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REVIEW

The emerging pharmacotherapeutic significance of the breast cancer resistance protein (ABCG2)

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The breast cancer resistance protein (also termed ABCG2) is an ATP-binding cassette transporter, which mediates the extrusion of toxic compounds from the cell, and which was originally identified in relation to the development of multidrug resistance of cancer cells. ABCG2 interacts with a range of substrates including clinical drugs but also substances such as sterols, porphyrins and a variety of dietary compounds. Physiological functions of ABCG2 at both cellular and systemic levels are reviewed. For example, ABCG2 expression in erythrocytes may function in porphyrin homeostasis. In addition, ABCG2 expression at apical membranes of cells such as hepatocytes, enterocytes, endothelial and syncytiotrophoblast cells may correlate to protective barrier or secretory functions against environmental or clinically administered substances. ABCG2 also appears influential in the inter-patient variation and generally poor oral bioavailability of certain chemotherapeutic drugs such as topotecan. As this often precludes an oral drug administration strategy, genotypic and environmental factors altering ABCG2 expression and activity are considered. Finally, clinical modulation of ABCG2 activity is discussed. Some of the more recent strategies include co-administered modulating agents, hammerhead ribozymes or antisense oligonucleotides, and with specificity in cell targeting, these may be used to reduce drug resistance and increase drug bioavailability to improve the profile of chemotherapeutic efficacy versus toxicity. While many such strategies remain in relative infancy at present, increased knowledge of modulators of ABCG2 could hold the key to novel approaches in medical treatment.
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Abbreviations: ABC, ATP-binding cassette; ABCP, placenta-specific ABC transporter; AhR, aryl hydrocarbon receptor; ALA-S, 5-aminolevulinate synthetase; AML, acute myeloid leukaemia; BCRP, breast cancer resistance protein; ER, oestrogen receptor; EST, expressed sequence tag; FPGS, folylpoly-γ-glutamate synthase; FTC, fumitremorgin C; MD, membrane domain; MDR, multidrug resistance; MRP1, multidrug resistance-associated P-glycoprotein 1; MTX, methotrexate; MX, mitoxantrone; NBD, nucleotide-binding domain; PPIX, protoporphyrin IX; SNP, single-nucleotide polymorphism; SP, side population

Introduction

ABCG2 is a member of the G subfamily of ATP-binding cassette (ABC) transporters (Krishnamurthy and Schuetz, 2006). Its endogenous expression at the apical membranes of hepatocytes, enterocytes, endothelial and syncytiotrophoblast cells is suggestive of basic physiological barrier functions that may protect tissues against toxicity of environmental or clinically administered substances (Leslie *et al*., 2005; Sarkadi *et al*., 2006). Identification of factors that can influence ABCG2 expression and activity might lead to the development of new strategies to clinically modulate ABCG2-mediated transport. This review first describes the initial characterization of ABCG2 as a multidrug transporter. The substrate specificity of ABCG2 is presented along with variations that can occur as a result from acquired mutations and/or polymorphisms in the protein. A range of physiological functions of ABCG2 at both a cellular and systemic level is discussed, and evidence is presented of how these influence the pharmacokinetics of administered drugs. Given that ABCG2 appears to contribute to the inter-patient variation in drug bioavailability, important genotypic and environmental factors modulating ABCG2 expression or function are reviewed. Looking to the future, the aim will be to harness our insights into the structure, function and modulation of ABCG2 to improve chemotherapeutic outcomes. Thus, some of the current and potential future strategies for clinical manipulation of ABCG2 expression and activity are mentioned.

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Initial discovery

Juliano and Ling (1976) first showed a link between the multidrug resistance (MDR) phenotype in mammals and the overexpression of a drug extrusion system, which they termed multidrug resistance P-glycoprotein. This protein is a member of the ABC protein superfamily (Higgins, 1992). To date, 49 ABC genes have been identified in the human genome, which are subdivided into seven families designated A to G. In this nomenclature, P-glycoprotein is also termed ABCB1. In addition to ABCB1, efflux-based MDR in mammals can be associated with the overexpression of the 'multidrug resistance-associated P-glycoprotein 1 (MRP1, also termed ABCC1). The characterization by Doyle *et al*. (1998) of the breast tumour cell line MCF-7/AdrVp, which displayed an efflux-based MDR phenotype in the absence of ABCB1 or ABCC1, led to the discovery of the breast cancer resistance protein (BCRP, also termed ABCG2). The *ABCG2* gene has a nearly identical sequence to expressed sequence tag (EST) 157481, previously identified as a potential ABC gene in an EST database search by Allikmets *et al*. (1996). In the literature, ABCG2 is also known as placenta-specific ABC transporter (ABCP) and mitoxantrone (MX) resistance protein. Work by Allikmets *et al*. (1998) revealed the highly expressed *ABCP* gene on the human 4q22 chromosome in placental syncytiotrophoblast cells. Two transcripts were found that differed at their 5′ end, but both encoded a 655-amino-acid ABCG2 protein that was predicted to be closely related to the *Drosophila* white genes. Finally, studies by Miyake *et al*. (1999) focused on a cDNA library from the MX-selected colon cell line S1-M1-80. Differential hybridization and Northern analysis identified two *ABCG2* transcripts, sharing over 98% homology with EST 157481.

