**:*    *.  ::::** *****:* ****:*****  **..*

Rat             KEILSDINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPRGLSGDVLINGAPQPANFKCS 119
Mouse           KEILSDINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPKGLSGDVLINGAPQPAHFKCC 119
Human           KEILSNINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPSGLSGDVLINGAPRPANFKCN 120
                *****:********************************* ************:**:***

Rat             SGYVVQDDVVMGTLTVRENLQFSAALRLPKAMKTHEKNERINTIIKELGLDKVADSKVGT 179
Mouse           SGYVVQDDVVMGTLTVRENLQFSAALRLPTTMKNHEKNERINTIIKELGLEKVADSKVGT 179
Human           SGYVVQDDVVMGTLTVRENLQFSAALRLATTMTNHEKNERINRVIQELGLDKVADSKVGT 180
                ****************************..:*..******** :*:****:*********

Rat             QFTRGISGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIF 239
Mouse           QFIRGISGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIF 239
Human           QFIRGVSGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIF 240
                ** **:******************************************************

Rat             SIHQPRYSIFKLFDSLTLLASGKLMFHGPAQKALEYFASAGYHCEPYNNPADFFLDVING 299
Mouse           SIHQPRYSIFKLFDSLTLLASGKLVFHGPAQKALEYFASAGYHCEPYNNPADFFLDVING 299
Human           SIHQPRYSIFKLFDSLTLLASGRLMFHGPAQEALGYFESAGYHCEAYNNPADFFLDIING 300
                **********************:*:******:** ** *******.**********:***

Rat             DSSAVMLNRGEQDHEANKTEEPSKREKPIIENLAEFYINSTIYGETKAELDQLPVAQKKK 359
Mouse           DSSAVMLNREEQDNEANKTEEPSKGEKPVIENLSEFYINSAIYGETKAELDQLPGAQEKK 359
Human           DSTAVALNR-EEDFKATEIIEPSKQDKPLIEKLAEIYVNSSFYKETKAELHQLSGGEKKK 359
                **:** *** *:* :*.:  **** :**:**:*:*:*:**::* ******.**. .::**

Rat             GSSAFREPVYVTSFCHQLRWIARRSFKNLLGNPQASVAQLIVTVILGLIIGALYFGLKND 419
Mouse           GTSAFKEPVYVTSFCHQLRWIARRSFKNLLGNPQASVAQLIVTVILGLIIGAIYFDLKYD 419
Human           KITVFKEISYTTSFCHQLRWVSKRSFKNLLGNPQASIAQIIVTVVLGLVIGAIYFGLKND 419
                  :.*:*  *.**********::*************:**:****:***:***:**.** *

Rat             PTGMQNRAGVFFFLTTNQCFTSVSAVELFVVEKKLFIHEYISGYYRVSSYFFGKLVSDLL 479
Mouse           AAGMQNRAGVLFFLTTNQCFSSVSAVELFVVEKKLFIHEYISGYYRVSSYFFGKVMSDLL 479
Human           STGIQNRAGVLFFLTTNQCFSSVSAVELFVVEKKLFIHEYISGYYRVSSYFLGKLLSDLL 479
                .:*:******:*********:*******************************:**::****

Rat             PMRFLPSVIYTCLLYFMLGLKRTVEAFFIMMFTLIMVAYTASSMALAIAAGQSVVSVATL 539
Mouse           PMRFLPSVIFTCILYFMLGLKKTVDAFFIMMFTLIMVAYTASSMALAIATGQSVVSVATL 539
Human           PMRMLPSIIFTCIVYFMLGLKPKADAFFVMMFTLMMVAYSASSMALAIAAGQSVVSVATL 539
                ***:***:*:**::*******  ..:***:*****:****:*********:**********

Rat             LMTISFVFMMLFSGLLVNLRTIGPWLSWLQYFSIPRYGFTALQHNEFLGQEFCPGLNVTM 599
Mouse           LMTIAFVFMMLFSGLLVNLRTIGPWLSWLQYFSIPRYGFTALQYNEFLGQEFCPGFNVTD 599
Human           LMTICFVFMMIFSGLLVNLTTIASWLSWLQYFSIPRYGFTALQHNEFLGQNFCPGLNATG 599
                ****.*****:******** **..*******************:*******:****:*.*

Rat             NSTCVNSYTICTGNDYLINQGIDLSPWGLWRNHVALACMIIIFLTIAYLKLLFLKKYS 657
Mouse           NSTCVNSYAICTGNEYLINQGIELSPWGLWKNHVALACMIIIFLTIAYLKLLFLKKYS 657
Human           NNPCN--YATCTGEEYLVKQGIDLSPWGLWKNHVALACMIVIFLTIAYLKLLFLKKYS 655
                *..*    *: ***::**::***:*******:*********:*****************
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Figure 3: Sequence alignment of human BCRP, rat, and mouse Bcrp1. The sequence alignment shows over 90% similarity between rat and mouse Bcrp1 ECL3, and around 80% similarity between human BCRP ECL3 and rat/mouse Bcrp1 ECL3. The conserved ^{569}QYFS motif is indicated in underlined red letter, while the three cysteines in ECL3 are indicated in underlined blue letter. The multiple sequence alignment is performed by ClustalW2 software (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalw2).

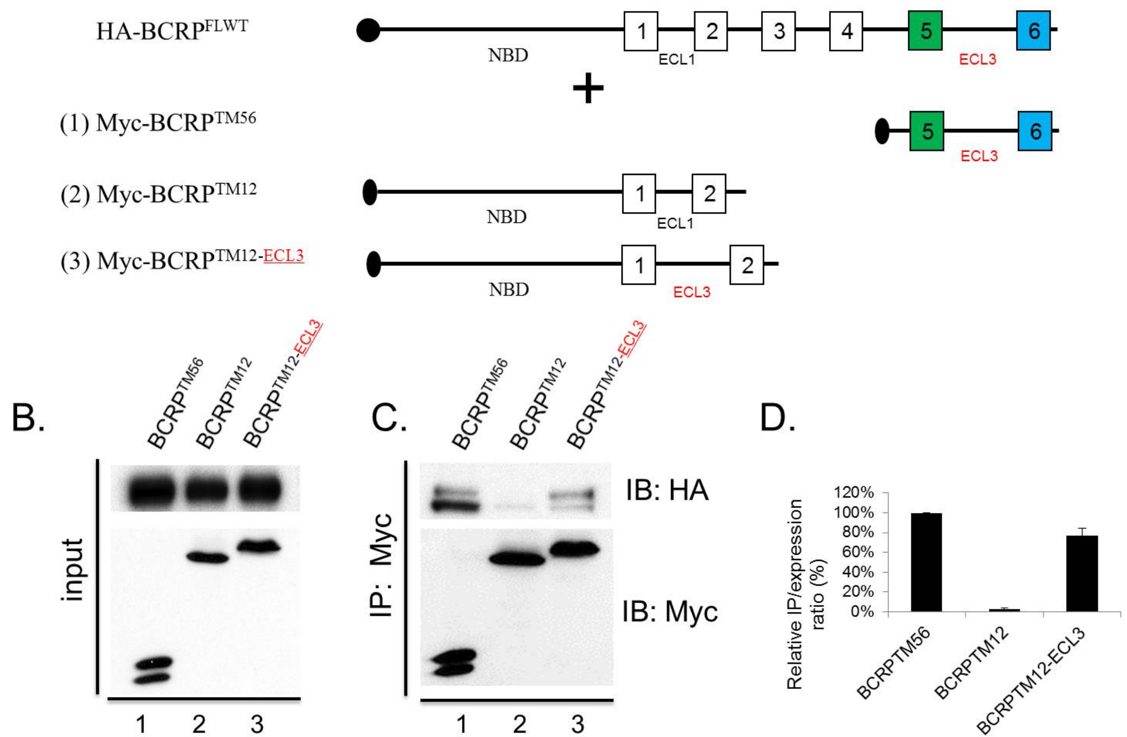


Figure 4: ECL3 is responsible for BCRP oligomerization in truncated BCRP construct. (A) Schematic diagram of HA-BCRP^{FLWT} and the truncated Myc-BCRP^{TM56} (1), truncated Myc-BCRP^{TM12} (2), and the domain-swapping Myc-BCRP$^{TM12\text{-}ECL3}$ (3) constructs. The TM segments are numbered and shown as boxes. The HA-tag and the Myc tag are shown as a circle and oval, respectively. NBD, ECL1 and ECL3 are also indicated. (B) Expression of the above BCRP constructs after transfection. (C) co-immunoprecipitation analysis of the above BCRP constructs. The Myc-tagged constructs were transiently transfected into HEK293 cells with stable expression of HA-BCRP^{FLWT}. Cell lysates were collected 24 hours after transient transfection and 400 μg total proteins was subjected to co-immunoprecipitation with anti-Myc antibodies followed by Western Blot analysis (IB). (D) Quantification of co-immunoprecipitation. The co-immunoprecipitation levels of constructs in (C) were quantified using ScnImage followed by calculation of the relative ratio of co-immunoprecipitation to expression level (B), N=3.

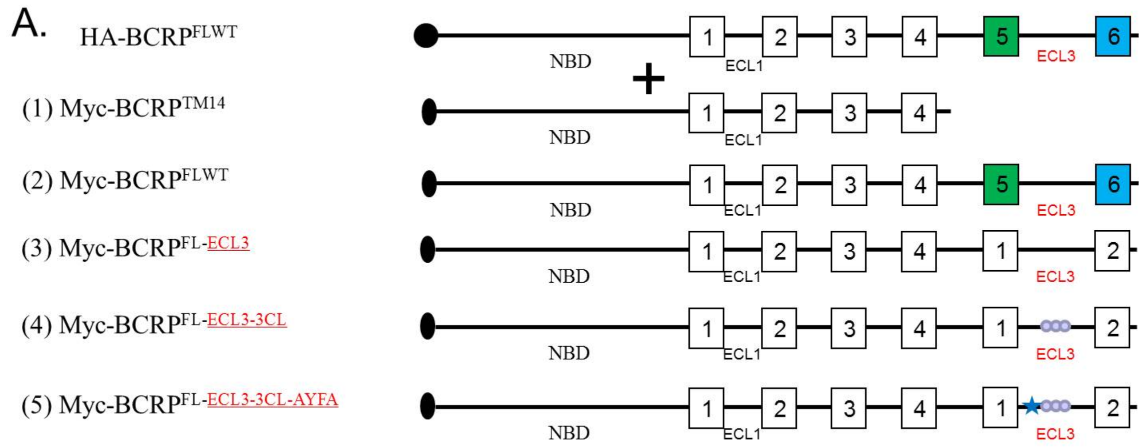
B. ECL3 is responsible for oligomerization in full-length BCRP

To further confirm ECL3 is an essential component of the oligomerization domain and to minimize the effect of domain truncation on the expression or function of BCRP, I engineered a second domain-swapping construct, Myc-BCRP$^{FL\text{-}ECL3}$ (Figure 5A-3), based on full-length BCRP. I have chosen TM1 and TM2 to replace TM5 and TM6 respectively, since TM1 and TM2 have been demonstrated to retain no oligomerization activity both in previous studies (Xu et al., 2007) and my studies (Figure 4C). Meanwhile, Myc-BCRP$^{FL\text{-}TM14}$ (Figure 5A-1), which has also been confirmed to abolish oligomerization activity (Xu et al., 2007), was used as a negative control. These two constructs, together with Myc-BCRP^{FLWT} (Figure 5A-2) as a positive control, were transiently transfected into HEK293 cells with stable expression of HA-BCRP^{FLWT}, respectively. All these contructs are well expressed as detected by Western Blot (Figure 5B). We next performed co-immunoprecipitation experiments with anti-HA antibodies (Figure 5C) followed by Western Blot analysis of the precipitates. As shown in Figure 5C, Myc-BCRP$^{FL\text{-}ECL3}$ co-precipitateS with HA-BCRP^{FLWT} (Figure 5C, lane 3), whereas Myc-BCRP$^{FL\text{-}TM14}$ did not (Figure 5C, lane 1), suggesting that ECL3 in full-length BCRP is responsible for the oligomerization of BCRP. Similar results were observed for co-immunoprecipitation with anti-Myc antibodies and Western Blot probed with anti-HA antibodies (Figure 5E). Quantification of the coprecipitated BCRP constructs after normalization to their expression levels shows that the relative level of coprecipitation of Myc-BCRP$^{FL\text{-}ECL3}$ is decreased compared to Myc-BCRP^{FLWT} (Figure 5D and 5F), which is consistent with the previous results

of Myc-BCRP$^{TM12–EC3}$ (Figure 4), confirming that ECL3 plays a partial role in BCRP oligomerization.

