Multidrug Resistance and Cancer: The Role of the Human ABC Transporter ABCG2

Karin F. K. Ejendal and Christine A. Hrycyna*

Department of Chemistry, Purdue University, 1393 Brown Laboratories of Chemistry, West Lafayette IN 47907-1393, USA

Abstract: A variety of human cancers become resistant or are intrinsically resistant to treatment with conventional chemotherapy, a phenomenon called multidrug resistance. This broad-based resistance results in large part, but not solely, from overexpression of members of the ATP-binding cassette (ABC) superfamily of membrane transporters, including P-glycoprotein, various members of the multidrug resistance associated proteins (MRPs), and ABCG2, also known as MXR1, BCRP, and ABCP. When overexpressed in cell lines, ABCG2 has the ability to confer high levels of resistance to anthracyclines, mitoxantrone, bisantrene, and the camptothecins topotecan and SN-38. This review focuses on the discovery, the biochemistry and the normal physiology of human ABCG2, a novel ABC half transporter expressed abundantly in placenta, as well as in liver, intestine and stem cells. ABCG2 may serve a protective function by preventing toxins from entering cells as well as potentially playing a role in regulating stem cell differentiation. We also discuss the involvement of ABCG2 in multidrug resistance in cancer, especially with regard to acute myeloid leukemia. The mechanism by which substrates are recognized by ABCG2 and how the energy of ATP hydrolysis is transduced into transport remain elusive. A complete understanding of the mechanism and biological function of ABCG2 will be important for understanding its normal physiology as well as potentially for the development of novel chemotherapeutic treatment strategies.

INTRODUCTION

The successful treatment of cancer is to a large degree dependent upon the effectiveness of cytotoxic anticancer drugs either alone or in combination with radiotherapy or surgery. Unfortunately, most cancers either are intrinsically resistant to any initial treatment with these therapeutic compounds or acquire resistance to a broad spectrum of these agents over time, a phenomenon called multidrug resistance [1-4]. Commonly used chemotherapeutic agents such as the *Vinca* alkaloids, the anthracyclines, the epipodophyllotoxins, taxol, and actinomycin D, have several different cytotoxic targets including microtubules, DNA, and RNA polymerase. Although these natural product compounds share little to none of the same chemistry, they all are amphipathic molecules with planar aromatic rings that preferentially partition to organic solvents. The question arises as to how cells become simultaneously resistant to such a wide variety of structurally unrelated compounds. Therefore, understanding the mechanisms of multidrug resistance in human cancers is crucial for developing novel strategies for treatment. It is now well established that multidrug resistance is largely, but not solely, due to the overexpression of proteins belonging to the ATP binding cassette (ABC) superfamily, most notably by two proteins, P–glycoprotein (P-gp) encoded by the *MDR*1 gene in humans, and the multidrug resistance associated protein, MRP1 [3, 5-10]. Overexpression of these transporters presents major problems for successful treatment [1]. In human cell lines, at least nine other ABC transporters are known to confer resistance to cytotoxic compounds including MRP2 (cMOAT; ABCC2), MRP3 (MOAT-D; ABCC3), MRP4 (MOAT-B; ABCC4), MRP5 (MOAT-C; ABCC5), MRP6 (MOAT-E; ABCC6), BSEP/SPGP (ABCB 11), MDR2 (ABCB4), ABCA2, and MXR1/BCRP/ABCP (ABCG2) [3, 11]. However, data supporting the involvement of many of these transporters in clinical multidrug resistance remain, for the most part, inconclusive.