Structure of ABCG2

ABCB1 shows the classical ABC transporter domain organization with four core domains: two membrane domains (MD), which form the drug translocation pathways across the phospholipid bilayer, and two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP to drive the transport reaction. These four domains are fused on a single polypeptide in the form of two homologous half-transporters, each consisting the N-terminal MD followed by the NBD. Initial characterization of ABCG2 by Doyle *et al*. (1998) identified a half-transporter with a reversed topology compared with the half-transporters in ABCB1. Thus, ABCG2 contains an N-terminal NBD followed by a C-terminal MD (Kusuhara and Sugiyama, 2006).

Some ABCG subfamily members such as the *Drosophila* white proteins (Sullivan and Sullivan, 1975) or the human ABCG5 and ABCG8 proteins (Graf *et al*., 2002) function in heterodimerized form, but the traditional view for ABCG2 has been based on a homodimer (Lorkowski and Cullen, 2002). Functional expression of ABCG2 in *Xenopus* oocytes (Nakanishi *et al*., 2003a), *Spodoptera frugiperda* insect cells (Ozvegy *et al*., 2001, 2002) and *Lactococcus lactis* bacterial cells (Janvilisri *et al*., 2003) argued against the necessity of an ABC partner protein for ABCG2. Miyake *et al*. (1999) found that exclusive upregulation of ABCG2 mRNA was sufficient to induce MDR in drug-selected cell lines. Consistent with the idea that ABCG2 might form a homodimer, Kage *et al*. (2002) observed that a transport-inactive ABCG2 mutant could exert a dominant-negative effect on co-expressed functional ABCG2. Analogous to previous work on the bacterial ABC half-transporter LmrA (an ABCB1 homologue in *L. lactis*) (Van Veen *et al*., 2000), Bhatia *et al*. (2005) constructed chimeric fusion proteins containing two ABCG2 proteins joined covalently either with or without a flexible linker peptide. Fusion proteins containing two wild-type units were transport-active. In contrast, the introduction of a D210N replacement in the Walker B region of the first unit, which inactivated the ATPase activity of the unit, imposed a dominant-negative phenotype on the fusion proteins. These data are consistent with the notion that the minimal functional unit in ABCG2-associated transport is a homodimer in which both halves are required for transport. Interestingly, there is also evidence for the existence of higher order ABCG2 oligomers. Xu *et al*. (2004) extracted ABCG2 from MCF-7.AdrVp3000 cells and found evidence for a tetrameric form on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Consistent with the higher order of oligomerization suggested by cross-linking experiments by Litman *et al*. (2002), they also found a less stable dodecameric assembly. Most recently, the structure of ABCG2 R482G (see below) was purified from *Trichoplusia ni* insect cells, and studied by cryonegatively stained electron microscopy and single-particle analysis. Evidence was obtained that ABCG2 R482 was extracted in an octameric form, as a tetramer of dimers (McDevitt *et al*., 2006). Although it is not yet clear whether the higher order oligomeric forms of ABCG2 are physiologically relevant, the formation of such complexes might potentially affect protein trafficking and transport activity by altering the number and/or functionality of molecules in the plasma membrane.

Transport of cytotoxic drugs

A mechanism of ATP-dependent drug efflux by ABCG2 was suggested from the inverse correlation between drug retention and cellular resistance, and inhibitory effects of the oxidative phosphorylation inhibitors such as sodium azide or 2,4 dinitrophenol (Nakagawa *et al*., 1992; Volk *et al*., 2000). Whereas half-transporters such as the transporter for antigen presentation 1 and 2 are typically found on intracellular membranes (Townsend and Trowsdale, 1993), ABCG2 is mainly localized in the plasma membrane (Litman *et al*., 2000), where it mediates extrusion of a wide variety of positively or negatively charged molecules, including cytotoxic compounds (MX, topotecan, flavopiridol, methotrexate (MTX)), fluorescent dyes (e.g., Hoechst 33342) and toxic compounds found in normal food (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, pheophorbide-a) (Sarkadi *et al*., 2006). Initial work resulted in conflicting reports as to the transport of some of these substances. For example, Dalton *et al*. (1988) reported little reduction in intracellular MX, whereas Taylor *et al*. (1991) found significant efflux of this compound. Discrepancies in results

