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ROLE OF ABCG2/BCRP IN BIOLOGY AND MEDICINE

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■ **Abstract** The protein variously named ABCG2/BCRP/MXR/ABCP is a recently described ATP-binding cassette (ABC) transporter originally identified by its ability to confer drug resistance that is independent of Mrp1 (multidrug-resistance protein 1) and Pgp (P-glycoprotein). Unlike Mrp1 and Pgp, ABCG2 is a half-transporter that must homodimerize to acquire transport activity. ABCG2 is found in a variety of stem cells and may protect them from exogenous and endogenous toxins. ABCG2 expression is upregulated under low-oxygen conditions, consistent with its high expression in tissues exposed to low-oxygen environments. ABCG2 interacts with heme and other porphyrins and protects cells and/or tissues from protoporphyrin accumulation under hypoxic conditions. Individuals who carry ABCG2 alleles that have impaired function may be more susceptible to porphyrin-induced toxicity. *Abcg2* knock-out models have allowed in vivo studies of Abcg2 function in host and cellular defense. In combination with immunohistochemical analyses, these studies have revealed how ABCG2 influences the absorption, distribution, and excretion of drugs and cytotoxins.

INTRODUCTION

It was first demonstrated in the 1970s that cancer cells become resistant to chemotherapeutic drugs by acquiring the ability to export multiple drugs by active efflux (1). The single gene believed responsible for this multidrug-resistance phenotype was subsequently identified and cloned from multiple mammals as *MDR1* [P-glycoprotein (Pgp) or ABCB1] (2–4). As knowledge of the number and types of substrates increased, it became apparent that MDR1 is expressed in normal tissues (5, 6). MDR1 was therefore proposed to play a general physiological role as a protector against toxins as well as drugs (6). MDR1-mediated protection from exogenous toxins was clearly illustrated by the knockout of *Mdrla* (mice have two Mdr genes—*Mdrla* and *Mdrlb*—that by function and expression complement the single human *MDR1* gene) (7, 8) and the serendipitous discovery of the role of murine Pgp in protecting the brain from accumulation of exogenous toxins (7, 9). The use of this model revealed how specific cells within an organ might be

protected from drugs and toxins in vivo by an efflux transporter located within those cells (e.g., Pgp in brain capillary endothelial cells).

The concept of a single drug efflux transporter (Pgp) was revised after multidrug-resistance protein 1 (Mrp1) was cloned in the laboratory of Cole & Deeley in 1992 (10). The almost concurrent development of the expressed sequence tag (EST) database and the human genome sequencing effort extended the list of ATP-binding cassette (ABCs) transporters. The ABC transporter superfamily is among the largest and most broadly expressed (11). A majority of its members actively transport an array of biological compounds, including drugs, bile acids, peptides, steroids, ions, and phospholipids. Although some of the currently recognized 48 human ABC transporters are known because of their close link to disease caused by deficient transport of a particular substrate, others have been discovered through the identification of a gene that mediates resistance to a chemotherapeutic agent (the following Web sites include information on the various ABC transporter genes: http://nutrigene.4t.com/humanabc.htm, http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.htm, http://www.humanABC.bio.titech.ac.jp/).

The eukaryotic ABC transporters are characterized by a highly conserved cytosolic domain, two hydrophilic cytosolic nucleotide-binding domains (NBDs), and at least two hydrophobic membrane-spanning domains (MSDs). The MSDs have been identified as the substrate-binding sites in studies using affinity probes and mutations within certain MSDs (12–14). Moreover, conformational changes within the transmembrane domains (located within the MSD) are believed to be responsible for the transport of molecules by these transporters. The binding and hydrolysis of ATP by the NBD provides energy for uphill movement of substrates across membranes. Each NBD has two sequence motifs, designated Walker A and Walker B. The Walker A motif contains a lysine that coordinates with the γ-phosphate of ATP, whereas the aspartate in Walker B interacts with Mg^{+2}. The ABC signature or Walker C motif is the third highly conserved sequence that distinguishes ABC proteins from other ATP-binding proteins. These domains are required to execute ABC transport activities.

CLONING BCRP/ABCG2

Breast cancer resistance protein (BCRP) was first cloned by Doyle et al. from a breast cancer cell line selected for its unique drug resistance in the presence of a Pgp inhibitor (verapmil) (15). This drug-resistant subline (MCF-7/AdrVp) displayed an ATP-dependent reduction in the intracellular accumulation of anthracyclines despite the absence of overexpression of known multidrug transporters, such as Pgp or the multidrug resistance protein Mrp1. Although not highly expressed in breast cancer, because it was isolated from the breast cancer cell line, the protein was termed BCRP. However, a cDNA essentially identical to that of *BCRP* was independently isolated from human placenta by Allikmets et al. using expression analysis and genomics [ATP-binding cassette placenta (ABCP), see below] (16) and by Miyake et al. from a highly mitoxantrone-resistant human colon carcinoma

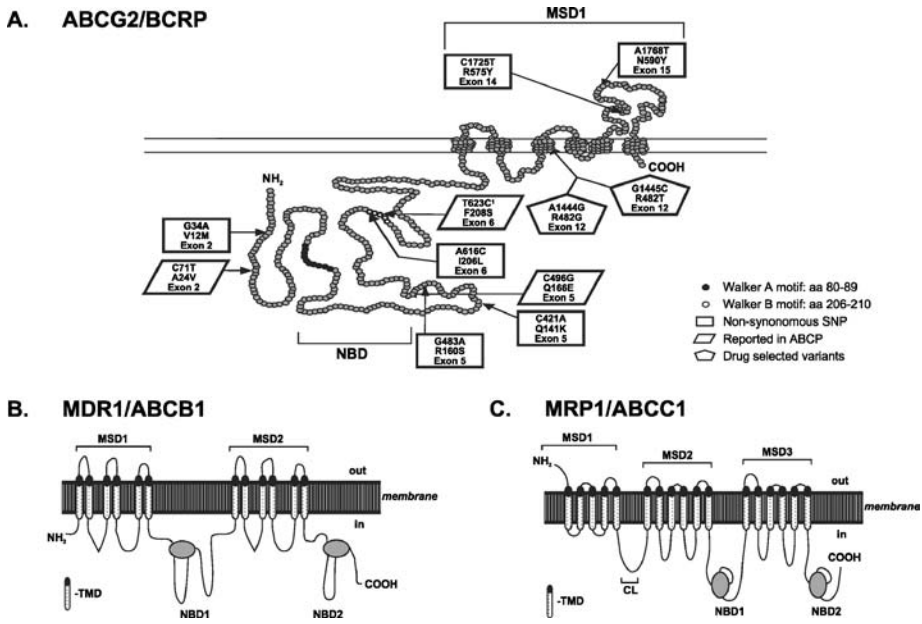

Figure 1 Polymorphisms and membrane topology of ABCG2 (45, 178) (*A*) compared with the membrane topology of the prototypical drug-resistance transporters MDR1/ABCB1 (*B*) and MRP1/ABCC1 (*C*). NBD, nucleotide-binding domain; MSD, membrane spanning domain; CL, cytoplasmic linker.