Collectively, the group of ATP binding Cassette (ABC) transporters represents one of the largest superfamilies of transport proteins known [12] and are found throughout nature in all organisms including bacteria, plants, yeasts and animals [13-18]. Complete, functional, full-length ABC transporters are comprised of four domains, two hydrophobic transmembrane regions and two soluble ATP-binding cassettes or nucleotide binding domains (NBD). These domains can be encoded either by a single gene or each domain can be the product of a separate gene. The proteins in the ABC superfamily were recently classified into seven subfamilies (A to G) according to different characteristics such as gene structure, order of the domains and sequence homology [19] (see also: http://www.humanabc.org/). For example, P-gp is classified as ABCB1 in the ABCB subfamily while the MRP proteins are members of the ABCC subfamily.

FUNCTIONAL DESIGN OF ABC TRANSPORTERS

The hydrophobic domains of ABC transporters are thought to form a pore, chamber or a channel-like structure across the membrane through which the substrate molecule

*Address correspondence to this author at the Department of Chemistry, Purdue University, 1393 Brown Laboratories of Chemistry, West Lafayette IN 47907-1393, USA; Tel: 765-494-7322; E-mail: hrycyna@purdue.edu

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is moved. These domains are not well conserved among ABC transporters and in many cases are the determinants of substrate specificity. The soluble nucleotide binding domain (NBD) is the most conserved part of ABC transporter proteins. There are several consensus motifs within each NBD including the Walker A and Walker B motifs and a region called the "signature region", or the "linker dodecapeptide". The Walker A nucleotide binding fold consensus sequence is G-$(X)_4$-G-K-(S/T)-$(X)_6$-I/V and the Walker B binding fold consensus sequence is R/K-$(X)_3$-G-$(X)_3$-L-(hydrophobic)$_4$-D where X is any amino acid [20]. The signature region is N-terminal of the Walker B motif and is defined by the consensus sequence LSGGQ or related sequences [21, 22]. Although many ATP binding proteins and ATPases, such as Rad50, myosin and F_1-ATPase also have the conserved Walker A and B sequences, it is the additional presence of the signature region that further defines an ABC transporter.

ABC transporters use the energy of ATP hydrolysis to extrude or import substrate molecules [1, 2, 4, 23-25]. In all ABC proteins studied to date, site-directed mutagenesis of the consensus sequences of the nucleotide binding domains suggest that both NBDs are essential for the proper functioning of the transporter, as inactivation of one ATP site abrogates activity [1, 18, 26-31]. Biochemical characterization of the catalytic ATP hydrolysis cycles of both human and hamster purified P-gps have determined that both ATP sites are capable of hydrolyzing ATP but not simultaneously [32-34], and that drug binding and ATP hydrolysis are intimately coupled [35]. These data led to a model of P-gp catalysis, originally proposed by Senior and colleagues, suggesting intimate cross-talk between the two ATP sites and alternating hydrolysis of ATP at each site [23, 25, 34, 36-44]. This model of the mechanism of action of P-gp will likely extend to all ABC transporters. However, the exact mechanism of how the energy of ATP hydrolysis is transduced in the system to result in the efficient transport of substrates in or out of cells remains elusive.

BIOLOGICAL ROLES OF ABC TRANSPORTERS

ABC transporters serve diverse biological roles in cells. For example, there are several well characterized importers in bacteria such as the $MalEK_2FG$ complex of *Eschericha coli* and the $HisJP_2MQ$ complex of *Salmonella typhimurium* that are responsible for the transport of maltose and histidine, respectively, across the inner membrane of gram negative bacteria [17, 21, 45-47]. In the yeast *Saccharomyces cerevisiae*, the Pdr5 (pleiotropic drug resistance) gene product is responsible for the efflux of a variety of potentially cytotoxic compounds out of plasma membrane to protect cells from toxins encountered in the environment [16, 48]. Additionally, the *Saccharomyces cerevisiae* ABC transporter Ste6p allows for the translocation of the pheromone **a**-mating factor, which is essential for mating formation of diploid yeast cells [49].