C. The cysteines in ECL3 are not essential for BCRP oligomerization

It has been shown that ECL3 of BCRP contains three cysteines, Cys603, Cys592 and Cys608, which are responsible for the formation of inter- and intra-molecular disulfide bonds (Wakabayashi et al., 2006). It has also been postulated that the disulfide bonds are possibly involved in the formation of BCRP dimer/oligomer (Kage et al., 2005). In order to investigate if these three cysteines are essential for BCRP oligomerization mediated by ECL3, I engineered a Myc-BCRP$^{FL-ECL3-3CL}$ construct (Figure 5A-4) with all three cysteines mutated to alanines. This construct was transiently transfected into HEK293 cells stably expressing HA-BCRP^{FLWT} and its expression was confirmed by Western Blot (Figure 5B, lane 4). Figure 5 demonstrates that following co-immunoprecipitation with anti-HA antibodies (Figure 5C, lane 4) and Western Blot analysis probed with anti-Myc antibodies, Myc-BCRP$^{FL-ECL3-3CL}$ co-precipitates with HA-BCRP^{FLWT} of a comparable level as Myc-BCRP$^{FL-ECL3}$ after normalization to protein expression levels (Figure 5D), indicating that these three cysteines or the disulfide bonds formed by them are not essential in BCRP oligomerization.



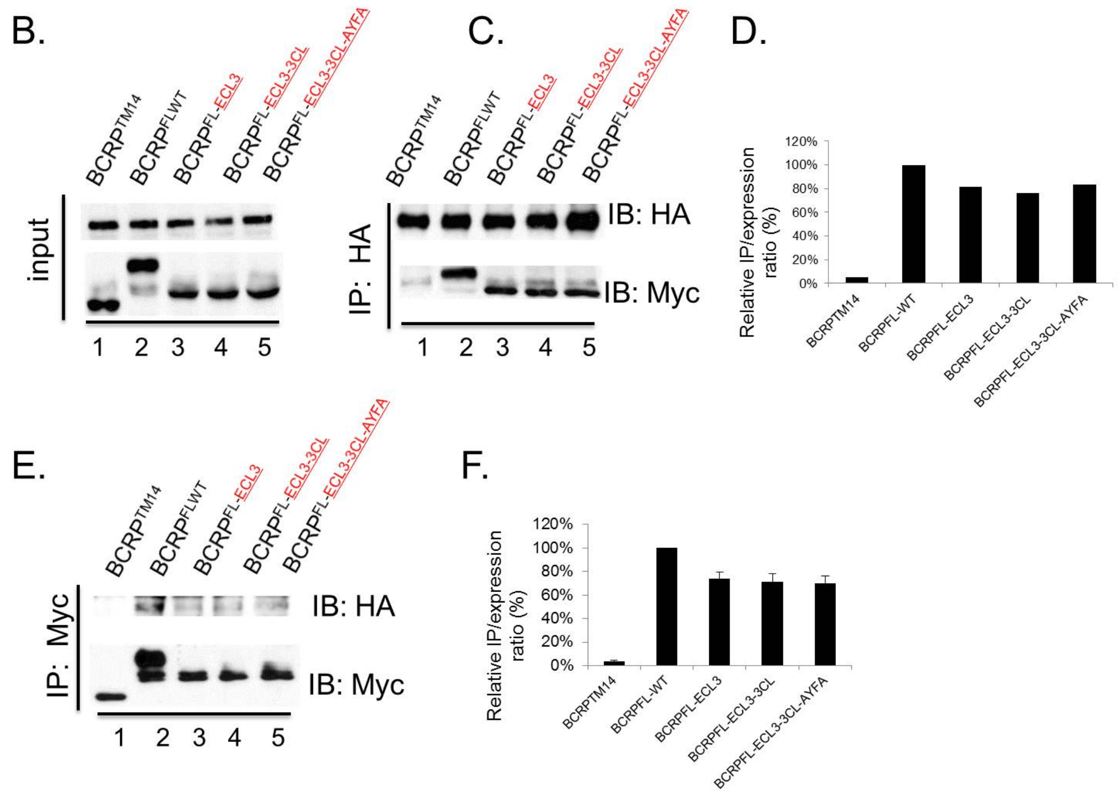


Figure 5: (Legend on next page).

Figure 5: The ECL3 is responsible for oligomerization in full-length BCRP and cysteines or the ^{569}QYFS motif in ECL3 is not essential for BCRP oligomerization. (A) Schematic diagram of HA-BCRP^{FLWT}, truncated Myc-BCRP^{TM14} (1), Myc-BCRP^{FLWT} (2) and the domain-swapping constructs Myc-BCRP$^{FL\text{-}ECL3}$ (3), Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL}$ (4), Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL\text{-}AYFA}$ (5). The TM segments are numbered and shown as boxes. The HA-tag and the Myc tag are shown as a circle and oval, respectively. NBD, ECL1 and ECL3 are also indicated. (B) Expression of the above BCRP constructs after transfection. (C) Co-immunoprecipitation of the above BCRP constructs. The Myc-tagged constructs were transiently transfected into HEK293 cells with stable expression of HA-BCRP^{FLWT}. 24 hours following transfection, cell lysates were collected and 400 µg total proteins was subjected to co-immunoprecipitation with HA (C) antibodies followed by Western Blot analysis. (D) Quantification of co-immunoprecipitation. The co-immunoprecipitation levels of constructs with anti-HA antibodies in (C) were quantified using ScnImage followed by calculation of the relative ratio of co-immunoprecipitation to expression level. (E) Co-immunoprecipitation of the above BCRP constructs with anti-Myc antibodies. (F) Quantification results of co-immunoprecipitation with anti-Myc antibodies and N=3.

Moreover, co-immunoprecipitation analysis with anti-Myc antibodies and Western Blot probed with anti-HA antibodies further confirmed the above findings (Figure 5E, lane 4).

D. The ^{569}QYFS motif is not essential for BCRP oligomerization

We next studied the involvement of the ^{569}QYFS motif (Sal-Man et al., 2005) in BCRP oligomerization mediated by ECL3. For this purpose, Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL\text{-}AYFA}$ construct (Figure 5A-5) was engineered, with the two essential polar amino acids (Q and S) in ^{569}QYFS motif mutated to nonpolar alanines. This Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL\text{-}AYFA}$ was transfected into HEK293 cells with stable expression of HA-BCRP^{FLWT}. As shown in Figure 5, Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL\text{-}AYFA}$ is similarly expressed (Figure 5B, lane 5) and coprecipitated by HA-BCRP^{FLWT} (Figure 5C, lane 5) as Myc-BCRP$^{FL\text{-}ECL3}$ after normalization to protein expression levels (Figure5D). This result was also verified by co-immunoprecipitation analysis with anti-Myc antibodies and Western Blot with anti-HA antibodies (Figure 5E, lane 5) and quantification analyses (Figure 5F). All these findings demonstrated that the ^{569}QYFS motif is also not involved in BCRP oligomerization.

E. TM5 and TM6 are essential for the oligomerization of BCRP

To further investigate the hypothesis that ECL3 is the sole responsible element for the oligomerization of BCRP, I designed a domain-swapping construct by replacing ECL3 in the Myc-BCRP^{FLWT} with ECL1. This new Myc-BCRP$^{FL\text{-}TM5TM6}$ construct (Figure 6A-1), was transiently transfected into HEK293 cells with stable expression of HA-BCRP^{FLWT}. All constructs were well

expressed as determined via Western Blot using anti-Myc antibodies (Figure 6B). Co-immunoprecipitation experiments with either anti-HA antibodies (Figure 6C) or anti-Myc antibodies (Figure 6E) were performed followed by Western Blot analysis of the precipitate. To our surprise, we found that Myc-BCRP$^{FL-TM5TM6}$ (Figure 6C and 6E, lane 1) could also co-precipitate with HA-BCRP^{FLWT}, with a slightly lower level as compared with Myc-BCRP^{FLWT} (Figure 6C and 6E, lane 2), suggesting that the combination of two flanking transmembrane segments of ECL3 is also essential for BCRP to oligomerize.

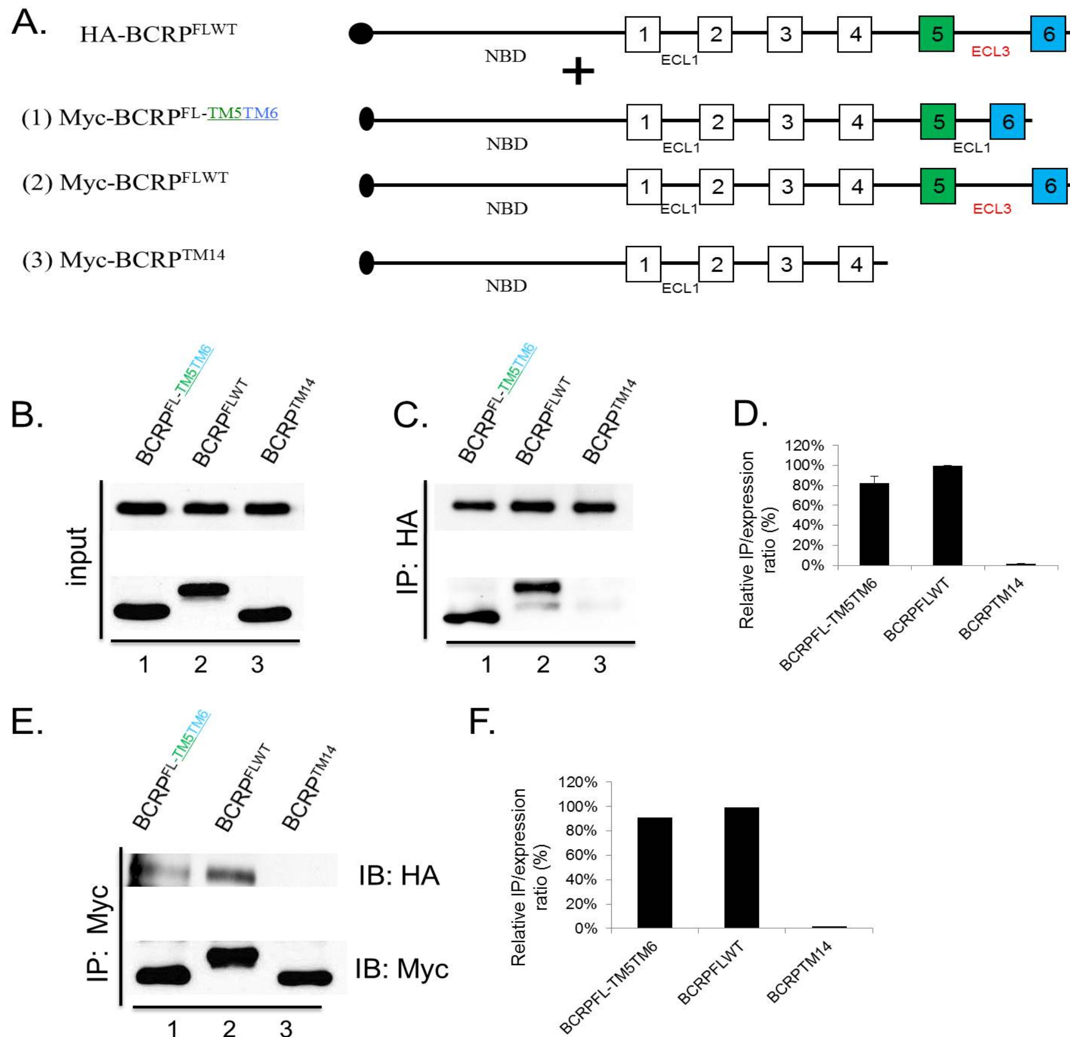


Figure 6: TM5 and TM6 are also essential for BCRP oligomerization. (A) Schematic diagram of the domain-swapping Myc-BCRP$^{FL-TM5TM6}$ construct (1), Myc-BCRP^{FLWT} (2) and Myc-BCRP^{TM14} (3). The TM segments are numbered and shown as boxes. The HA-tag and the Myc tag are shown as a circle and oval, respectively. These constructs were transiently transfected into HEK293 cells stably expressing HA-BCRP^{FLWT}. 24 hours following transfection, cell lysates were collected and expression were tested (B). 400 µg total protein was used for co-immunoprecipitation with anti-HA (C) and anti-Myc antibodies (E) followed by Western Blot analysis probed using the same two antibodies. The co-immunoprecipitation levels of constructs were quantified using ScnImage followed by calculation of the relative ratio of co-immunoprecipitation to expression level. (D) Quantification results of co-immunoprecipitation with anti-HA antibodies and N=3. (E) Quantification results of co-immunoprecipitation with anti-Myc antibodies.