cell line by [mitoxantrone resistance (MXR)] (17). Analysis of the phylogenetic relationship of BCRP to other members of the ABC transporter superfamily revealed that BCRP is only distantly related to Pgp and MRP1 but is closely related to ABCG1, a human ortholog of the *Drosophila* white gene (18) (See Figure 1). BCRP belongs to the ABCG gene family of proteins, which includes, among others, the *Drosophila* proteins white, brown, and scarlet, which play a concerted role in eye pigment import (19–22). The white-brown heterodimer transports guanine, whereas the substrate of the white-scarlet complex is tryptophan (19–22). It is notable that homodimers of these proteins do not appear to have transport activity (23–25). BCRP/MXR/ABCP is the second member of the G subfamily of ABC transporter proteins, and the Human Gene Nomenclature Committee has recommended that *BCRP* be renamed *ABCG2*; therefore, we use the term ABCG2 throughout the remainder of this review. The G subfamily of ABC transporters has a reverse domain arrangement as compared with other drug-resistance ABC transporters, wherein the NBD is located toward the N terminus of the polypeptide chain. Figure 1 shows the suggested membrane topology (based on various prediction models) of the human ABCG2 and compares it with that of two prototypical drug-resistance transporters (Pgp and Mrp). Recent studies have identified the murine, porcine, and rhesus orthologs of ABCG2 (26–28).

ABCG2 CHROMOSOMAL LOCALIZATION AND GENE REGULATION

Cytogenetic analysis determined that *ABCG2* resides on chromosome band 4q22 between the markers D4S2462 and D4S1557 (29). Fluorescence in situ hybridization studies with a bacterial artificial chromosome probe containing *ABCG2* confirmed localization of the gene at 4q21–4q22 in cells with a normal chromosome 4 (29). Notably, in cells with acquired drug resistance to ABCG2 substrates, a gene amplification peak was observed at 4q21–4q22 in cells with acquired resistance to ABCG2 drug substrates, but it was not observed in cells that did not overexpress ABCG2 (30). The absence of cytogenetic evidence of coamplification in other chromosomal regions was interpreted as consistent with the absence of an ABCG2 half-transporter partner and suggested that two ABCG2 molecules form a homodimer (see below) to create an active transporter.

The *ABCG2* gene spans more than 66 kb and consists of 16 exons (ranging from 60 to 532 bp) and 15 introns (31). As in several other ABC transporters (ABCB1, ABCB4, etc.), the translational start site is found in the second exon, with the majority of the 5′ untranslated region in exon 1. The *ABCG2* promoter is predicted to be TATA-less and to have several Sp1, AP1, and AP2 sites and a CCAAT box downstream of a putative CpG island (31). Although the role of these *cis*-elements in *ABCG2* transcription has not been assessed, more recent studies have demonstrated functional hormone (32) and hypoxia (33) response elements in the *ABCG2* promoter [see Figure 2 and below]. Basal promoter activity was conferred by a sequence 312-bp upstream of the transcriptional start site (31), and both positive and negative *cis*-regulatory elements have been identified in the 5′-regulatory region of *ABCG2* (31). Because S1-M1–80 cells overexpress ABCG2 but have no increase in the *ABCG2* gene copy number (29), other alterations may account for the increased expression. One possible explanation is the simple reciprocal translocation between chromosomes 4 and 17 in this cell line. It is possible that this rearrangement in the *ABCG2* gene causes overexpression through loss of a *cis*-acting inhibitory region.

ABCG1 is related to ABCG2 and is transcriptionally regulated by the nuclear receptor LXR (liver X receptor) that is important for control of lipid homeostasis (34, 35). This regulation of ABCG1 by LXR fits with ABCG1 transport of cholesterol. Surprisingly, ABCG2 expression is not upregulated by LXR agonists (36). Because ABCG2 participates in cellular detoxification, we might hypothesize, by analogy, that it is regulated by nuclear receptors that upregulate genes involved in cellular detoxification (37). However, ABCG2 expression is only minimally affected by PXR (pregnane X receptor) or CAR (constitutively active receptor) activators, such as valproate, phenytoin, dexamethasone, and carbamazepine. These findings are consistent with the fact that ABCG2 is not induced by a prototypical activator of the nuclear receptor CAR (38). Therefore, although ABCG2 is localized to the plasma membrane and apical surfaces of polarized cells (see below), it appears unlikely to be activated by PXR and CAR as part of a cellular detoxification cascade.

Despite the above findings, ABCG2 is expressed at high levels in normal cellular milieus in which oxygen tension is reduced (e.g., venules and stem cells) (39). Recent studies by Krishnamurthy et al. demonstrated that hypoxia increases the expression of ABCG2 (33). A central mediator of the hypoxic response is the bHLH-PAS transcription factor complex hypoxia-inducible factor 1 (HIF-1), which is composed of the heterodimeric partners Hif-β and Hif-α. Under normoxia, Hif-α is inactivated by a proteosomal degradation pathway, but when hypoxia develops, Hif-α is stabilized (40). A computational analysis of the *ABCG2* promoter revealed several potential hypoxia response elements (HRE); however, only one was demonstrated to be functional in DNA-binding and promoter assays (see Figure 2). Mutation or deletion of this HRE in ABCG2 promoter-luciferase assays attenuated specific activation by the HIF-1 complex. Moreover, electrophoretic mobility shift assays revealed specific binding of the HIF-1 complex to the *ABCG2* promoter through the functional HRE (position –116) and not through the other upstream HRE sites. Because ABCG2 is activated by HIF-1, and Hif-α is overexpressed in many primary and metastatic human cancers (41) as a result of either hypoxia or loss of Hif-α-inactivating tumor suppressor genes (e.g., *VHL* or *PTEN*) (41), it is possible that activated HIF-1 increases ABCG2 expression in some tumors to facilitate drug resistance. It is also possible that ABCG2 is overexpressed in renal neoplasms that are highly resistant to chemotherapy and that often have mutations in pVHL (Von-Hippel Lindau).