arose, in part, from differences in cell context (e.g., rates of drug uptake) as well as differences in assay methods for drug efflux. For example, in the case of studies in intact cells using fluorescent substrates versus radiolabelled substrates, the intracellular metabolism of the compound might cause a loss of fluorescence, whereas radioactivity might still be retained. More importantly, reported differences in substrate specificity were found to be due to variations in the *ABCG2* sequence. High anthracycline resistance was reported in doxorubicin-selected cell lines (Doyle *et al*., 1995), but MX-selected (Rabindran *et al*., 1998) or topotecan-selected cells (Maliepaard *et al*., 1999) showed variable and usually modest resistance when compared with MX. Although Robey *et al*. (2001) were developing a flow cytometric assay for ABCG2 detection, they noted a disparity in rhodamine 123 transport that led them to assess the DNA sequence in different cell lines (published in Honjo *et al*., 2001). Amino-acid variations at position 482 were found to underlie the altered cross-resistance patterns. Arginine at position 482 is considered to be the wild-type residue, found in parental cell lines and early drug selection, whereas later drug selection can result in replacement of this arginine by glycine, threonine or other residues. Allen *et al*. (2002a) investigated several murine cell lines from *Abcb1a/1b* (−/−) knockout mice during drug selection and found substitution of arginine-482 in ABCG2 by either serine or methionine, with similar functional consequences to those seen in human cell lines. As mutations are acquired during the course of selection, they represent an example of a gain-of-function mutation in ABC multidrug transporters that enables the mutant form to transport certain anticancer drugs, and hence, confer resistance on cells. Studies on ABCG2 expressed in insect cells also suggested that amino-acid replacements at position 482 induce major alterations in the apparent substrate specificity of the transporter (Ozvegy-Laczka *et al*., 2005). Similarly, a study by Janvilisri *et al*. (2005) on the R482 and G482 variants of ABCG2 expressed in *L. lactis* pointed to major changes in the transport of charged compounds. On the other hand, the transport of neutral molecules was found not to be different between the variants in *L. lactis* pointing to a lack of interaction between R482 and neutral substrates during transport, or to the interaction of these substrates with regions in ABCG2 not including R482. Consistent with this finding, recent work by Ejendal *et al*. (2006) and Pozza *et al*. (2006) suggested that the effect of R482 replacements on ABCG2-mediated transport is not mediated by changes in drug binding but that residue 482 might affect the ATPase reaction and/or transport cycle of ABCG2 for particular substrates. In addition to residue 482, other residues in ABCG2 have been implicated in drug specificity. For example, Miwa *et al*. (2003) suggested significant roles for transmembrane residues E446, R482, N557 and H630 in ABCG2, following generation of 32 mutant proteins with amino-acid substitutions in the MD. It is likely that, similar to ABCB1 and other multidrug transporters (Shilling *et al*., 2006), drug–protein interactions in ABCG2 will be organized in drug-binding sites or surfaces. Recently, equilibrium drug-binding experiments on ABCG2 point to the presence of at least two symmetric drug-binding sites per ABCG2 dimer (Clark *et al*., 2006). It is interesting to note that a similar conclusion was reached for equilibrium drug-binding to the bacterial, homodimeric ABCB1 homologue LmrA in *L. lactis* (Van Veen *et al*., 2000).

ABCG2 and clinical MDR

Although compelling data exist, indicating an important role for ABCB1 in determining efficacy of chemotherapy of tumours (Gottesman *et al*., 2002), information on the relevance of ABCG2 in clinical MDR is still limited (Leonard *et al*., 2003; Szakacs *et al*., 2006). Expression of ABCG2 in the relatively small number of tumour samples, examined to date, appears to be variable (Table 1). Expression of ABCG2 before drug selection may underlie some cases of innate tumour resistance, and this may have particular relevance for acute myeloid leukaemia (AML) treatment with ABCG2 substrates daunorubicin, MX and topotecan. For example, Ross *et al*. (2000) used quantitative reverse transcriptase-polymerase chain reaction to determine ABCG2 mRNA expression in blast cells taken from 20 AML patients and observed that although the levels of ABCG2 expression

Table 1 Results from several studies conducted since 2000 on the expression of ABCG2 in a variety of solid tumour and leukaemia samples