F. Either TM5 or TM6 plays a partial role in BCRP oligomerization

As shown above, Myc-BCRP$^{FL\text{-}TM5TM6}$ is sufficient to interact with the full-length BCRP. It is of interest to determine if either TM5 or TM6 is essential for BCRP oligomerization. For this purpose, two additional domain-swapping constructs, Myc-BCRP$^{FL\text{-}TM5}$ (Figure 7A-1), with ECL3 and TM6 replaced by ECL1 and TM2, and Myc-BCRP$^{FL\text{-}TM6}$ (Figure 7A-2), with TM5 and ECL3 replaced by TM1 and ECL1, were engineered to investigate the responsibility of TM5 or TM6 in BCRP oligomerization. These two constructs were transiently transfected into HEK293 cells stably expressing HA-BCRP^{FLWT}, respectively. All constructs were well expressed as determined via Western Blot using Myc antibodies (Figure 7B). I next performed co-immunoprecipitation using the anti-HA antibodies (Figure 7C) or Myc antibodies (Figure 7E) followed by Western Blot analysis probed by HA or Myc antibodies. It is clearly shown that either Myc-BCRP$^{FL\text{-}TM5}$ (Figure 7C and 7E, Lane 1) or Myc-BCRP$^{FL\text{-}TM6}$ (Figure 7C and 7E, Lane 2) could co-precipitate with HA-BCRP^{FLWT}, suggesting that either TM5 or TM6 alone can interact with full-length BCRP. However, compared to Myc-BCRP^{FLWT} (Figure 7C and 7E, Lane 3), both Myc-BCRP$^{FL\text{-}TM5}$ and Myc-BCRP$^{FL\text{-}TM6}$, showed a decreased ability to interact with HA-BCRP^{FLWT} after normalization to protein expression level (Figure 7D for co-immunoprecipitation with anti-HA antibodies and 7F for co-immunoprecipitation with anti-Myc antibodies), indicating that TM5 and TM6 each has only partial contribution in BCRP oligomerization.

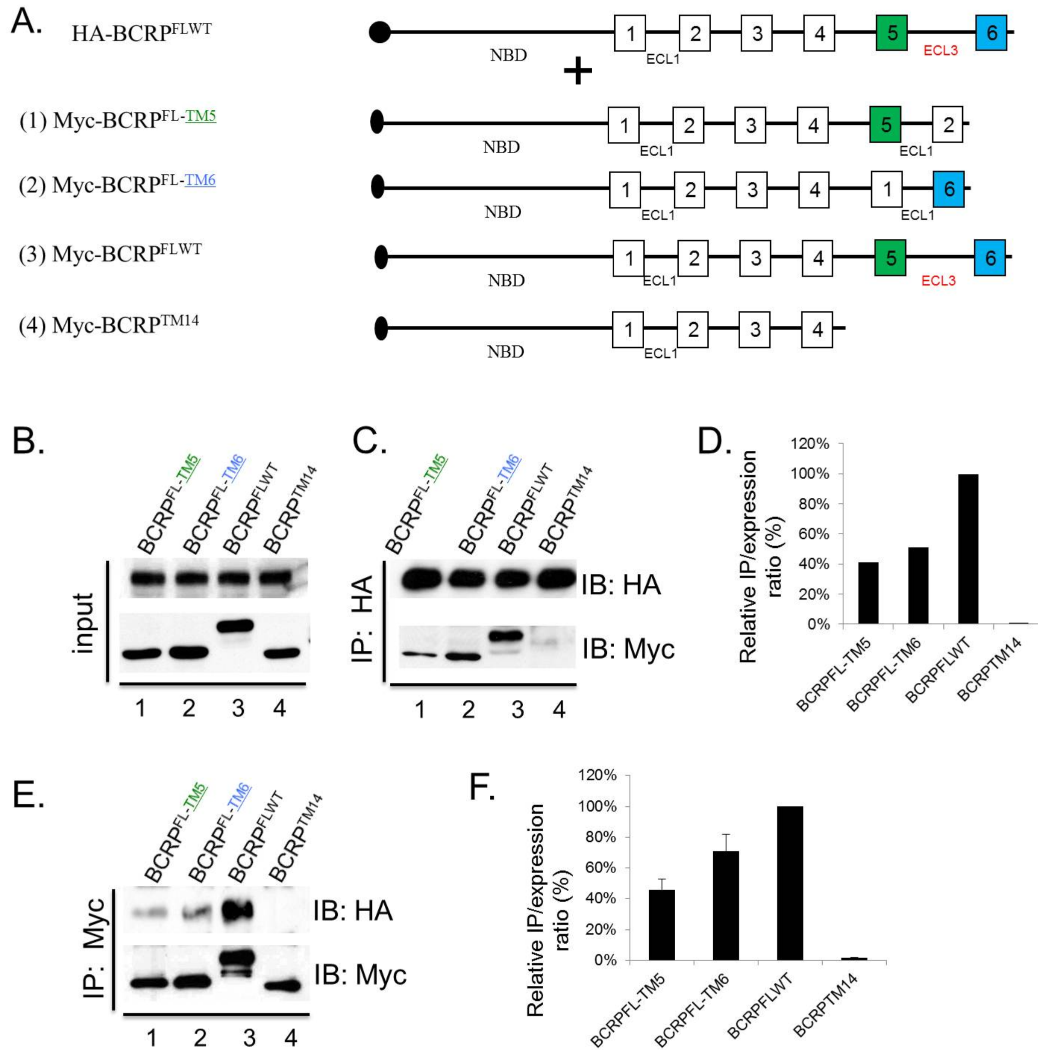


Figure 7: Either TM5 or TM6 in full-length BCRP is partially responsible for oligomerization. (A) Schematic diagram of HA-BCRP^{FLWT} and Myc-BCRP^{FL-TM5} (1), Myc-BCRP^{FL-TM6} (2), Myc-BCRP^{FLWT} (3), Myc-BCRP^{TM14} (4) constructs. The HA-tag and the Myc tag are shown as a circle and oval, respectively. NBC, ECL1 and ECL3 are also indicated. These constructs were transiently transfected into HEK293 cells stably expressing HA-BCRP^{FLWT}. 24 hours following transfection, cell lysates were collected and expression were tested (B). 400 µg total protein was used for co-immunoprecipitation with HA (C) and anti-Myc antibodies (E) followed by Western Blot analysis probed using the same two antibodies. The co-immunoprecipitation levels of constructs were quantified using ScnImage followed by calculation of the relative ratio of co-immunoprecipitation to expression level. (D) Quantification results of co-immunoprecipitation with anti-HA antibodies. (E) Quantification results of co-immunoprecipitation with anti-Myc antibodies and N=3.

G. Chemical cross-linking analyses of domain-swapping BCRP constructs in living cells

We next determined if BCRP domain-swapping constructs Myc-BCRP$^{FL\text{-}ECL3}$, Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL}$, Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL\text{-}AYFA}$, Myc-BCRP$^{FL\text{-}TM5}$ or Myc-BCRP$^{FL\text{-}TM6}$ could form oligomers by themselves in living cells. For this purpose, chemical cross-linking experiments of living cells using disuccinimidyl suberate (DSS) were conducted as previously described (Xu et al., 2004). HEK293 cells stably expressing Myc-BCRP^{FLWT}, domain-swapping Myc-BCRP$^{FL\text{-}ECL3}$, Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL}$, Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL\text{-}AYFA}$, Myc-BCRP$^{FL\text{-}TM5}$ or and Myc-BCRP$^{FL\text{-}TM6}$ were established and used for the cross-linking assay.

Following 2 mM DSS treatment, plasma membranes were isolated and subjected to SDS-PAGE and Western Blot analyses with anti-Myc antibodies. As shown in Figure 8A, three bands of Myc-BCRP^{FLWT} with molecular weight greater than that of the monomeric BCRP were detected following corss-linking by DSS, as compared with the control without DSS. The estimated sizes of these bands are close to 292, 219, 146 and 73 kD, corresponding to the sizes of tetrameric, trimeric, dimeric and monomeric Myc-BCRP^{FLWT}. This is consistent with the previously reported results (Xu et al., 2004).

On the other hand, Myc-BCRP$^{FL\text{-}ECL3}$ (Figure 8B, right panel), Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL}$ (Figure 8B, middle panel) and Myc-BCRP$^{FL\text{-}ECL3\text{-}3CL\text{-}AYFA}$ (Figure 8B, left panel) stable cells showed identical cross-linking products profiles with two major cross-linked products. The estimated molecular weights of these

products are close to 292, 146kD, corresponding to the size of tetrameric and dimeric Myc-BCRP$^{FL-ECL3-3CL-AYFA}$, Myc-BCRP$^{FL-ECL3-3CL}$, or Myc-BCRP$^{FL-ECL3}$. The chemical cross-linking results confirmed that ECL3 alone plays a partial role in the oligomerization of BCRP and that neither three cysteines in the ECL3 nor the ^{569}QYFS motif is necessary for the oligomerization of BCRP.

In addition, chemical cross-linking analyses with DSS were also performed in living Myc-BCRP^{FL-TM5} and Myc-BCRP^{FL-TM6} stable cells to further determine if Myc-BCRP^{FL-TM5} or Myc-BCRP^{FL-TM6} each forms oligomers in living cells. As shown in Figure 8, Myc-BCRP^{FL-TM5} (Figure 8C) and Myc-BCRP^{FL-TM6} (Figure 8D) expressed on the plasma membrane have two major cross-linked products. The estimated molecular weights of these products are close to 264 and 132kD, corresponding the sizes of tetrameric and dimeric Myc-BCRP^{FL-TM5} or Myc-BCRP^{FL-TM6}. The non-crosslinked monomeric Myc-BCRP^{FL-TM5} or Myc-BCRP^{FL-TM6} has an apparent molecular weight of ~64 kD on SDS-PAGE. These results clearly suggest that either TM5 or TM6 is partially responsible for the oligomerization of BCRP.

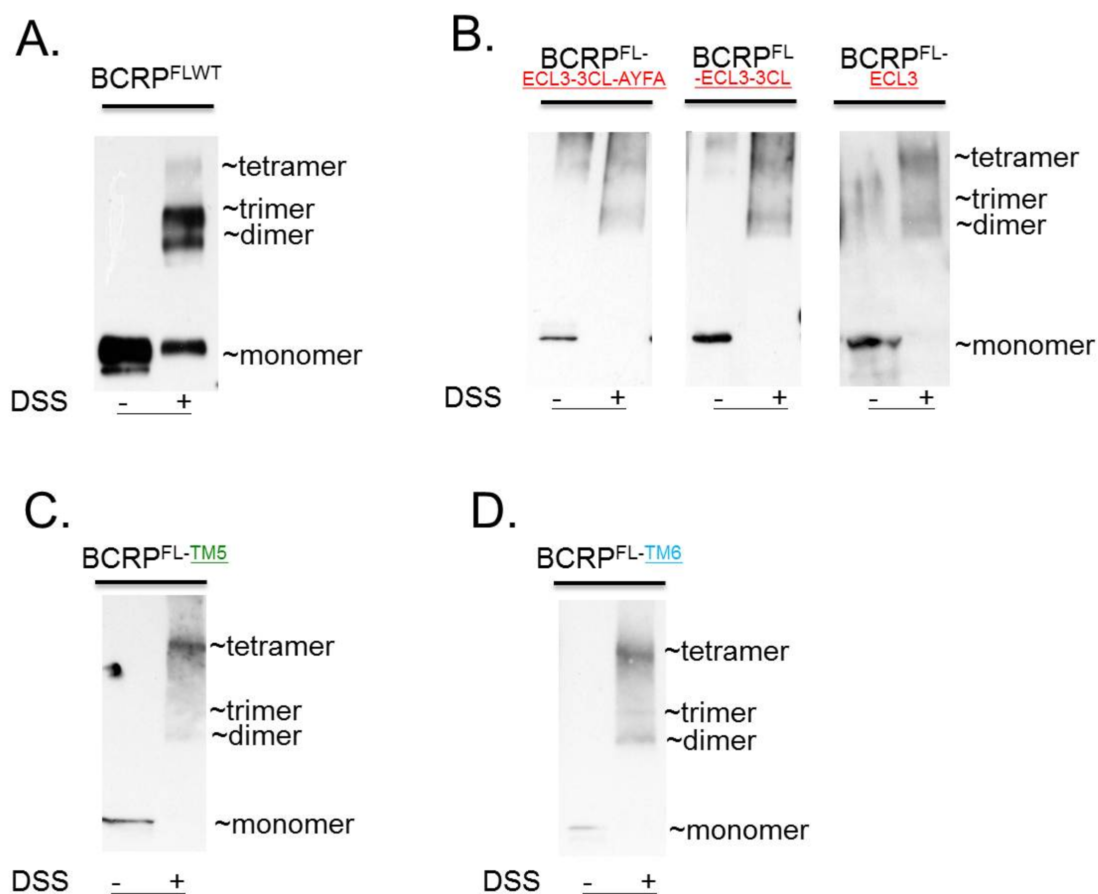


Figure 8: Chemical cross-linking analyses of BCRP domain-swapping constructs.10 μg of plasma membranes isolated from the stably transfected HEK293 cells with Myc-BCRP^{FLWT}, domain-swapping Myc-BCRP$^{FL-ECL3}$, Myc-BCRP$^{FL-ECL3-3CL}$, Myc-BCRP$^{FL-ECL3-3CL-AYFA}$, Myc-BCRP^{FL-TM5} or Myc-BCRP^{FL-TM6}, and truncated Myc-BCRP^{TM14} treated with or without 2mM DSS were separated by SDS-PAGE followed by Western Blot analysis. The sizes of all the constructs were estimated based on linear regression of the protein markers used.