The high level of ABCG2 expression in placenta suggested that ABCG2 is regulated by sex hormones (16). Recent studies investigated whether this process explains gender differences in *ABCG2* gene expression. Tanaka et al. found in rats that male-predominant expression of Abcg2 in the kidney reflected the suppressive effect of estradiol in females (42). In contrast, the predominance of Abcg2 in male liver was attributed to the inductive effect of testosterone. Although gender differences in growth hormone release patterns may contribute to the gender difference in liver expression of Abcg2, the higher hepatic expression in male mice was recently reported to be correlated with observed effects of gender on the pharmacokinetics of Abcg2 substrates (the plasma level of Abcg2 substrates was lower in males) (43). Interestingly, male humans were reported to have higher hepatic levels of ABCG2 (44), although this finding was not corroborated by other studies (45). This discrepancy may be attributable to the type of tissue evaluated (liver versus intestine). Despite their limitations, these studies suggest that at least two different hormone receptor pathways regulate ABCG2 expression. One may involve the androgen receptor and the other, the estrogen receptor. ABCG2 is also upregulated in the mammary gland during lactation (see below) (46). Ee et al. (32) have taken the first steps toward elucidating the molecular mechanism of hormonal regulation of ABCG2. They used a recently developed computational approach to scan for estrogen-receptor-responsive DNA binding sites (47) and identified an estrogen response element (ERE) in the *ABCG2* gene (see Figure 2) between -161 and -190 bp upstream (5′–3′) of the transcription start site. They extended this study to demonstrate that the endogenous *ABCG2* mRNA is

upregulated by physiologically relevant quantities of estradiol. Moreover, this effect was readily antagonized by an estrogen receptor antagonist. Further analysis showed that the ERE in the ABCG2 promoter bound estrogen receptor and was responsive to nanomolar concentrations of estrogen. These findings are consistent with the high ABCG2 expression in placental syncytiotrophoblasts in which estrogen concentrations can reach the high nanomolar range. In contrast, Imai et al. (48) reported that *ABCG2* mRNA was not increased in the estrogen-responsive human breast cancer MCF-7 cells. One explanation for this discrepancy in estrogen response may be the culture conditions. The expression of estrogen receptors (alpha and beta) is regulated by the estrogens in the culture medium (49). For example, to guarantee reduction of exogenous estrogens, Imai et al. (48) used media without phenol-red [a known estrogen (50, 51)] and charcoal-stripped the serum, whereas Ee et al. (32) used only charcoal-stripped serum.

Other transcription factors may regulate ABCG2 expression. For example, the high expression of ABCG2 in both erythroblasts (52, 53) and mature erythroid cells (54) may reflect the presence of Gata1 and 2 and Aml1a sites in the ABCG2 promoter (see Figure 2). Interactions that involve the Aml1a sites may explain the high expression of ABCG2 in leukemias with translocations that create the *TEL-AML* fusion (55).

Epigenetic factors and/or regulatory polymorphisms can influence the selective expression of alleles (56). For example, the expression of one allele may predominate as a result of its preferential activation by a *cis*-regulatory single nucleotide polymorphism (SNP) (57). Although current studies have yet to find evidence of functional *cis*-regulatory SNP in ABCG2, there is evidence of monoallelic expression (allelic imbalance) that suggests parental imprinting (58). Because this type of allele-specific imprinting may be tissue dependent, it would be informative to determine whether ABCG2 is imprinted in a tissue-specific pattern. This possibility may be especially important as we have identified ABCG2 alleles with different functional capabilities (see below).

TISSUE DISTRIBUTION OF ABCG2

The level of expression of ABCG2 mRNA can provide insight into the potential role of ABCG2 in different tissues. A recent study that assessed ABCG2 mRNA expression in 50 human tissues found no *ABCG2* transcripts in heart, lung, skeletal muscle, kidney, pancreas, spleen, thymus, or peripheral blood leukocytes (30). It was notable that placenta showed the highest expression of ABCG2 mRNA, and expression was also high in liver and small intestine (30). Unlike humans, mice have high levels of *Abcg2* mRNA in kidney and only moderate levels in placenta (26). These species differences suggest that other transporters perform the function of *Abcg2* in these tissues. Nevertheless, immunohistochemical analysis reveals specific cell types within an organ and provides functional insights. Two monoclonal antibodies (BXP21 and BXP34) have been used to confirm that ABCG2 is expressed in the plasma membrane of placental syncytiotrophoblasts

(39). Because these cells are in contact with maternal blood, these findings suggest that ABCG2 serves to protect the human fetus by causing the efflux of drugs and toxins from the placenta back into the maternal circulation. Expression of ABCG2 in the canalicular membrane of hepatocytes is consistent with the reported ability of ABCG2 to transport sulfated conjugates of steroids and xenobiotics, and the recent demonstration of gall bladder expression suggests that it may play a role in excretion of bile acid conjugates into the gut (59). In both murine and human intestine, ABCG2 is expressed in the villous tip of the intestinal cells, at the apical membrane (39, 54). This location is ideal for a role in limiting the intestinal absorption of ingested compounds. ABCG2 expression in the ducts of breast lobules appears to be hormonally regulated (see above).

ABCG2 is consistently expressed in the endothelial cells of venules and capillaries (39). In contrast, only sporadic ABCG2 staining is seen in the endothelium of arterioles (39). The prominence of ABCG2 in the endothelial cells of veins may be related to ABCG2 regulation by the level of oxygen (see above). In humans, ABCG2 is located mainly in the luminal surface of the endothelial cells in the CNS vasculature, suggesting that it functions in the blood-brain barrier to protect the brain against drugs (60). However, in mice, the expression of ABCG2 in the brain capillary endothelial cells is low and perhaps not functionally important (61); in pigs, however, an ABCG2 ortholog was identified in these cells and is thought to play a role in brain penetration (62). These findings underscore how the use of different model systems reveals species differences.

FUNCTION OF ABCG2 IN STEM CELLS

Evidence now indicates that ABC transporters play a crucial role in protecting hematopoietic stem cells and possibly other stem cells. ABC transporters were identified in these cells by their low fluorescence after staining with fluorescent dyes such as rhodamine 123 and Hoechst 33342 (referred to hereafter as Hoechst). Subsequently, a technique utilizing the red and blue fluorescent properties of Hoechst and cell sorting characterized a subpopulation of hematopoietic stem cells (63). This population, referred to as the side population (SP), contained approximately 0.05% mature nucleated bone marrow cells (63). SP cells are highly enriched in hematopoietic stem cells that are characterized as long-term culture-initiating cells (64). Because Pgp transports Hoechst, initial attention was drawn to Pgp as the transporter responsible for Hoechst exclusion by the SP cells. This conclusion was supported by the hematopoietic stem cell expansion and myeloproliferative disease observed in response to enforced overexpression of Pgp in hematopoietic cells (65). However, mice deficient in Pgp were found to have normal numbers of SP cells in the bone marrow, thus ruling out Pgp as a contributor to the SP phenotype (52, 66).

An early clue to the importance of ABCG2 was the fact that its expression in hematopoietic stem cells is greater than that of other well-established ABC transporters expressed in human SP cells (MDR1 and MRP1) (52). Moreover,

because ABCG2 is strongly down-regulated at the time of lineage commitment in both murine and human hematopoietic cells, it may play a functional role in these uncommitted cells (53). However, the reexpression of ABCG2 in some mature lineages (e.g., erythroid cells) suggests an endogenous function in these cells. Furthermore, overexpression of ABCG2 in multiple cell types produces cell lines that strongly export Hoechst, and flow cytometry analysis reveals a strong SP population (27); these findings suggest that a subpopulation of stem cells exists in many cell types.