In humans, to date, there are approximately 50 identified members of the ABC transporter superfamily, many of which are becoming increasingly important in both the etiology and treatment of disease [11, 19, 50]. As noted above, P-gp and the MRP proteins can confer drug resistance to cancer cells when overexpressed [2, 3, 51]. Another clinically important ABC transporter is the cystic fibrosis transmembrane regulator protein (CFTR/ABCC7) [52, 53]. The CFTR protein is expressed in the plasma membrane of epithelial cells and is involved in chloride and water excretion. Mutations in the CFTR gene are directly responsible for various forms of the disease cystic fibrosis, the most deleterious of which involves the deletion of a phenylalanine residue at position 508 [54, 55]. Several ABC transporters have important roles in cholesterol homeostasis and have been linked to various cholesterol disorders, such as ABCA1, mutated in Tangier Disease [56] and ABCG5 and ABCG8, implicated in sitosterolemia [57-60]. Other clinically relevant ABC transporters are the ALDP (ABCD1) [61-64] and PMP70 (ABCD3) [65, 66] proteins involved in the peroxisomal disorders adrenoleukodystrophy and Zellweger syndrome respectively, ABCR (ABCA4) involved in the macular degenerative disease Stargardt syndrome [67-69], MRP6 (ABCC6) defective in the connective tissue disorder Pseudoxanthoma elasticum [70-75], and cMOAT/MRP2 (ABCC2) involved in the bile transport disorder, Dubin-Johnson syndrome [76].

ABCG2, A NOVEL MULTIDRUG TRANSPORTER

Recently, a new ABC transporter, ABCG2, has been cloned [77-80]. ABCG2 is a member of the ABCG subfamily that consists of eight potential transporters. Proteins in this subfamily all share a similar domain structure, i.e. a single ATP binding cassette (ABC) precedes the hydrophobic domain (Fig. **1**). These are so-called "half-transporters" and are generally thought to form dimers. The ABCG subfamily of ABC transporters is also called the "white" subfamily after the *Drosophila* white gene, a homologue of the human ABCG1 gene. In *Drosophila*, the white protein forms heterodimers with either the scarlet or the brown gene products [81]. These ABC transporters translocate precursors of eye pigments from the cytoplasm into pigment granules of the cells [82, 83]. To date, other members of the ABCG white gene subfamily have been cloned (ABCG1, ABCG3, ABCG4, ABCG5 and ABCG8) and two (ABCG6 and ABCG7) have been identified based on expressed sequence tags (EST) [84]. ABCG1, ABCG5 and ABCG8 are involved in cholesterol and sterol transport, as evidenced by the involvement of ABCG5 and ABCG8 in sitosterolemia [57-60, 84, 85]. Expression of ABCG4 has been detected in liver tissue and macrophages [86, 87] and ABCG3 is highly expressed in thymus and spleen but has been found only in rodents [88, 89]. However, the functions of ABCG3 and ABCG4 are not yet well understood.

ABCG2 was originally cloned and sequenced from genomic DNA, from highly mitoxantrone-resistant S1-M1-80 human colon carcinoma cells and from MCF-7 AdVp human breast cancer cells selected in doxorubicin (Adriamycin) [77-79]. These selected cell lines overexpress ABCG2 at high levels and show reduced accumulation of mitoxantrone, daunorubicin, rhodamine 123, and bisantrene. This gene is designated ABCG2 by the new nomenclature system but is also referred to as BCRP (breast cancer resistance protein) [79], MXR1 (mitoxantrone resistance