H. ECL3 is insufficient for BCRP transport activity or drug resistance mediated by BCRP

As shown above, Myc-BCRP$^{FL-ECL3}$ plays a partial role in the oligomerization of BCRP. Next, I proceeded to investigate the functional significance of ECL3. For this purpose, I tested and compared the drug resistance cytotoxicity of HEK293 cells stably expressing vector control, Myc-BCRP^{FLWT}, Myc-BCRP$^{FL-ECL3}$ and Myc-BCRP$^{FL-TM5TM6}$ to either mitoxantrone or Adriamycin. As shown in Figure 9-10, the cells expressing Myc-BCRP^{FLWT} alone are resistant to both mitoxantrone (Figure 9A) and Adriamycin (Figure 10A) compared with cells transfected with vector alone. However, compared with Myc-BCRP^{FLWT} stable cells, Myc-BCRP$^{FL-ECL3}$ stable cells have significantly reduced mitoxantrone (Figure 9B) and Adriamycin (Figure 10B) resistance with a decrease in RRF (relative resistance factor) by 50% after normalization to the average plasma membrane expressions. On the other hand, Myc-BCRP$^{FL-TM5TM6}$ stable cells, without ECL3, have shown similar mitoxantrone (Figure 9B) and Adriamycin (Figure 10B) resistance as the wildtype BCRP after normalization to the expression levels of these constructs on the plasma membranes, indicating TM5 and TM6 are responsible for BCRP functions. Figure 11 shows the plasma membrane expression level of the above BCRP constructs as determined by Western Blot analyses with anti-Myc antibodies.

To further confirm the results from drug resistance cytotoxicity assays, drug accumulation activity of HEK293 cells stably expressing vector control, Myc-BCRP^{FLWT}, Myc-BCRP$^{FL-ECL3}$ and Myc-BCRP$^{FL-TM5TM6}$ were tested. Figure 12

(A) shows typical profiles of mitoxantrone accumulation in these cells as determined by FACS. The relative accumulation of mitoxantrone reduced markedly in both Myc-BCRP^{FLWT} and Myc-BCRP$^{FL-TM5TM6}$ stable cells, while Myc-BCRP$^{FL-ECL3}$ expressing cells showed similar drug accumulation as vector expressing cells (Figure 12B), suggesting that ECL3 alone is insufficient to retain the drug efflux activity of BCRP and drug resistance mediated by BCRP. Furthermore, disrupting the oligomerization activity mediated by ECL3 alone (shown by results of Myc-BCRP$^{FL-TM5TM6}$) do not affect BCRP activity, indicating the inter-domain interactions between ECL3s, which is partially responsible for the formation of BCRP oligomers, is not necessary for BCRP transport activities or drug resistance mediated by BCRP.

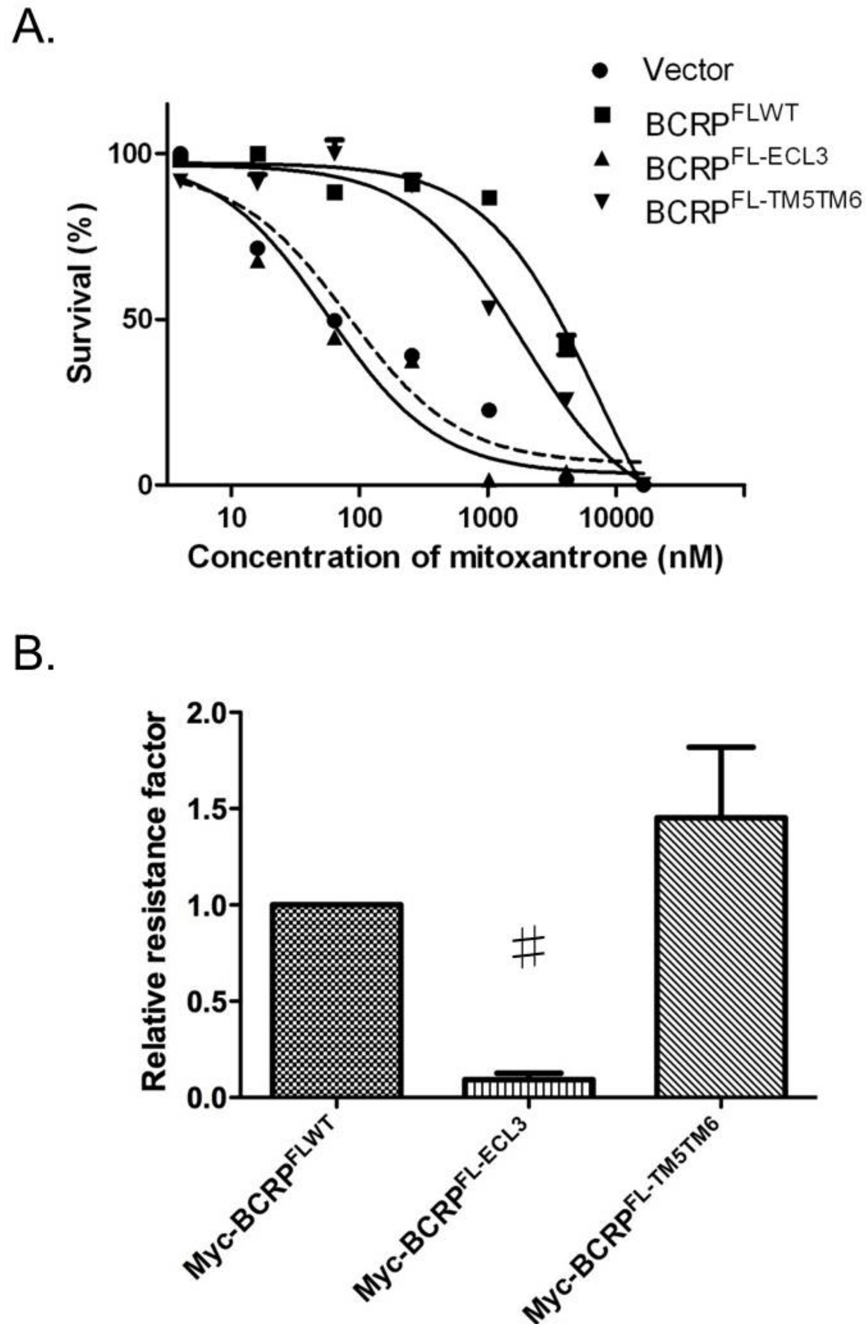


Figure 9: Effect of ECL3 on mitoxantrone resistance. Vector-transfected, Myc-BCRP^{FLWT}, Myc-BCRP$^{FL-ECL3}$ and Myc-BCRP$^{FL-TM5TM6}$ stable cells were treated with various concentrations of mitoxantrone followed by analysis using MTT assy. Dose-survival curves (A) were fitted using Prism software (version 3.02). Relative resistance factor (RRF) (B) was derived by normalizing the IC50 of Myc-BCRPFLWT, Myc-BCRP$^{FL-ECL3}$ or Myc-BCRP$^{FL-TM5TM6}$ to the IC50 of the vector transfected stable cells and further to the average plasma membrane expression of the above constructs. # $p<0.01$.

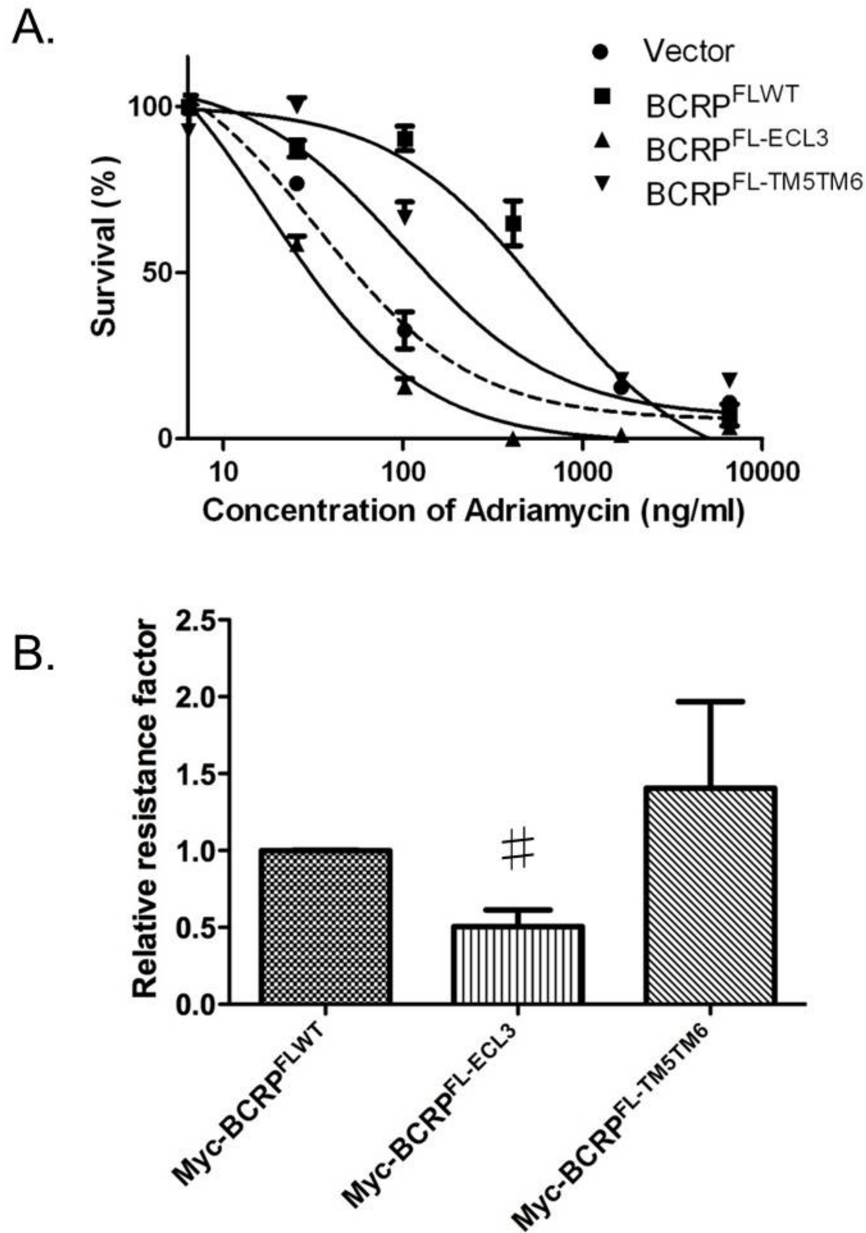
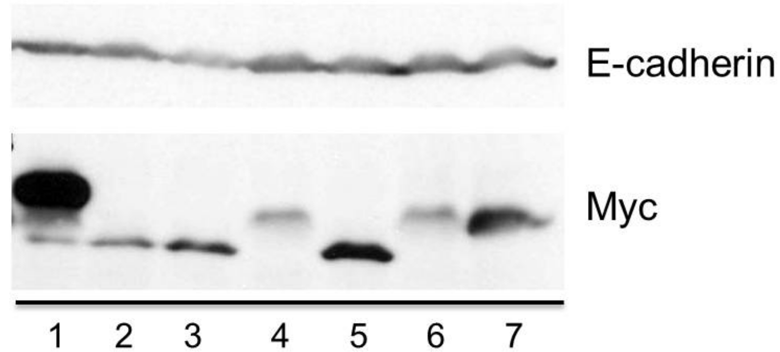


Figure 10: Effect of ECL3 on Adriamycin resistance. Vector-transfected, Myc-BCRP^{FLWT}, Myc-BCRP$^{FL-ECL3}$ and Myc-BCRP$^{FL-TM5TM6}$ stable cells were treated with various concentrations of Adriamycin followed by analysis using MTT assy. Dose-survival curves (A) were fitted using Prism software (version 3.02). Relative resistance factor (RRF) (B) was derived by normalizing the IC50 of Myc-BCRPFLWT, Myc-BCRP$^{FL-ECL3}$ or Myc-BCRP$^{FL-TM5TM6}$ to the IC50 of the vector transfected stable cells and further to the average plasma membrane expression of the above constructs. # $p<0.01$.