Does ABCG2 play a role in stem cells? Its high expression in SP cells from hematopoietic sources and from nonhematopoietic sources, such as muscle, pancreas, brain, and embryonic stem (ES) cells, suggests that it does. In fact, the frequency of chimeric mice formed from ES cells increases when ES cells with the SP phenotype are used (52). This finding suggests that ABCG2 has a unique function in stem cells and that its role is to maintain progenitor cells in an undifferentiated state. This hypothesis was supported by the results of bone marrow transplantation studies (52). Transplantation of ABCG2-overexpressing bone marrow cells into lethally irradiated mice resulted in a lower number of mature hematopoietic cells (52). This finding was interpreted as an indication that ABCG2 causes the efflux of a substance important for differentiation (52). Alternatively, overexpressed ABCG2 may have exported a substance important for the growth or survival of committed lineages. Therefore, the level of ABCG2 expression may be tightly regulated. In any case, high ABCG2 expression in stem cells suggested that it has a functional role in stem cell survival and that it can be used as a tool for stem cell enrichment (67, 68). After the *Abcg2* knockout mouse was developed and the *Mdr1a*-null mouse was analyzed, ABCG2 was confirmed as the sole contributor to the SP transport phenotype in hematopoietic cells (69). Moreover, ABCG2 protected hematopoietic stem cells against the toxicity of the chemotherapeutic agent mitoxantrone (69). Thus, in stem cells, ABCG2 may function as a general protectant against endogenous and exogenous substances. If such is the case, *Abcg2*-null mice would be expected to develop a higher proportion of gastrointestinal disorders because of the inability to export toxins, as has been reported in *Mdrla*-deficient null mice (70).

The ability of stem cells to thrive under low-oxygen conditions led us to evaluate whether ABCG2 is regulated by the hypoxia-inducible transcription factor (HIF) complex, which we showed to be the case (see above). Subsequent analysis of $Abcg2^{+/+}$ and $Abcg2^{-/-}$ progenitor cells revealed markedly impaired survival of $Abcg2^{-/-}$ cells under hypoxic conditions. Further, inhibition of Abcg2 function in $Abcg2^{+/+}$ progenitor cells with the ABCG2 inhibitors fumitremorgin C and reserpine reduced cell survival under hypoxic growth conditions (71, 72). The reduced survival of $Abcg2^{-/-}$ progenitor cells was caused by the accumulation of heme (porphyrin) molecules, as shown by the rescue of the cells by an inhibitor of heme biosynthesis. Collectively, the regulation of ABCG2 by HIF and the ability of ABCG2 to bind and interact with porphyrins suggest that this molecule is important for the maintenance of heme homeostasis in progenitor cells under low-oxygen conditions (33).

ABCG2 PROTEIN: SUBCELLULAR LOCALIZATION, STRUCTURE, AND FUNCTION

Immunohistochemical studies with monoclonal and polyclonal antibody probes to ABCG2 demonstrate that ABCG2 is predominantly localized to the plasma membrane in both drug-selected and *ABCG2*-transfected cells (39, 73, 74). Fluorescence confocal microscopy studies confirmed these observations, showing mitoxantrone fluorescence around the plasma membrane of MXR HCT-116 cells that was attenuated by membrane-fluidizing agents (75). The function of ABCG2 as an export pump for cytotoxins is consistent with this localization. ABCG2 is unique among the ABC half-transporters in its localization to the plasma membrane rather than to an intracellular membrane of a subcellular organelle, such as the mitochondrion (M-ABC1 and ABC-7) (76, 77), endoplasmic reticulum (TAP 1 and 2) (78), or peroxisome (ALDP/ ABCD1) (79). When ABCG2 was ectopically expressed in a cell line that polarized in culture, the apical membrane, but not the basolateral membrane, showed positive staining for ABCG2 (74). Similarly, when murine ABCG2 was ectopically expressed in polarized canine or porcine epithelial cell lines, it mediated apically directed transport of its drug substrates (80, 81). These results indicate that human and mouse ABCG2 localize to the apical membrane in polarized cells.

The predicted structure of ABCG2 differs from those of Pgp- and MRP-family proteins (ABCB and ABCC, respectively) in that ABCG2 has only one NBD and is a single peptide with six putative transmembrane domains; it is therefore referred to as a half-transporter (see Figure 1). ABCG2 exists as a glycoprotein and has three N-linked glycosylation sites (82). Studies using the insect cell system have shown that ABCG2 membrane expression and transport function do not require glycosylation (83), and recent studies by Diop & Hrycyna (84) indicate that glycosylation of the conserved asparagine 596 is not required for function or localization. However, it is possible that other asparagines or additional post-translational modifications may affect optimal ABCG2 function or localization in mammalian cells, as is the case with Pgp (85, 86).

Many of the ABC half-transporters function by forming homodimers or heterodimers. The *Drosophila* brown and scarlet proteins heterodimerize with the white protein to transport substrates. Similarly, the G family members ABCG5 and ABCG8 heterodimerize but lack the capacity to functionally homodimerize (87). The functional activity of ABCG2 in two heterologous systems argues for its function as a homodimer. For example, ABCG2 expressed in insect cells is functional despite the absence of any likely heterodimeric partner. Moreover, a nonfunctional ABCG2 mutant (bearing a K86M mutation in the Walker A NBD) acted as an effective dominant-negative inhibitor of ABCG2 when cotransfected with a nonmutated ABCG2 (83, 88). A functional homodimer was further suggested in a prokaryote transfected with ABCG2 (89). Kage et al. demonstrated physical interaction that confirmed homodimer formation (90). These authors used two separate ABCG2 molecules, each tagged differently, and revealed their homodimerization by coimmunoprecipitation.

What drives the formation of ABCG2 homodimers? The fact that the 140-kDa ABCG2 complex can dissociate into 70-kDa monomeric bands under reducing conditions in the presence of a sulfhydryl reducing agent suggests that intermolecular disulfide bonds are formed. A molecular mass shift from a low molecular weight under denaturing conditions to a high molecular weight after treatment with crosslinking agents was also noted by Litman et al. (91), who used polyclonal antibodies directed against peptide epitopes. These results show that ABCG2 forms a homodimer, and they suggest that homodimerization occurs through the formation of disulfide bonds. However, studies in model systems have suggested that ABC transporters do not use cysteines for dimer formation (92). For example, the bacterial ABC half-transporter LmrA has no cysteines but still transports substrates (92).