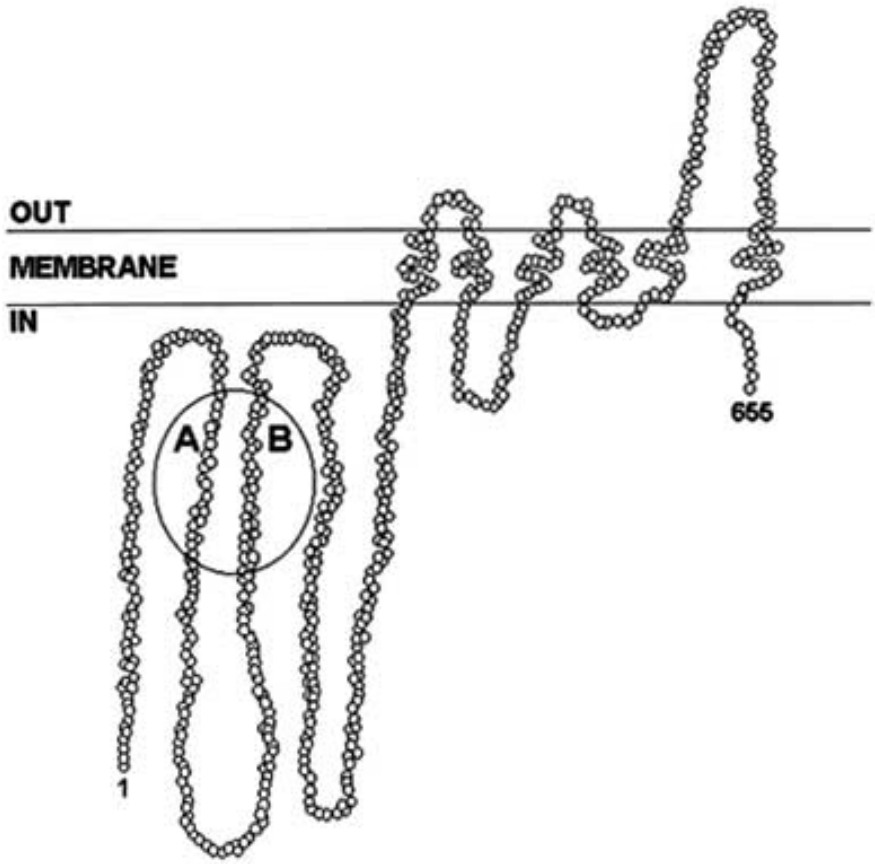

Fig. (1). Two-dimensional hypothetical model of the topological structure of human ABCG2 based on hydropathy analysis of the primary amino acid sequence [77-79]. Potential transmembrane domains and loops are based on the SwissProt database (ABCG2 primary accession number Q9UNQ0) and PHD (Profile network prediction HeiDelberg). Human ABCG2 is 655 amino acids in length with six predicted transmembrane domains and one soluble nucleotide binding domain (NBD). The NBD is circled and the Walker A and Walker B sequence motifs are labeled A and B. ABCG2 has been localized primarily to the plasma membrane in both normal and drug resistant cells [98, 103-104, 122]. (Figure adapted from [18]).

protein) [77], or ABCP (placenta-specific ABC transporter) [78]. Subsequently, human ABCG2 overexpression has also been detected in topotecan-resistant breast carcinoma cells [90, 91], flavopiridol-resistant breast cancer cells [92], several NB-506 (a topoisomerase I inhibitor) resistant colon and lung cell lines [93], a mitoxantrone-resistant HT29 colon carcinoma cell line [94], topotecan- or mitoxantrone-selected human IGROV1 ovarian cancer cell lines [95], mitoxantrone-selected fibrosarcoma, colon and breast cancer cell lines [96] and SN-38-resistant breast, colon and PC-6 small-cell lung cancer cells [91, 97].