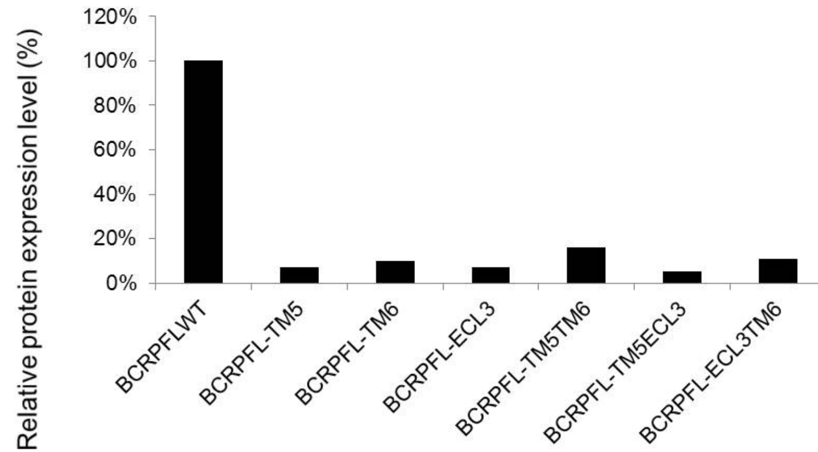
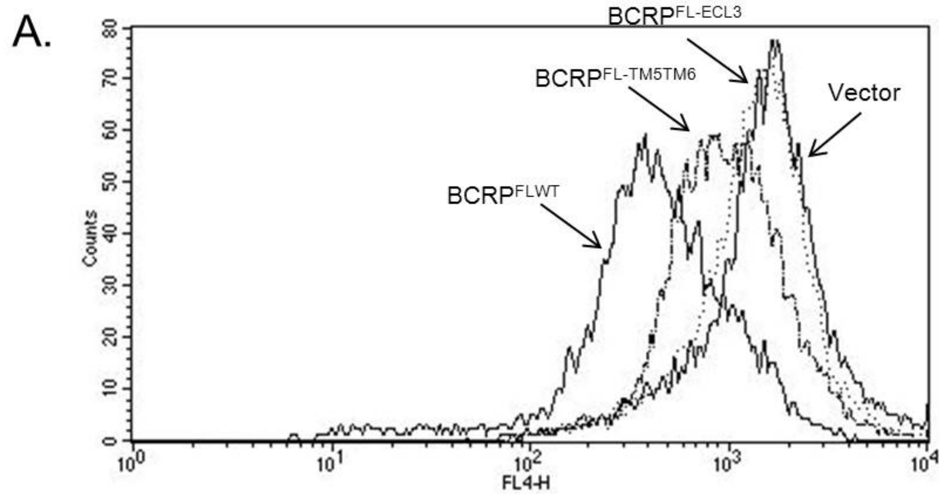


Figure 11: Expression levels of BCRP constructs in the plasma membrane vesicles extracted from HEK293 stable cell clones. (A) 5μg plasma membrane extracted from HEK293 cells stably expression Myc-BCRP^{FLWT} (1), Myc-BCRP^{FL-TM5} (2), Myc-BCRP^{FL-TM6} (3), Myc-BCRP$^{FL-ECL3}$ (4), Myc-BCRP$^{FL-TM5TM6}$ (5), Myc-BCRP$^{FL-TM5ECL3}$ (6), and Myc-BCRP$^{FL-ECL3TM6}$ (7) were probed with anti-Myc and anti-E-cadherin antibodies as internal control to determine the expression of these constructs on the plasma membranes. (B) The relative protein expression level was normalized to BCRP^{FLWT}.

A.



B.

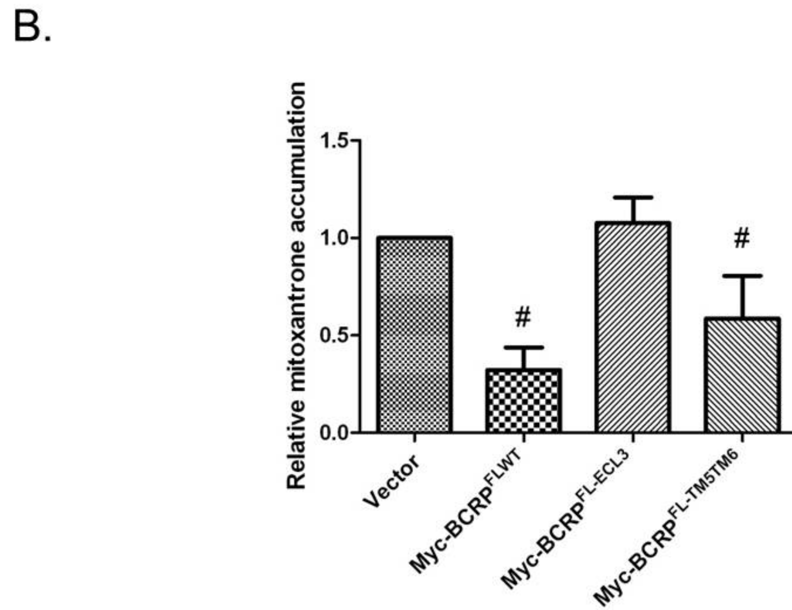


Figure 12: Effect of ECL3 on mitoxantrone accumulation acitvity. (A) Vector-transfected, Myc-BCRP^{FLWT}, Myc-BCRP$^{FL-ECL3}$ and Myc-BCRP$^{FL-TM5TM6}$ stable cells were subject to FACS analysis of mitoxantrone efflux for transport activity of BCRP. (B) Relative motixantrone accumulations were normalized to vector expressing cells. # $p<0.01$.

I. TM5 is a major contributor for the drug transport and drug resistance mediated by BCRP

Next, we investigated the contribution of TM5 to drug resistance mediated by BCRP. MTT assays with either mitoxantrone or Adriamycin were performed with Myc-BCRP^{FL-TM5}, Myc-BCRP$^{FL-ECL3TM6}$ (TM5 replaced by TM1) (Figure 13A) stable cells. As shown in Figure 13-14, compared with Myc-BCRP^{FLWT} stable cells, Myc-BCRP^{FL-TM5} stable cells have a trend of increased mitoxantrone (Figure 13B) and Adriamycin (Figure 14B) resistance. In contrast, Myc-BCRP$^{FL-ECL3TM6}$ stable cells, which abolished the activity conferred by TM5, showed a significantly decreased mitoxantrone (Figure 13B) and Adriamycin (Figure 14B) resistance with a reduction in RRF (relative resistance factor) by more than 50%, indicating that TM5 alone is sufficient and necessary for BCRP functions.

Drug accumulation assays with FACS were also performed in HEK293 cells stably expressing vector control, Myc-BCRP^{FLWT}, Myc-BCRP^{FL-TM5}, and Myc-BCRP$^{FL-ECL3TM6}$ cells to confirm the above findings. Figure 15A shows typical profiles of mitoxantrone accumulation in these cells as determined by FACS. The relative accumulation of mitoxantrone is reduced significantly in both Myc-BCRP^{FLWT} and Myc-BCRP^{FL-TM5} stable cells, while Myc-BCRP$^{FL-ECL3TM6}$ expressing cells showed similar drug accumulation as vector expressing cells (Figure 15B). These results strongly suggest that TM5 is a major contributor for BCRP functions.

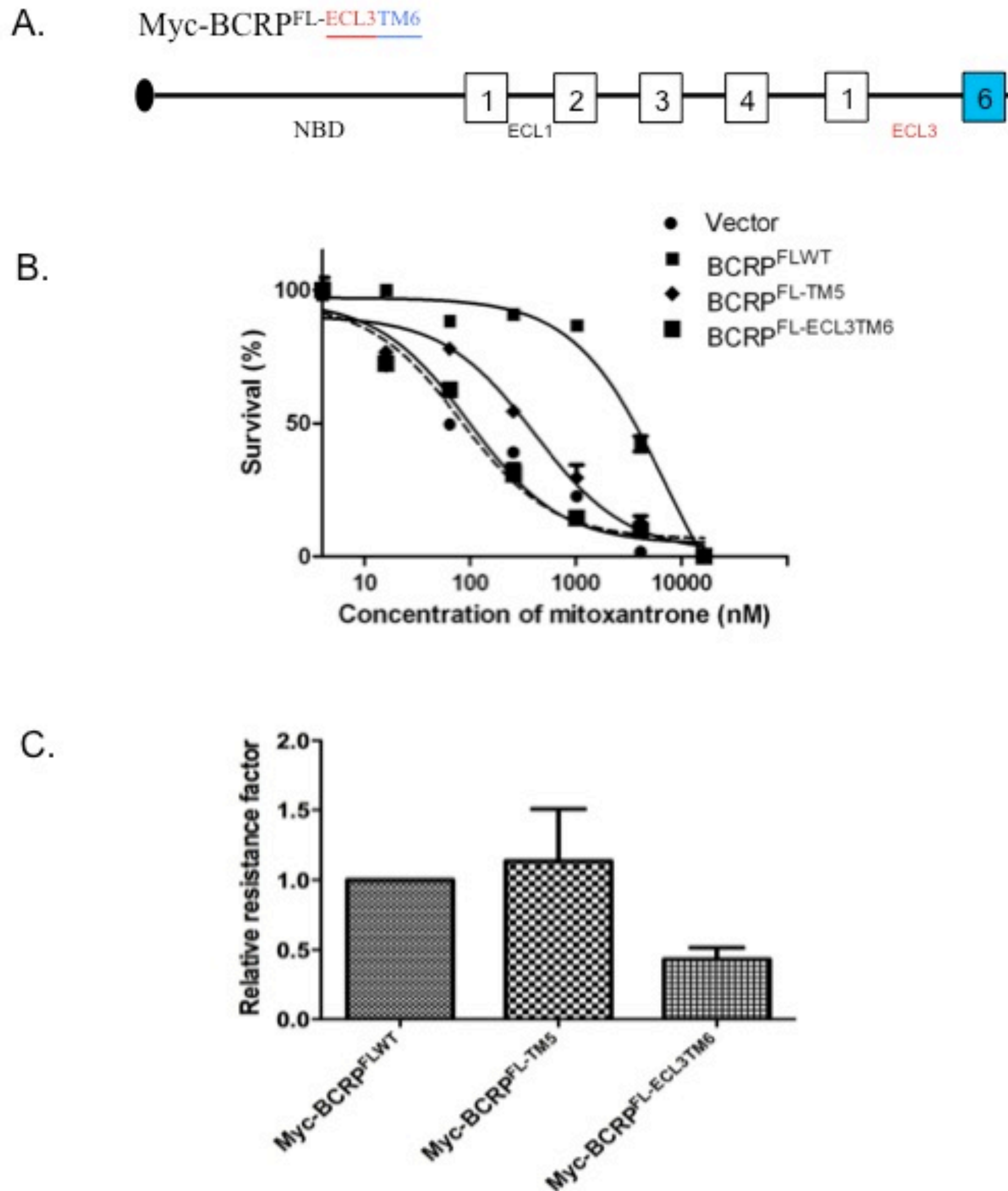


Figure 13: Effect of TM5 on mitoxantrone resistance. Vector-transfected, Myc-BCRP^{FLWT}, Myc-BCRP^{FL-TM5} and Myc-BCRP$^{FL-ECL3TM6}$ stable cells (A) were treated with various concentrations of mitoxantrone followed by analysis using MTT assy. Dose-survival curves (C) were fitted using Prism software (version 3.02). Relative resistance factor (RRF) (A) was derived by normalizing the IC50 of Myc-BCRPFLWT, Myc-BCRP^{FL-TM5} or Myc-BCRP$^{FL-ECL3TM6}$ to the IC50 of the vector transfected stable cells and further to the average plasma membrane expression of the above constructs. # p<0.01.