What are the signals governing ABCG2 dimerization? Does ABCG2 function depend on dimerization? A recent report suggested that a conserved $G(X)_3G$ motif, which is reportedly important in helix self-association (93), is involved in the dimerization of the ABCG2 transmembrane segments and leads to the formation of a functional ABCG2 homodimer. However, Diop (84) found that mutation of essential glycine residues in the $G(X)_3G$ motif did not impair either plasma membrane localization or dimerization. Unexpectedly, mutation of this conserved motif abolished the substrate transport activity of ABCG2. This finding implies that ABCG2 dimerization and transport activity are separable phenomena. Irrespective of the mechanism of dimerization, it is clear that ABCG2 functions as a homodimer but that homodimerization alone does not ensure function. Whether ABCG2 can also interact with other proteins, of the ABCG class or others, is an open question, although it is incapable of forming an appropriate heterodimer with family members ABCG5 and ABCG8 (87). Further, it is not yet clear whether ATP binding is required for ABCG2 dimerization or whether dimerization is a prerequisite for ABCG2 membrane localization. Dimerization may require ATP binding activity, as is the case with the half-transporter Atmlp (94). Interestingly, recent reports suggest that ABCG2 oligomers exist and that the tetramer is the functional form. Triton X-100 extraction of ABCG2 from MCF-7/AdrVp 3000 cells that overexpress ABCG2 showed that the predominant form of ABCG2 was a homotetramer rather than a monomer or homodimer (95). However, the relation of ABCG2 transport activity to the proportion existing as a homotetramer was not investigated. Future studies should help us better understand the oligomeric state of ABCG2 under physiological conditions and the relation between oligomerization and function.

The localization of some transport proteins to membranes is regulated by a signaling pathway. Croce et al. found that ABCG2 was diffusely localized in the cytoplasm of drug-sensitive HT 29 cells and localized almost exclusively in the plasma membrane of drug-resistant HT29 cells (96). In contrast, in some malignant tissues, ABCG2 is found mainly in the cytoplasmic compartment (97). Interestingly, some human variants of ABCG2 (ABCG2-V12M) have impaired membrane localization (98). Signaling by the phosphatidylinositol-3 kinase (PI3K)/Akt

pathway may explain these differences in localization. Akt activation is correlated with translocation of Glut 4 to the plasma membrane and enhanced glucose transport (99). Similarly, bile-salt export protein (BSEP/abcb11) traverses a path to the canalicular membrane that requires PI3K activation (100). Takada et al. demonstrated that inhibition of PI3K activity by wortmannin caused translocation of ABCG2 to an intracellular compartment in human gall bladder cells (101). Similarly, the presence of the PI3K inhibitor LY294002 in hematopoietic cells resulted in translocation of ABCG2 from the plasma membrane to an intracellular compartment (102). Further, the role of Akt1 in ABCG2 localization was confirmed by the fivefold reduction of the capacity of Akt1-deficient hematopoietic cells to export the ABCG2 substrate Hoechst (102). This result was specific to Akt1, as the Akt2-null cells had not lost the capacity to export Hoechst. These results suggest that intracellular distribution of ABCG2 may depend on upstream signaling events and that this signaling may complement the transcriptional regulation of ABCG2 by HIF-1 (Figure 3). Moreover, ABCG2 translocation from the plasma membrane to an intracellular site may restrict ABCG2-mediated efflux of endogenous substrates such as folate; under low-folate growth conditions, ABCG2 localizes to an intracellular compartment and folic acid accumulates in the cell (103). This relocalization was proposed to be a means of conserving folates that could be exported by ABCG2 (103). The role of signaling in the subcellular localization of ABC transporters was first detailed by Misra and colleagues in their description of the effect of PI3K activation on Abcb11 (BSEP) subcellular localization (104). The findings with ABCG2 suggest that this regulation of intracellular localization is a conserved, although not widely investigated, property of ABC transporters.

CLINICAL SIGNIFICANCE OF ABCG2 IN CANCER

After ABCG2 was identified in drug-resistant cells, its presence in cancer cells was scrutinized to determine its role in acquired drug resistance and the effect of its level of expression in normal tissues on therapeutic response. In immunohistochemical studies of ABCG2 in adult lymphoblastic leukemia, shorter disease-free survival was associated with the detection of ABCG2 (105). The Ross laboratory found that approximately 30% of patients with acute myeloid leukemia (AML) had high levels of *ABCG2* mRNA in their malignant cells and that this level of expression was sufficiently high to justify investigation of its role in resistance to therapy (106). The relation of ABCG2 expression to chemoresistance of AML blast cells in vivo has also been examined (107, 108). Several studies have found a positive relationship between ABCG2 expression and drug resistance in AML (109–112). Sargent et al. found that in 6 of 22 AML samples, more than 10% of cells stained positively for ABCG2 (107). This finding raised the possibility that ABCG2 expression is related to poor response to agents that are ABCG2 substrates. Such a possibility was supported by findings that ABCG2 mRNA levels are correlated

with in vitro sensitivity to flavopiridol, an ABCG2 substrate, in AML cells (111). Nonetheless, conclusions about the impact of ABCG2 overexpression on clinical outcome or on prognosis in AML require caution. A few studies have failed to find significant expression of ABCG2 in malignant blast cells; instead, they found that expression in these cells was low and, when present, was confined to the subpopulation of immature blast cells (113, 114). This finding may reflect the heterogeneity of hematopoietic cells, but because most blast cells that express ABCG2 have characteristics of leukemic stem cells (CD 34^+ $CD38^-$ $CD33^-$), these cells may be refractory to therapy because of their ABCG2 expression. Moreover, if these cells are like normal hematopoietic stem cells, then ABCG2 expression may identify a leukemic stem cell in this cell population. These leukemic stem cells are a potential reservoir of resistant malignant cells that can persist because of their resistance to ABCG2 substrates. A side population of Hoechst-exporting cells has been described in AML (115). These cells were phenotypically immature and had the capability to engraft in NOD/SCID mice and to produce leukemia, thus meeting the definition of leukemic stem cells.

Analysis of ABCG2 expression in a panel of 150 untreated tumors revealed ABCG2 immunoreactivity in more than 40%, with prominent expression in carcinomas of the digestive tract (colon, esophagus, stomach) (97, 116). Osteosarcomas and carcinomas of the bladder showed a low frequency of ABCG2 expression. The increased expression of ABCG2 in hepatic metastases from colon tumors after irinotecan chemotherapy (a ABCG2 substrate, see Table 1) suggests that expression of ABCG2 in a subpopulation of tumor cells [perhaps tumor stem cells (117)] might lead to therapeutic failure (118). The finding that 65% of neuroblastomas had a Hoechst-transport side population phenotype that was correlated with high ABCG2 levels suggested that ABCG2 expression may typify a cancer stem cell population. These neuroblastomas also had a greater capacity to export the ABCG2 substrate mitoxantrone and showed enhanced survival when challenged with a cytotoxic dose of mitoxantrone (119). It is notable that a number of human solid cancers have a Hoechst-export side population (e.g., ovarian carcinoma, small-cell lung carcinoma, Ewing sarcoma, prostate cancer). Taken together, these results are consistent with the putative role of ABCG2 in clinical drug resistance in solid tumors and suggest that ABCG2 expression has prognostic value. This conclusion is supported by the report of Yoh et al. that ABCG2-negative non-small-cell lung carcinomas had a better rate of response to therapy (44%) than did ABCG2-positive tumors (24%) (120).