The human *ABCG*2 gene is located on chromosome 4q22 between the markers *D4S2462* and *D4S1557* [78] and encodes a 655 amino acid polypeptide with a predicted molecular weight of 72 kDa [77, 79, 98]. ABCG2 is proposed to be a half-transporter, containing only one set of six putative transmembrane domains preceded by one ATP site (Fig. **1**). The functional consequences of the molecular architecture of ABCG2 are unclear, however, it is generally thought that homodimers/homooligomers or heterodimers/ heterooligomers must form to create the active transporter. Stable clonal expression of ABCG2 in drug sensitive MCF-7 cells confers resistance to multiple drugs including mitoxantrone, doxorubicin and daunorubicin [79]. In addition to resistance, these cells also show decreased accumulation of daunorubicin [79] and mitoxantrone [99]. Functional ABCG2 has also been transiently expressed in a human HeLa cell line that has low endogenous ABCG2 and P-gp expression [100]. Taken together, these data

demonstrate that the ABCG2 gene product mediates substrate efflux, however, it remains unclear whether it functions as a monomer or a homomultimer or as a heteromer with an as yet unidentified highly expressed endogenous protein. Recently, dominant-negative inhibition of ABCG2 function was shown, suggesting that homodimerization is necessary for function [101].

In drug-resistant selected cell lines as well as normal tissues, ABCG2 has been shown to be localized to the plasma membrane [98, 102, 103]. In MCF-7 AdVp3000 and S1-M1-80 drug-resistant cells that overexpress ABCG2, both plasma membrane and cytoplasmic staining has been observed, consistent with the possibility of intracellular localization with redistribution to the plasma membrane [99]. Using flow cytometry with a monoclonal antibody specific for a cell-surface epitope [104], we have determined that ABCG2 is expressed at the cell surface when transiently overexpressed in non-drug selected HeLa cells (Hrycyna, C.A. unpublished results). Localization of ABCG2 to the plasma membrane is unique amongst half-transporters. All other half-transporters evaluated have been found exclusively in intracellular membranes [105], such as the TAP1/TAP2 complex which is localized to the endoplasmic reticulum [106].

ABCG2 SUBSTRATE SPECIFICITY

ABCG2 effectively confers drug resistance to selected or transiently transfected cell lines. Despite differences in domain organization, the multidrug resistant phenotype associated with ABCG2 expression is overlapping with but distinct from that associated with expression of P-gp and MRPs, giving cell-lines, and potentially even tumors, unique drug transport phenotypes that may ultimately be exploited clinically [99]. ABCG2 confers resistance to several P-gp substrates such as mitoxantrone, the anthracyclines such as daunorubicin and doxorubicin, the camptothecins, bisantrene, topotecan, rhodamine 123 and prazosin and SN-38 [77, 79, 91, 97, 99]. In contrast, ABCG2 does not efflux other known P-gp substrates such as taxol, colchicine, verapamil, vinblastine and calcein-AM, nor the MRP substrates calcein and glutathione-conjugated monochlorobimane [99]. At present, the exact molecular nature of these substrate specificity differences remains unknown, though it is likely that the determinants will lie near or within the transmembrane domain themselves or in their specific interactions with each other to form drug binding sites. It is well established that the substrate binding sites for P-gp are located within and near the transmembrane segments and the loops that connect the hydrophobic segments. By photoaffinity labeling studies, mutagenesis and chemical modification of cysteine residues of P-gp it has been shown that amino acid residues of segments 5 and 6 and of segments 11 and 12 are primarily involved in substrate binding [2, 18, 107-112]. Additionally, substrate specificity could be dictated by the manner in which the transmembrane domains interact upon drug binding to transmit the signal to the ATP sites.

The *ABCG2* gene cloned from two differentially selected resistant cell lines was originally designated *MXR*1 and *BCRP*. *MXR*1 was cloned from a mitoxantrone (MXR) selected S1 human colon carcinoma cell line (S1-M1-80) and *BCRP* from an MCF-7 breast cancer cell line selected in doxorubicin and verapamil (MCF-7/Adr/Vp), both of which overexpress the ABCG2 protein [77, 79]. Upon examination of the sequences, it was noted that these genes encoded proteins with either a threonine or glycine substituted for the wild-type arginine at amino acid 482 [78]. These substitutions were found to increase the spectrum of substrates recognized by ABCG2, including the ability to transport doxorubicin and the dye rhodamine 123 [93, 100]. These results suggest that amino acid 482, predicted to lie at or near the cytosolic interface of transmembrane domain three (Fig. **1**), has a crucial role in ABCG2 function and that mutation of a single amino acid residue can significantly change substrate specificity, as has been well-documented for P-gp [1, 4]. Importantly, these alterations represent some of the first examples of gain of function mutations occurring in an ABC drug transporter upon drug selection [100]. Three mouse cell lines similarly selected in doxorubicin also acquired mutations at position 482, substituting the native arginine with either methionine or serine which resulted in comparable phenotypes to the human cell lines including increased resistance to anthracyclines, lower resistance to topotecan and enhanced efflux of rhodamine 123 [113].