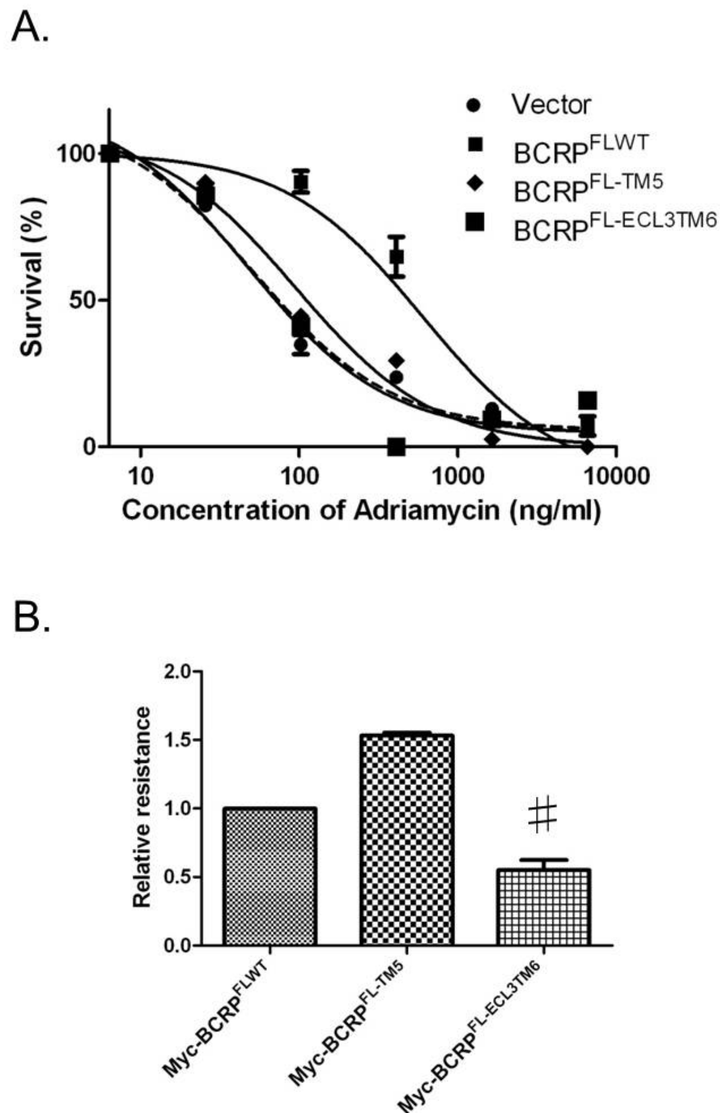


Figure 14: Effect of TM5 on Adriamycin resistance. Vector-transfected, Myc-BCRP^{FLWT}, Myc-BCRP^{FL-TM5} and Myc-BCRP$^{FL-ECL3TM6}$ stable cells were treated with various concentrations of Adriamycin followed by analysis using MTT assy. Dose-survival curves (A) were fitted using Prism software (version 3.02). Relative resistance factor (RRF) (B) was derived by normalizing the IC50 of Myc-BCRPFLWT, Myc-BCRP^{FL-TM5} or Myc-BCRP$^{FL-ECL3TM6}$ to the IC50 of the vector transfected stable cells and further to the average plasma membrane expression of the above constructs. # p<0.01.

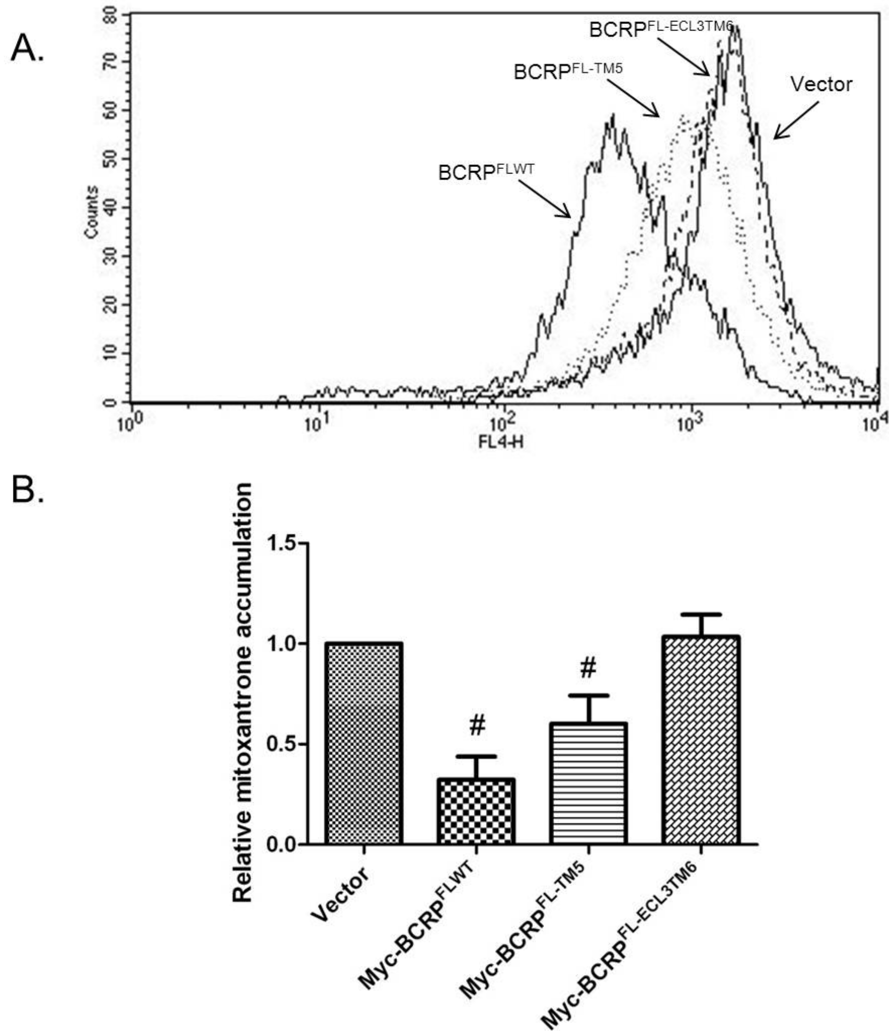


Figure 15: Effect of TM5 on mitoxantrone accumulation acitvity. (A) Myc-BCRP^{FLWT}, Myc-BCRP$^{FL\text{-}TM5}$, Myc-BCRP$^{FL\text{-}ECL3TM6}$ stable cells were subject to FACS analysis of mitoxantrone efflux for transport activity of BCRP. (B) Relative mitoxantrone accumulations were normalized to vector expressing cells. # $p<0.01$.

J. TM6 is insufficient for the drug transport and drug resistance mediated by BCRP

It has been demonstrated that TM6 plays a partial role in the oligomerization of BCRP. Here, the effects of TM6 on BCRP functions were investigated. Figure 15 and 16 show that Myc-BCRP^{FL-TM6} stable cells have significantly decreased mitoxantrone (Figure 16B) and Adriamycin (Figure 17B) resistance with RRF reduced by over 50% compared to Myc-BCRP^{FLWT} stable cells (Figure 16C and 17C). On the other hand, Myc-BCRP$^{FL-TM5ECL3}$ (TM6 replaced by TM2) (Figure 16A) stable cells, which abolishes the contribution of TM6, shows a slightly increased or similar resistance to mitoxantrone (Figure 16B) and Adriamycin (Figure 17B), suggesting that TM6 alone is insufficient for the functions of BCRP.

Drug accumulation activity of HEK293 cells stably expressing vector control, Myc-BCRP^{FLWT}, Myc-BCRP^{FL-TM6} and Myc-BCRP$^{FL-TM5ECL3}$ were also tested. Figure 18A shows typical profiles of mitoxantrone accumulation in these cells as determined by FACS. The relative accumulation of mitoxantrone was not reduced in either Myc-BCRP^{FL-TM6} stable cells compared to vector-transfected cells (Figure 18B), further confirming that TM6 alone is not sufficient for BCRP functions. Interestingly, Myc-BCRP$^{FL-TM5ECL3}$ stable cells also showed similar mitoxantrone accumulation as vector-transfected cells.

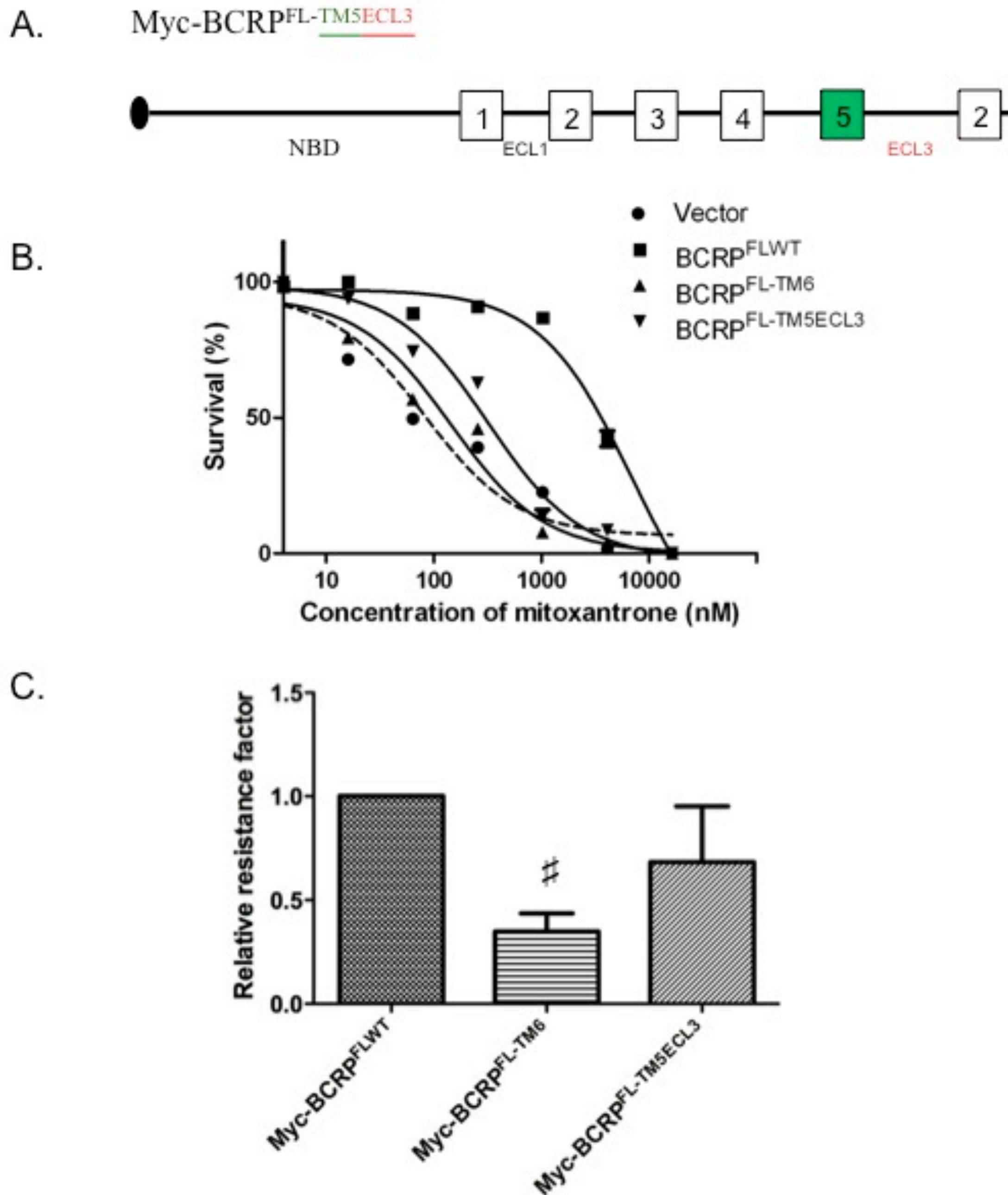


Figure 16: Effect of TM6 on mitoxantrone resistance. Vector-transfected, Myc-BCRP^{FLWT}, Myc-BCRP$^{FL\text{-}TM6}$ and Myc-BCRP$^{FL\text{-}TM5ECL3}$ (A) stable cells were treated with various concentrations of mitoxantrone followed by analysis using MTT assy. Dose-survival curves (B) were fitted using Prism software (version 3.02). Relative resistance factor (RRF) (C) was derived by normalizing the IC50 of Myc-BCRPFLWT, Myc-BCRP$^{FL\text{-}TM6}$ or Myc-BCRP$^{FL\text{-}TM5ECL3}$ to the IC50 of the vector transfected stable cells and further to the average plasma membrane expression of the above constructs. # $p<0.01$.