PHYSIOLOGICAL AND PHARMACOLOGIC IMPACT OF ABCG2

The normal function of ABCG2 may be to prevent the accumulation of both extracellular and intracellular toxins in cells. These two functions are not mutually exclusive and are compatible with the broad tissue expression of ABCG2, from the

TABLE 1 ABCG2 substrates

Anticancer drugs	Natural compounds
9-Aminocamptothecin (156, 157)	Flavonoids (158–160)
Bisantrene (161, 162)	Pheophorbide a (33, 130)
Diflomotecan (153)	PhiP (2-amino-1-methyl-1–6-phenyl-imidazo[4,5-b]pyridine) (121, 163)
Doxorubicin (15, 17, 26, 161, 162)	Porphyrins (33, 4)
*Daunomycin (17, 107)	*Sulfated estrogens (126)
*Daunorubicin (15, 17, 26, 161)	
*Epirubicin (157)	Fluorescent substrates
*Etoposide (26, 162, 164, 165)	*Hoechst 33342 (52, 53)
Flavopiridol (167)	Lysotracker (168)
GV 196771 (170)	*Rhodamine 123 (15, 83, 161)
Irinotecan (CPT-11) (156, 157, 162, 164)	
J-107088 (164)	
*Methotrexate and its polyglutamates (147, 172)	Inhibitors
Mitoxantrone (15, 26, 146, 157, 161, 162, 167)	Anti-ABCG2 monoclonal antibody (5D3) (173)
	HIV protease inhibitors (179)
NB-506 (10, 164)	CI1033 (139)
Quinazoline (23)	Futremorgrin C (FTC) (162)
*SN-38 (6, 7, 9)	GF120998 (165)
*Imatinib (ST1–571, Gleevec) (135, 171)	K0143 (174)
Teniposide 3	Hammerhead ribozyme (175, 176)
*Topotecan (2, 3, 5–7, 9, 10)	Imatinib (ST1–571, Gleevec) (135)
	Pantoprazole (171)
Nucleoside drugs	Prazosin (62, 83, 161)
Lamivudine (3TC) (166)	*Reserpine
Zidovudine (AZT) (166, 169)	ZD 1839 (gefitinib, Iressa) (134, 177)

*Overlap with Pgp and/or Mrp1.

canalicular membrane of the hepatocyte to the epithelia of small intestine, colon, and kidney, to the endothelia of veins and capillaries (39).

In the blood-brain barrier (BBB), expression of ABCG2 mRNA is comparable to that of Pgp, another ABC transporter known to protect the brain from accumulation of drugs and cytotoxins (28, 60, 62). Because ABCG2 is expressed at the apical surface of polarized cells (74) and is capable of vectoral transport, it seems likely that ABCG2 would limit brain penetration by its substrates. However, Sugiyama and colleagues, using in situ brain perfusion of *Abcg2*-knockout mice, found that efflux of the chemotherapeutic agent mitoxantrone at the BBB was unaffected by the absence of ABCG2 (61). This result agrees with those of van Herwaarden et al. (121), who recently reported that although the dietary carcinogen 2-amino-1-methy1–6-phenylimidazo [4,5-b] pyridine (PhIP) is an ABCG2 substrate, there was no significant difference between wild-type and *Abcg2*-knockout mice in the

brain penetration of PhIP. Therefore, although ABCG2 is expressed in the human BBB, its function at that site in mammals is unknown.

The expression of ABCG2 in the apical membrane of small intestine and colon epithelial cells and in the canalicular membrane of hepatocytes suggests that it plays a role in reducing absorption of ingested toxins. This distribution pattern supports the hypothesis that ABCG2 functions both as a Phase 0 (inhibition of cellular uptake) and Phase III (acceleration of removal) defense against xenobiotics through direct inhibition of uptake in the gut and rapid elimination in bile. If so, decreased levels of ABCG2 might reduce hepatic elimination of ABCG2 substrates and increase their systemic levels, although decreased absorption from the gut might counter-balance this effect. Studies using the *Abcg2*$^{-/-}$ mouse generated by the laboratory of Schinkel revealed that Abcg2 protects against the gastrointestinal absorption of a potent phototoxic agent (pheophorbide a) that is a product of chlorophyll degradation (54). These authors also demonstrated that Abcg2 is important for absorption of orally administered topotecan: The bioavailability of the agent was increased sixfold in the absence of Abcg2. An extension of these studies found that Abcg2 not only limits the gastrointestinal uptake of the dietary carcinogen PhIP but also profoundly affects its hepatobiliary elimination (121). Abcg2 exports glucuronide and sulfate conjugates from enterocytes in vitro, suggesting that it exports these Phase II metabolites (conjugates) in vivo. Moreover, the high level of glucuronyl transferases in the gastrointestinal tract would facilitate the formation of these conjugates and further limit absorption. These findings indicate that ABCG2, like Mrp2 [another conjugate transporter expressed in the gastrointestinal tract (122)], may coordinate the removal of metabolites after their conjugation with glucuronides or sulfates.

ABCG2 was found to be highly expressed in mammary gland only in epithelium and only during late pregnancy and lactation (46). Because lactogenesis requires the orchestration of a number of transport processes and because some ABCG2 substrates may be found at high levels in breast milk [e.g., cimetidine (123, 124)], the possible transport of these substances into breast milk was investigated (46). These authors showed that active secretion of topotecan, PhIP, and cimetidine (all ABCG2 substrates) into breast milk was markedly reduced in *Abcg2*-null animals. This finding raised the possibility that although Abcg2 may facilitate the inadvertent transfer of toxins to offspring, it may also play a role in supplying nutrients to suckling offspring. However, the identity of these nutrients is unknown, and, in view of the viability of mice born to *Abcg2*$^{-/-}$ females, this appears to be an auxiliary route of nutrient transfer.

ABCG2 SUBSTRATES AND MODULATION OF FUNCTION

As seen in Table 1, ABCG2 is capable of transporting a diverse array of substrates, which overlap those of Pgp and Mrp1 to a certain extent. It is notable that strong resistance to mitoxantrone characterizes most cell lines that overexpress ABCG2.

Moreover, like other multidrug transporters, ABCG2 shows drug-induced ATPase activity and transports hydrophobic positively and negatively charged molecules, although in some substrates it is selective for specific structures. However, it should be noted that ABCG2, like Pgp, does not require glutathione to transport substrates. The latter is required for optimal Mrp1 transport of some substrates (125). These diverse capabilities of ABCG2 may, in part, reflect the different model systems used to characterize them. For example, ABCG2 has been shown to only transport sulfated steroids in mammalian vesicle systems (126). In contrast, unconjugated estradiol and the bile acid cholate are substrates of ABCG2 expressed in the gram-positive bacterium *Lactococcus lactis* (89). The difference may reflect disparate membrane composition in *L. lactis* and mammalian cells or differences in the assay systems (cells versus membrane vesicles).