Several compounds have been shown to be effective ABCG2 reversing agents. The acridonecarboxamide GF120918, a compound originally developed as a P-gp inhibitor, is also a potent inhibitor of ABCG2 [114-116]. Additionally, the *in vitro* ATPase activity of ABCG2 has been shown to be inhibited by sodium orthovanadate, and N-ethylmaleimide [117]. Fumitremorgin C (FTC), a fungal metabolite from *Aspergillus fumigatus*, specifically inhibits the activity of ABCG2 but does not affect the ability of P-gp or MRP to transport substrates [92, 118]. Due to its specificity, FTC is being used as a diagnostic agent for measuring ABCG2 expression and activity in both selected and unselected cells and cell lines [119, 120].

PHYSIOLOGICAL ROLE OF ABCG2

ABCG2 is endogenously expressed at high levels in human placenta and to a lesser extent in liver, small intestine and colon, ovary, vein and capillary endothelia, kidney, adrenal and lung, with little to no expression in brain, heart, stomach, prostate, spleen and cervix [25, 78, 79, 96, 98, 102, 121]. ABCG2 protein expression has been further localized to the surface of placental syncytiotrophoblasts, to the apical epithelium of the colon and small intestine, to the liver canalicular membrane and to the surface of cells in breast ducts and lobules [98, 122]. In addition ABCG2 is expressed at high levels in the human jejunum [121] at levels considerably higher than the those of many other ABC transporters, among them P-gp, which has also been found to be primarily expressed in the intestinal epithelia and in cells of the blood-brain barrier [3]. Taken together, these data suggest that the physiological roles of P-gp and ABCG2 may be to protect cells from potentially toxic substances and to prevent absorption of xenobiotics ingested in our diet by actively transporting compounds from cells [18]. Given the

liver and intestinal localization pattern, ABCG2 and P-gp may act as barriers to uptake and absorption and limit the oral bioavailabilty of drugs as well as mediating hepatobiliary excretion of drugs, an additional factor to consider when administering or developing therapeutic agents [3, 122]. In the placenta, ABCG2, along with P-gp and members of the MRP family, is thought to protect the fetus from xenobiotics by creating a maternal-fetal barrier [98, 122-124].

Recently, ABCG2 has been shown to be expressed at the cell surface in immature hematopoietic stem cells and is a major determinant of the "side-population phenotype" [104, 125, 126]. The localization of ABCG2 exclusively to the hematopoietic side population stem cells and neural stem/progenitors suggests that ABCG2 can be used as a selectable marker for isolating these cells [126]. Interestingly, expression of ABCG2 is sharply downregulated in these cells upon differentiation [104, 125]. Although the function of ABCG2 in this cell type is not clear, several hypotheses have been offered. The role of ABCG2 may simply be to protect sensitive stem cells from toxins [125]. Another compelling hypothesis is that ABCG2 may maintain immature cells or regulate stem cell differentiation, apoptosis or growth by actively removing regulatory lipophilic molecules from the cells [125]. Due to their biological importance, the role of ABCG2 in stem cells should prove to be a fertile area of investigation in future years.