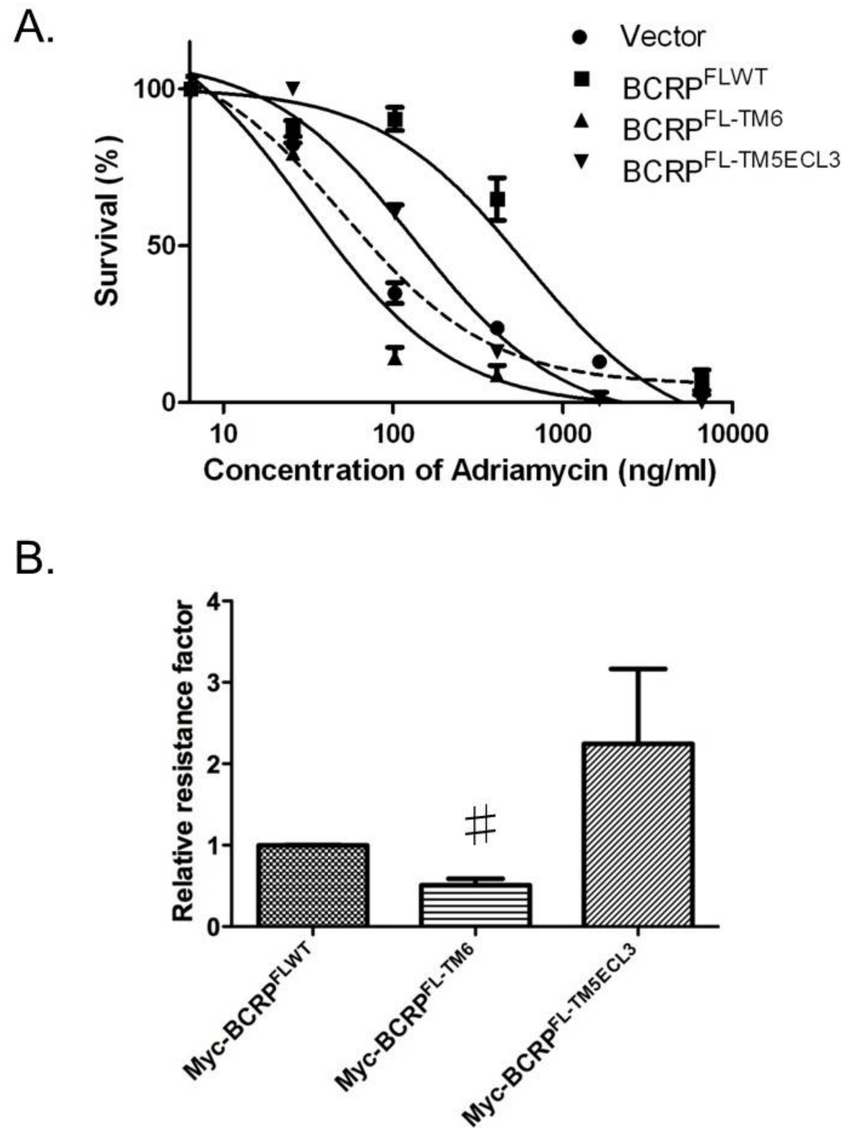
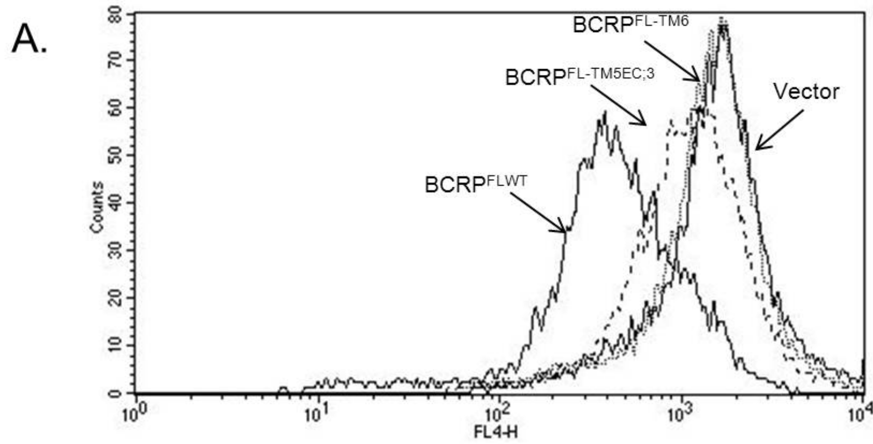


Figure 17: Effect of TM6 on Adriamycin resistance. Vector-transfected, Myc-BCRP^{FLWT}, Myc-BCRP^{FL-TM6} and Myc-BCRP$^{FL-TM5ECL3}$ stable cells were treated with various concentrations of Adriamycin followed by analysis using MTT assy. Dose-survival curves (A) were fitted using Prism software (version 3.02). Relative resistance factor (RRF) (B) was derived by normalizing the IC50 of Myc-BCRPFLWT, Myc-BCRP^{FL-TM6} or Myc-BCRP$^{FL-TM5ECL3}$ to the IC50 of the vector transfected stable cells and further to the average plasma membrane expression of the above constructs. # $p<0.01$.



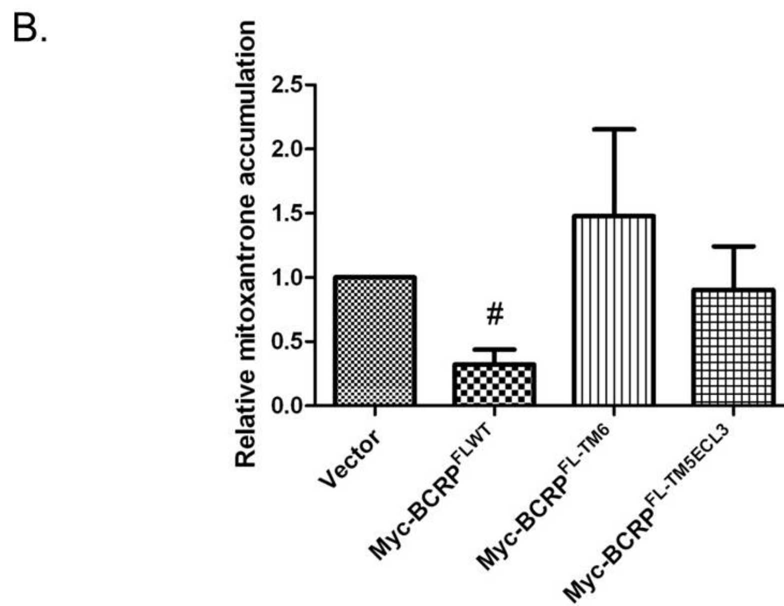


Figure 18: Effect of TM6 on mitoxantrone accumulation acitvity. (A) Myc-BCRP^{FLWT}, Myc-BCRP^{FL-TM6}, Myc-BCRP$^{FL-TM5ECL3}$ stable cells were subject to FACS analysis of mitoxantrone efflux for transport activity of BCRP. (B) Relative motixantrone accumulations were normalized to vector expressing cells. # $p<0.01$.

IV. Discussion

Human BCRP is considered as a half transporter and exists on the plasma membrane as a homo-oligomer. Although there is no consistent conclusion concerning the composition of oligomeric BCRP complex, the region that is solely responsible for the oligomerization of BCRP has been mapped to C-terminal TM5-ECL3-TM6. In addition, introduction of the peptide containing TM5-ECL3-TM6 resulted in a marked inhibition of the drug transport activities and drug resistance phenotype of BCRP, indicating that the oligomerization of BCRP might be functionally significant. Therefore, it is worthwhile to further characterize the subdomains that are responsible for BCRP oligomerization and explore possible mechanisms.

In the present studies, we dissected TM5-ECL3-TM6 into three distinct domains, including ECL3, TM5 and TM6, to investigate the contribution of each domain to the oligomerization and functions of BCRP. We engineered several domain-swapping constructs (Figure 5A, 6A, 7A) by substituting ECL3, TM5 or TM6 with ECL1, TM1 or TM2, respectively. We chose this domain-swapping design because the truncated construct Myc-BCRP^{TM12} has neither the ability to interact with full-length BCRP nor the oligomerization activity by itself in living cells (Xu et al., 2007), indicating that TM1 or TM2 is not important for the oligomerization activity of BCRP; therefore, replacing TM5/ECL3/TM6 with TM1/ECL1/TM2 is less possible to promote artificial domain interactions or to complicate the formation of BCRP oligomers. In addition, we also compared the estimated hydrophobicity of each TM domain (Table 5) to investigate the

involvement of hydrophobic interactions in the oligomerization of BCRP. We have demonstrated that Myc-BCRP$^{FL-TM5TM6}$ (Figure 6) interacts with full-length BCRP at a similar level to wild-type BCRP, while Myc-BCRP^{FL-TM6} (Figure 7) showed a decreased ability to interact with full-length BCRP. Since substitution of TM5 by TM1 does not change the hydrophobicity between Myc-BCRP$^{FL-TM5TM6}$ and Myc-BCRP^{FL-TM6}, other protein-protein interactions possibly account for the oligomerization activity mediated by TM5. Moreover, we have also shown that Myc-BCRP^{FL-TM5} (Figure 7) has a markedly decreased ability to interact with full-length BCRP compared to Myc-BCRP$^{FL-TM5TM6}$ and replacement of TM6 by TM2 greatly reduced the hydrophobicity of Myc-BCRP^{FL-TM5} (Table 5). This observation may indicate that one of the major factors of TM6 contributing to BCRP oligomerization is due to its hydrophobicity.

Table 5: The GRAVY score of TM domains of BCRP for engineering domain-swapping full-length BCRP constructs.

TM domain	No. of aa	Amino acids sequence	GRAVY score
TM1	21	IAQIIVTVVLGLVIGAIYFGL	2.400
TM5	21	VATLLMTICFVFMMIFSGLLV	2.505
TM2	20	LFFLTTNQCFSSVSAVELFV	1.260
TM6	21	VALACMIVIFLTIAYLKLLFL	2.581

Grand average of hydropathicity (GRAVY: indicates the solubility of the proteins: positive GRAVY (hydrophobic), negative GRAVY (hydrophilic) (Kyte and Doolittle, 1982) (http://www.expasy.ch/tools/protparam.html).

ECL3 appears to be an essential domain for BCRP oligomerization (Figure 4B and Figure 5B). However, neither the three cysteines nor the ^{569}QYFS motif are necessary for the interaction between Myc-BCRP$^{FL\text{-}ECL3}$ and full-length BCRP (Figure 5B) or the oligomerization activity of ECL3 by itself in living cells (Figure 8B). Among the three cysteines predicted on the extracellular face of BCRP, Cys603 might be involved in the formation of an intermolecular disulfide bond, while Cy592 and Cy608 are responsible for the formation of an intramolecular disulfide bond within ECL3 of BCRP (Wakabayashi et al., 2006). In order to investigate the functional relevance of cysteines in BCRP, Liu et al. created a series of cysless mutants and expressed them in Sf9 insect cells (Liu et al., 2008). The results demonstrated that Cys592, Cys603 and Cys608 in ECL3 are not important for either expression or function of BCRP expressed in insect cells. Additionally, a recent study using fluorescence resonance energy transfer (FRET) analysis has also demonstrated that Cys603Ala mutant of BCRP does not affect the dimer/oligomer formation of BCRP *in vivo* (Ni et al., 2010). Therefore, our results are consistent with the above observation that Cys592, Cys603 and Cys608 are not essential for BCRP oligomerization.