One example clearly reveals how ABCG2 recognizes differences in substrate structure. Cancer cells that overexpress ABCG2 are not resistant to the camptothecins or to camptothecin analogues (e.g., BCP1350, DX8951f, or NX211), yet nucleophilic groups at the ninth or tenth position of the camptothecin A ring facilitate interaction with ABCG2 (see Figure 4). For example, ABCG2 expression confers resistance to 9-aminocaptothecin but not to 9-nitrocamptothecin (127). Some studies have found that the camptothecin E-ring (128) as well as the polarity of the molecule explains the reduced accumulation of camptothecin analogues in cells overexpressing ABCG2 (129). These studies suggested that highly polar camptothecin analogues are good ABCG2 substrates and are therefore effectively exported from cancer cells. Conversely, low-polarity camptothecin analogues may overcome ABCG2-mediated drug resistance.

ABCG2 interacts with porphyrin and porphyrin-like compounds (Figure 4), and recent reports suggest that these compounds are natural substrates of ABCG2 (33, 54). Pheophorbide a is a chlorophyll metabolite used as a photosensitizer in the experimental treatment of tumors and has been shown to be a substrate of ABCG2 (130). Elevated levels of protoporphyrin IX (PPIX) in erythroid progenitors of ABCG2-deficient mice support the idea that porphyrins are endogenous ABCG2 substrates (54). Further analysis has revealed that ABCG2 prefers certain porphyrin structures and that it may interact with more than one endogenous substrate, as does Mrp2 (bile acids, bilirubin conjugates) (131, 132).

Inhibition of ABCG2 function may be a viable pharmacologic anticancer strategy from two perspectives. First, in cancers that naturally have high levels of ABCG2 (e.g., germ cell tumors, brain tumors) (http://cgap.nci.nih.gov), ABCG2 inhibition can increase the intracellular level of the chemotherapeutic agent, thereby increasing cytotoxicity. Second, the viability of the *ABCG2* knockout mice and the increased bioavailability of some orally administered drugs in the knockout mice suggest that inhibition of ABCG2 function in vivo would increase systemic drug levels.

Two recently developed specific tyrosine kinase inhibitors, imatinib (Gleevec, ST1–571) and gefitinib (Iressa, ZD1839), interact with high affinity with ABCG2, although they were developed to selectively interact with their intracellular targets,

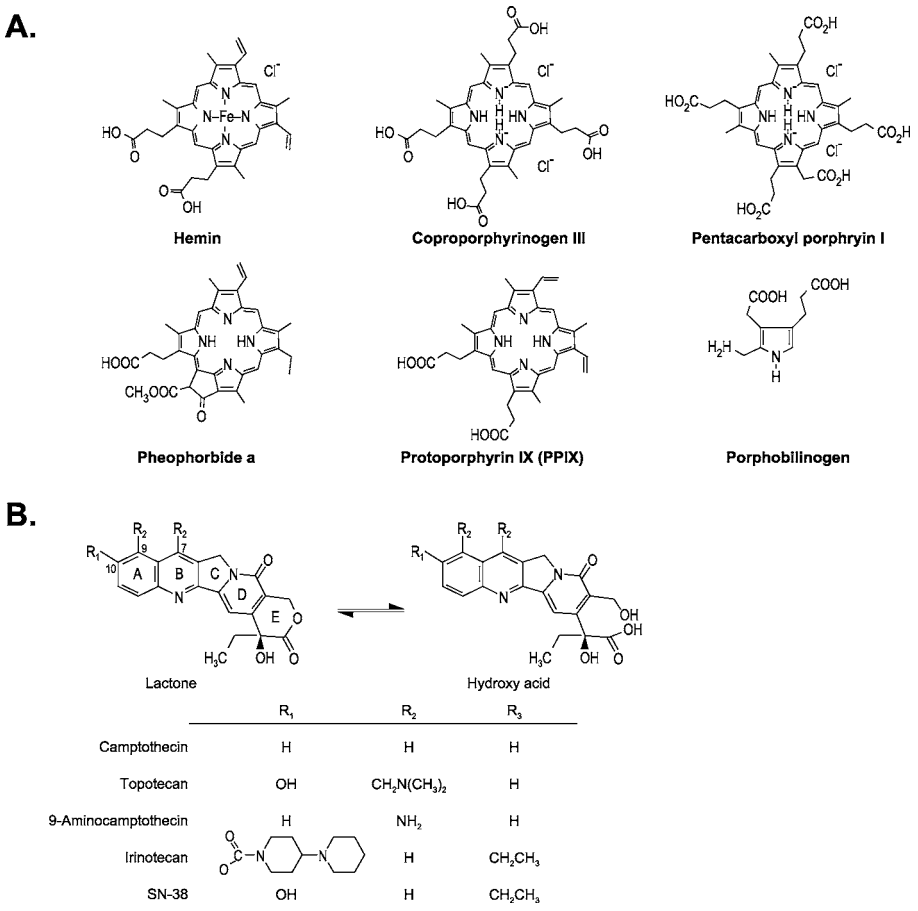

Figure 4 ABCG2 specifically interacts with porphyrins (*A*) and camptothecins (*B*).

Bcr-Abl and ErB1 kinases, respectively. Although gefitinib appears only to inhibit ABCG2 (133, 134), imatinib appears to be an inhibitor and a substrate (135). Thus, at low concentrations (<1 μM), imatinib is transported by ABCG2 and is able to stimulate ABCG2 vanadate-sensitive ATPase activity (136). At higher concentrations, imatinib inhibits ABCG2 ATPase activity and shows no propensity to behave as an ABCG2 substrate (136). This effect is analogous to the action of cyclosporine as both an inhibitor and a substrate of Pgp (ABCB1) (105, 137, 138).

Recent studies by Houghton & Stewart showed that systemic levels of the anticancer drug irinotecan (an ABCG2 substrate; Table 1) can be effectively increased by coadministration of gefitinib (133). Moreover, gefitinib increased the sensitivity of ABCG2-overexpressing cells to SN-38 (the active metabolite of irinotecan),

which is an ABCG2 substrate. These results showed that gefitinib is an ABCG2 inhibitor. Gefitinib is similar in structure to the ABCG2 inhibitor CI-1033, a 4-anilino quinazoline (139). In a proof-of-principle experiment, the authors showed that the antitumor activity of the irinotecan-gefitinib combination was greater than that of irinotecan alone (133). It is notable that these tumors lacked the ERBB1 kinase (the target of gefitinib). Therefore, the enhanced activity of the drug combination was attributed solely to gefitinib's ability to inhibit ABCG2 and increase systemic exposure to irinotecan. The mechanism of this effect was presumably the inhibition of gastrointestinal ABCG2 function and increased intestinal irinotecan absorption.