ABCG2 AND CANCER

Since ABCG2 is expressed in a variety of cell-lines and tissues, ABCG2 overexpression may ultimately prove to be clinically important in a variety of cancers such as breast, gastric, ovarian, intestinal and colon cancers. However, although it is clear that ABCG2 is overexpressed in drug-selected tumor cell lines, to date, evidence linking high level expression in tumor samples is limited. ABCG2 was found in low or undetectable amounts in a panel of human tumor samples using BXP-34 monoclonal antibody staining [102]. Increased expression of the protein has also not been detected in clinical samples from numerous naïve and anthracyline-treated breast tumors [127]. In fact, ABCG2 expression tended to be higher in the samples taken from patients before treatment and ABCG2 expression was not associated with decreased response to treatment or survival.

On the other hand, evidence is mounting that substantiates ABCG2 expression in tumor cells from patients with acute myeloid leukemia (AML) in addition to MRP1, the lung-resistance-related protein LRP, and P-gp, expression of which is generally considered to correlate with prognosis [3, 120, 128-134]. Ross *et al.* used quantitative RT-PCR to determine ABCG2 mRNA expression in blast cells taken from AML patients and observed that although the levels of ABCG2 expression varied considerably, high expression was detected in about a third of the samples relative to MCF-7 reference cell which express quantitatively high levels of *ABCG2* mRNA [131]. Further immunohistochemical studies demonstrated expression of the ABCG2 protein in fresh blast cells from patients. Importantly, the presence of ABCG2 correlated with daunorubicin resistance *in vitro*, a drug used commonly in the treatment of AML [130].

In a study examining differences in ABCG2 expression from the time of AML diagnosis to relapse or refractory disease, quantitative real-time PCR was used to detect ABCG2 mRNA in paired clinical samples from 20 AML patients [132]. These results demonstrated that the levels of ABCG2 mRNA increased significantly from diagnosis to relapse or refractory disease, suggesting that ABCG2 expression may be a major determinant in clinical resistance in AML. In another recent study, ABCG2 protein expression, as determined by flow cytometry using the monoclonal antibodies BXP-34 and BXP-21, was also examined in 20 paired samples from AML patients at time of diagnosis and relapse [120]. The results showed that ABCG2 protein expression is low but variable in AML and is expressed in most $CD34^+$ cells, consistent with recent reports of expression of ABCG2 in immature hematopoietic stem cells [98, 104, 125, 126]. Using flow cytometric analysis and mitoxantrone accumulation assays, it was also shown that ABCG2 expression in blast cells correlated with low mitoxantrone accumulation, again suggesting a role for ABCG2 in conferring drug resistance in AML. In contrast, however, no consistent ABCG2 overexpression or increase in activity was observed at the time of relapse [120]. It should be noted that both of these studies were based on a very small and selected number of clinical samples (n=20), further emphasizing the need for larger scale studies of clinical samples from AML patients.

FUTURE DIRECTIONS

During therapy, many cancers become resistant to cytotoxic drugs. This observed resistance is likely multifactorial, involving not only ABC transporters but many other mechanisms including activation of detoxifying enzymes, activation of repair mechanisms for damaged intercellular targets, and disruptions in apoptotic signaling pathways [3]. To complicate the problem further, many of the ABC drug transporters such as P-gp, the MRPs and ABCG2 can be expressed concomitantly, leading to a complex multidrug resistance phenotype. The development of inhibitors of these ABC transporters remains a promising avenue in the treatment of cancers. A more complete understanding of the distribution of these proteins with an appreciation of their relative contribution to clinical drug resistance may potentially help with the successful development of unique and effective combination therapies including cytotoxic drugs, reversing agents, and transport inhibitors. The recent publication of the three-dimensional structures of two complete ABC transporters BtuCD [135] and MsbA [136] as well as the future elucidation of other ABC transporter structures will greatly help our understanding of the mechanism of action of these transport proteins. This information may, in time, aid in the development of more rational based drug design for this important family of proteins.

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