The short polar motif QXXS was first identified in the bacterial Tar-1 homodimer TM domain and has been found sufficient to induce stable TM-TM interactions (Sal-Man et al., 2004). The two polar residues (Q and S) are crucial for the dimerization of the Tar-1 TM domain *in vivo* by creating symmetric hydrogen bonds that promote and/or stabilize the dimeric state of Tar-1. Replacement of these two polar residues by nonpolar residues markedly impaired

the self-association ability of the TM domain. The QXXS motif is commonly present in a bacterial TM database, suggesting a general role for this motif in TM assembly (Sal-Man et al., 2005). In our studies, we replaced these two polar residues with the nonpolar residue alanine to disrupt the potential inter-helical hydrogen bond formation. However, results from both the co-immunoprecipitation and the chemical cross-linking studies suggest that the ^{569}QXXS motif is not essential for the formation of BCRP oligomer. This result could possibly be explained by the fact that the putative ^{569}QXXS motif is located in the extracellular loop of BCRP, instead of transmembrane domains, therefore losing the ability to promote hydrogen bond formation and dimer formation. In conclusion, ECL3 is an essential element in BCRP oligomerization, and the detailed mechanisms of interactions need to be further explored.

Chemical cross-linking, a biochemical approach used in our studies, takes advantage of specific features of various cross-linkers and provides covalent binding among adjacent subunits in a protein complex in living cells, which allows the detection of the cross-linked complex on SDS-PAGE for size estimation (Das and Fox, 1979). The results of chemical cross-linking are largely dependent on the distance between active groups and concentrations of the cross-linker as well as the availability of adjacent reactive groups in the neighboring protein subunits of a complex. DSS, a noncleavable and membrane permeable crosslinker that reacts with primary amines, has a spacer arm length of 11.4Å, which is within the intermolecular distance of BCRP obtained from purified protein studies (McDevitt et al., 2006, Dezi et al., 2010) and therefore is able to

detect possible interactions between the transmembrane domains and the extracellular loop of BCRP. Indeed, Myc-BCRP^{FLWT} showed four major bands corresponding to tetramer, trimer, dimer and monomer, with less than 50% of BCRP monomers cross-linked (Xu et al., 2004). In contrast, most of the domain-swapping mutants in our studies displayed higher cross-linking efficiency, suggesting that domain-swapping might affect the conformation of the resulting proteins, therefore possibly increasing the proximity of the interacting subdomains and increasing cross-linking of higher forms. Nevertheless, oligomers higher than tetramer could not be detected for both wild-type and mutant BCRP, consistent with our previous findings (Xu et al., 2004).

Two anticancer agents used in our functional studies are mitoxantrone and Adriamycin. Mitoxantrone is a type II topoisomerase inhibitor, which disrupts DNA synthesis and DNA repair by intercalating into DNA. Selection with mitoxantrone usually results in overexpression of BCRP, and resistance to mitoxantrone is the most distinctive feature of the phenotype conferred by BCRP. On the other hand, Adriamycin, also called doxorubicin, is an anthracycline antibiotic, and its cytotoxicity is mainly mediated by inhibiting topoisomerase II, intercalating DNA and inducing reactive oxygen species production. All our BCRP constructs harbor 482T mutations, therefore maintaining the ability to transport both mitoxantrone and Adriamycin effectively. So far, the exact binding sites of mitoxantrone or Adriamycin on BCRP are not clear, which creats some uncertainty in explaining the altered transport activity and resistance phenotype of BCRP domain-swapping constructs. However, early studies have suggested that the

interactions of substrate with BCRP involve multiple binding sites in the protein (Giri et al., 2009). Moreover, at least one amino acid, H630 (Miwa et al., 2003), which is predicted to be close to or in TM6, has been shown to be associated with mitoxantrone and Adriamycin recognition. Nevertheless, a recent study using purified ECL3 peptide has demonstrated that ECL3 constitutes a porphyrin-binding domain interacting with heme, but it does not bind to mitoxantrone or doxorubicin (Desuzinges-Mandon et al., 2010). Therefore, the loss of transport function of Myc-BCRP$^{FL\text{-}TM6}$ is possibly not attributed to loss of substrate binding, and the observation that Myc-BCRP$^{FL\text{-}ECL3}$ does not retain BCRP transport activity might be partially related to loss of substrate binding, given that TM6 might be crucial for recognition of mitoxantrone or Adriamycin.

TM5, but not TM6 or ECL3, has been shown in our studies to be a major contributor to transport activity and resistance mediated by BCRP. This is consistent with previous reports that TM5 contains several amino acids that are essential for the membrane trafficking and transport activity of BCRP. Kage et al. have found that a leucine to proline mutation at residue 554 in the putative TM5 of BCRP could partially reverse the resistance of wild-type BCRP to SN-38 and mitoxantrone, indicating L554 might be important for transport activity (Kage et al., 2002). Moreover, N557 might play an important role in the proper routing of BCRP (Mohrmann et al., 2005). One conserved residue G553 in TM5, the corresponding residue of which is involved in the dimerization of the Drosophila white protein (Mackenzie et al., 1999), has been examined as well. G553 does not seem to contribute to the formation of BCRP dimer/oligomer; instead, G553L mutant

reduced protein expression and abolished N-glysosylation and the membrane trafficking of BCRP (Polgar et al., 2006). In addition, a steroid binding element has recently been found in TM5 (Velamakanni et al., 2008), further verifying the signifance of TM5 in substrate binding of BCRP. These data suggest that TM5 harbors several amino acids or elements that are important for the substrate binding, membrane trafficking and transport activity of BCRP, which is consistent with our findings that TM5 is a major contributor to BCRP functions.

Based on the quantification analyses of our co-immunoprecipitation results, it has been shown that the oligomerization activity of BCRP is mediated by ECL3, TM5 and TM6, while each domain contributes partially to form the oligomeric BCRP complex. Nevertheless, both drug resistance assays and the drug accumulation assay have shown that Myc-BCRP$^{FL\text{-}TM5}$ retains BCRP functions, while Myc-BCRP$^{FL\text{-}ECL3TM6}$, a construct without TM5, totally lost BCRP activities. Furthermore, I also showed that both Myc-BCRP$^{FL\text{-}ECL3}$ and Myc-BCRP$^{FL\text{-}TM6}$ lost BCRP functions, while their counterparts Myc-BCRP$^{FL\text{-}TM5TM6}$ or Myc-BCRP$^{FL\text{-}TM5ECL3}$, which have been assisted by the existence of TM5, recovered both drug efflux of and drug resistance mediated by BCRP. These results suggest that the oligomerization activity of BCRP, at least the part mediated by ECL3 or TM6, is not directly correlated with the functions of BCRP.

The existence of BCRP oligomers might provide another level of regulation for this drug resistance transporter (Mo and Zhang, 2009). One possibility is that different oligomeric states may be coupled with the catalytic cycle and promote transporter activity. In the nucleotide-free state, BCRP may exist as low

oligomerization state such as monomer. ATP/substrate binding would trigger conformational changes that bring the adjacent transporters closer and induce the formation of higher order oligomers. This oligomer formation would create a complex with a larger central pore, allowing a higher degree of substrate transport. Following completion of the transport process with ATP hydrolysis, the conformation of the ABC transporter restores, and oligomers dissociate. Another possibility is that the formation of dimers or higher order oligomers may simply play a regulatory role either as a functional unit or reservoir of non-functional molecules waiting to be activated for function. The finding that monomeric and dimeric ABCB1 tend to have a higher degree of phosphorylation compared to higher order oligomeric ABCB1 (Poruchynsky and Ling, 1994) suggests that post-translational modifications may regulate this activation/inactivation process. Given that co-expression of protein kinase C could promote substrate binding and ATPase activity of ABCB1 (Ahmad et al., 1994), phosphorylation may activate dissociation of the oligomeric ABCB1. In contrast, disruption of phosphorylation of BCRP resulted in impaired plasma membrane trafficking and reduced oligomerization, indicating that the oligomerization of BCRP probably happens on the plasma membrane; therefore, reduction in the amount of protein trafficked to the membrane leads to less formation of BCRP oligomers.

As the number of documented oligomerization of ABC transporters rapidly grows (see (Mo and Zhang, 2009) for comprehensive review), it is important to understand the functional significance of these complexes. Studies of G-protein coupled receptor oligomerization have brought advances in technology to clarify

the composition, plasma membrane targeting, and function of oligomeric protein complexes (Gurevich and Gurevich, 2008). For example, FRET experiments could be used to track protein-protein interactions in time and space, while Bioluminescence resonance energy transfer (BRET) assays could be used to measure the relative affinities of protein-protein interactions. Moreover, a variety of computational studies based on sequence analysis and docking experiments have been performed towards the understanding of membrane protein complexes' structures (Simpson et al.). Indeed, Ni et al. have applied FRET assay to wild-type BCRP and directly demonstrated that BCRP could form dimer/oligomer in cellular levels (Ni et al., 2010). This result provides a basis for further applying new technologies to investigate the oligomerization and function of BCRP and related ABC transporters.

In conclusion, we have demonstrated in our studies that ECL3, TM5 and TM6 all play a partial role in BCRP oligomerization, while only TM5 is a key element for BCRP function. These findings suggest that oligomerization of BCRP might not be directly required for transport or resistance phenotype mediated by BCRP. Instead, BCRP might work as a monomer, while the existence of BCRP oligomers on the plasma membrane is a form of regulation, as discussed above. Still, the physiological and pharmacological significance of BCRP oligomers remains to be fully elucidated.

V. Summary and conclusion

The experimental results of this dissertation can be summarized as follows:

1. ECL3, TM5 and TM6 all play a partial role in BCRP oligomerization, and each domain might contain at least one interacting site responsible for the formation of oligomeric BCRP.

2. The oligomerization activity of BCRP is partially mediated by ECL3. Nevertheless, disruption of this oligomerizaition mediated by ECL3 does not significantly affect the drug transport activity of or drug resistance conferred by BCRP.

3. TM5 plays a less important role in the formation of BCRP oligomers. However, TM5 is a major contributor of the drug transport activity of or drug resistance conferred by BCRP.

4. TM6 also contributes in part to BCRP oligomerization. Meanwhile, this domain is also not essential for BCRP functions.

5. The findings of the relationship between BCRP oligomerization and function have enriched our understanding of the structure-function relationship of BCRP and will help to set the basis for the development of a better chemosensitizing regimen targeting BCRP-mediated MDR in cancer chemotherapies in the future.

VI. Future plans

Based on the present work, we conclude that ECL3, TM5 and TM6 all play a partial role in BCRP oligomerization, while only TM5 is a key element for the drug transport of and drug resistance mediated by BCRP. Future directions that may extend the current work are:

1. To further determine via alanine scanning assays which amino acids in ECL3, TM5 and TM6 are crucial for the oligomerization of BCRP.
2. To verify via FRET analysis whether the domain-swapping constructs of BCRP do form oligomers in intact cells.
3. To characterize via FRET experiments the oligomer formation of BCRP and its domain-swapping constructs at tissue level.
4. To identify the cellular compartment(s) where BCRP assembles into higher order oligomers.
5. To further identify the contribution of TM5 to the drug binding and ATPase activity of BCRP.

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Curriculum vitae

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Education

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Research Experiences

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Thesis project: Targeting the oligomerization domain of human BCRP for chemosensitization

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Master's Research: Institute of Clinical Pharmacology, Central South University, 2002-2005

Project 1: Genetic polymorphisms of human beta-adrenergic receptor genes and their association with obesity in Chinese hypertension populations

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Honors, Awards and Fellowships

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2007 Outstanding Master's Thesis in Hunan Province

2006 Outstanding Master’s Thesis in Central South University

2005-2006 University Fellowship of Indiana University

2004 Outstanding Poster in the 2nd Japan-China Joint Meeting of Basic and Clinical Pharmacology

2004 Outstanding Report in the National Meeting on Pharmacology of Young Pharmacologists

1999-2004 Chen Jia-Ju Fellowship of Changsha Tsinghua University Alumni Association

1998-2000 Excellent Undergraduate Award, Xiangya School of Medicine, Central South University

Invited Journal Reviewer

- Expert Opinion On Drug Metabolism and Toxicology
- International Journal of Biochemistry and Molecular Biology

Professional Societies

- American Association for Cancer Research
- European Association for Cancer Research
- Biophysical Society
- American Association for the Advancement of Science
- Chinese Pharmacological Society

Publications

Liu R, Dong Z, Liu J, Yin JY, Zhou L, Wu X, Yang Y, Mo W, Huang W, Khoo SK Chen J, Petillo D, Teh BT, Qian CN, Zhang JT (Role of eIF3a in regulating cisplatin sensitivity and nucleotide excision repair of nasopharyngeal carcinomas. Oncogene May 30 2011)

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