SIGNIFICANCE OF ABCG2 VARIANTS

Single amino acid changes in key residues of ABC transporters can alter their substrate specificity. Two well-known examples are acquired Pgp mutations (a G to V substitution at amino acid 185), which alter substrate specificity, and an amino acid residue in a key domain of Mrp1 (12, 140–143), which defines species differences in substrate transport capability. For ABCG2, conversion of arginine to threonine or glycine at position 482 alters the substrate specificity (80, 144, 145). Transfection studies with a vaccinia virus expression system confirmed that ABCG2 transport of rhodamine 123 depends on the specific amino acid at position 482 (144). A glycine or threonine at that position allows rhodamine 123 transport, whereas arginine does not (144). This result suggests that a single amino acid mutation (a gain-of-function mutation) can enhance transport capability. This concept is illustrated by the acquired ability of ABCG2 with 482 glycine or threonine to export anthracyclines. However, although these mutants acquire the ability to transport some substrates, they lose the ability to export methotrexate (146, 147). The role of the amino acid substitution at position 482 has been extensively studied. Mitomo et al. found that cells expressing these variants had various levels of increased mitoxantrone resistance (glycine 482, 47-fold; threonine 482, 54-fold) as compared with cells expressing wild-type ABCG2 (arginine 482, 15-fold) (148). Allen et al. found similar differences in the cross-resistance to doxorubicin resulting from mutations acquired at 482 in the mouse ABCG2 transporter (145). It is notable that ABCG2 with arginine at 482 and ABCG2 with glycine at 482 have similar transport capability for antibiotics, unconjugated sterols, and primary bile acids, whereas ABCG2 with glycine at 482 loses the capability to transport methotrexate (149). The authors speculate that the intracellular charge at position 482 is important for electrostatic interactions with some substrates at the membrane-cytosol interface. Miwa et al. created amino acid substitutions in transmembrane domains 2, 3, 5, and 6 in ABCG2 and discovered that single amino acid substitutions in each of these domains affected substrate specificity (150).

With respect to natural nonsynonymous polymorphisms in human *ABCG2*, no drug resistance–associated substitutions of uncharged amino acids at position 482

have been detected in the human populations analyzed to date; therefore, arginine at position 482 is the wild-type residue and substitutions at this position are a drug-selected alteration. However, because ABCG2 can transport natural substrates, functional variations of ABCG2 may exist within humans. Allelic variations have been reported. Nine SNPs, including four nonsynonymous and three synonymous coding SNPs and two intronic SNPs, were reported by Zamber et al. (45). The two SNPs most frequently identified were in exon 2 (G34A, resulting in a V12M change) and exon 5 (C421A, resulting in a Q141K substitution). Two other SNPs produced amino acid changes in exon 6 (A616C and I206L) and exon 15 (a 1768T and N590Y) (45). These common natural allelic variations of ABCG2 did not influence the expression of ABCG2 mRNA in human intestine (45). However, it should be pointed out that these authors did not assess which ABCG2 allele was present in the mRNA (i.e., whether allelic imbalance was present, see above). Itoda et al. reported 8 additional SNPs in Japanese patients that were administered irinotecan (151). Three of these resulted in the amino acid substitutions F431L, F489L, and S441N. Konda et al. extended these studies and suggested that the SNP resulting in the S441N substitution may affect the expression level and cellular localization of ABCG2 (152). Other studies have shown that ABCG2 variants have altered transport properties. Depending on their functional properties, these variants may alter the pharmacokinetics of ABCG2 substrates. For example, Sparreboom et al. (153) found that the ABCG2 C421A variant affected the pharmacokinetics of diflomotecan, a synthetic derivative of camptothecin. They discovered that patients with a wild-type allele had plasma drug levels only one third those of patients with the C421A allele. This finding is consistent with in vitro evidence that the C421A allele is less functional. Although there is no direct evidence that 5′-regulatory polymorphisms affect ABCG2 transcriptional activity, several reports identify a deletion (CTCA) in the ABCG2 promoter as well as a SNP in the CpG island (154, 155).

CONCLUSIONS AND PERSPECTIVES

Much progress has been made since the discovery of ABCG2 (BCRP/MXR/ABCP). We have witnessed an explosion of information about the role of ABCG2 in multidrug resistance, especially regarding the types of drug substrates it transports. However, some very early studies suggested that ABCG2 has two substrate binding sites. If this is true, some interesting drug interactions may occur in vivo, perhaps between endogenous substrates and drugs. *ABCG2*-knockout animals from two separate labs are proving invaluable in elucidating the roles of ABCG2 in vivo. However, much remains to be learned. We know only a little about the regulation of ABCG2 and its function in vivo. It would be interesting to know whether genetic background modifies the phototoxicity caused by chlorophyll degradation products (i.e., pheophorbide a) because both sets of knockout mice (on different genetic backgrounds) have elevated porphyrin levels in the absence of ABCG2 (54, 69). The knowledge that Akt1 regulates ABCG2 migration from the plasma

membrane to an intracellular site seems to be the first step in elucidating the dynamic regulatory process that moves ABCG2 between these two sites. It would, of course, be important to know whether a specific protein interaction mediates this process or whether a domain in ABCG2 is required. Finally, it would be important to know whether natural variants of ABCG2 in humans are associated with inherent susceptibility to some xenobiotic toxicants or pophyrias.

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Figure 2 The human ABCG2 promoter has multiple transcription factor sites that are important for regulation. "T" indicates the transcription start site. The green oval represents the first untranslated exon.

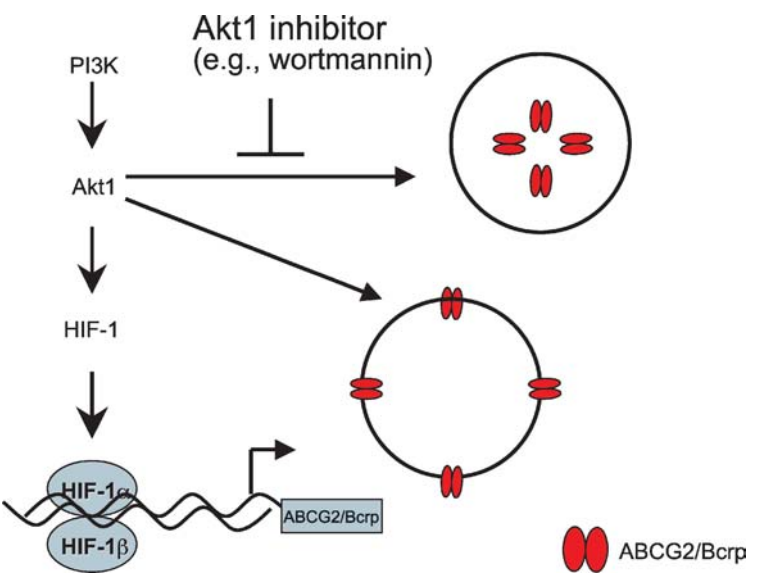

Figure 3 Transcription of ABCG2 is regulated by the hypoxia-inducible factor 1 (HIF-1), and the localization of ABCG2 to the plasma membrane is determined by Akt1, an upstream regulator of HIF-1.

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