Samples tested	*Results*	*Method*	*Reference*
43 breast cancer tumours	Uniformly low levels	RT-PCR	Kanzaki *et al*. (2001)
52 breast cancer tumours	ABCG2 not detected	IHC	Faneyte *et al*. (2002)
150 variable solid tumours	Frequent expression in astrointestinal adenocarcinomas, endometrial and lung carcinoma and myeloma	IHC	Diestra *et al*. (2002)
20 AML samples	Variable expression but high in seven samples	RT-PCR	Ross *et al*. (2000)
20 AML samples	Variable expression	IHC	Sargent *et al*. (2001)
59 childhood AML samples	High levels detected at relapse	RT-PCR	Steinbach *et al*. (2002)
20 paired AML samples	Increased expression in relapsed disease	RT-PCR	Van Den Heuvel-Eibrink *et al*. (2002)
20 paired AML samples	No correlation with relapsed disease	IHC	Van Der Kolk *et al*. (2002)
40 AML samples	Low levels of expression	RT-PCR	Abbott *et al*. (2002)
67 childhood ALL samples	ABCG2 of no prognostic significance	RT-PCR	Sauerbrey *et al*. (2002)
21 AML samples	ABCG2 expression correlated with *in vitro* flavopiridol toxicity	RT-PCR	Nakanishi *et al*. (2003b)

Abbreviations: ALL, acute lymphoid leukaemia; AML, acute myeloid leukaemia; IHC, immunohistochemistry; RT-PCR, reverse transcriptase-polymerase chain reaction.

varied considerably, high expression was detected in about a third of the samples. In a subsequent study, Nakanishi *et al*. (2003b) observed a strong correlation between ABCG2 mRNA and blast cell viability in the presence of the cyclin-dependent kinase inhibitor flavopiridol. Raaijmakers *et al*. (2005) showed preferential expression of ABCG2 in both normal and leukaemic primitive CD34+38− haematopoietic cells. Hence, ABCG2-mediated resistance may lead to incomplete eradication of leukaemic cells and therefore contribute to relapse. This conclusion is consistent with observations by Steinbach *et al*. (2002) and Van Den Heuvel-Eibrink *et al*. (2002) that levels of ABCG2 mRNA were significantly increased in relapsed AML.

Physiological roles of ABCG2

Even though ABCG2 was initially characterized as a multidrug transporter in drug-selected tumour cells overexpressing the protein, evidence is increasing that the protein serves specific roles in normal cell physiology. Consideration of such roles is important not only in terms of the impact of ABCG2 on drug distribution and therefore efficacy or toxicity but also for potential side effects if ABCG2 is to be clinically modulated to reduce tumour resistance.

Cellular protection

Similar to its role in drug resistance in tumour cells, ABCG2 might function in the defense of normal cells against cytotoxic agents. For example, multiple studies have characterized the expression of ABCG2 in stem cells of both haematopoietic and non-haematopoietic origin (Zhou *et al*., 2001, 2002; Kim *et al*., 2002; Scharenberg *et al*., 2002). In these studies, ABCG2 has been shown to account for the 'side population (SP) phenotype', defined by the characteristically low level of accumulation of Hoechst 33342 (Zhou *et al*., 2001). SP cells are highly enriched in haematopoietic stem cells that are characterized as long-term culture initiating cells (Goodell *et al*., 1997; Storms *et al*., 2000). Mice with deletion of the *Abcg2* gene had normal haematopoiesis, marked by absence of the characteristic Hoechst-dim SP in bone marrow (Zhou *et al*., 2002). The enforced expression of the murine *Abcg2* gene was sufficient for SP phenotype, and there was a reduction in endogenous levels of Abcg2 expression during cell maturation. Abcg2 also protected haematopoietic stem cells against the toxicity of the chemotherapeutic agent MX (Zhou *et al*., 2002). These findings were interpreted as an indication that ABCG2 causes the efflux of a substance, which is important for differentiation of the stem cells. Alternatively, overexpressed ABCG2 may export a substance important for the growth or protection of committed lineages, given the need for potentially life-long survival of these cells (Krishnamurthy and Schuetz, 2006).

Role in cellular homeostatic mechanisms

The *Abcg2* (−/−) knockout mouse was generated by two independent groups (Jonker *et al*., 2002; Zhou *et al*., 2002) by disruption of exon 3 that encodes the essential Walker A motif in the NBD. Before specific challenge, these mice essentially had no major phenotypic aberrations, but both groups did observe elevated levels of erythrocyte protoporphyrin IX (PPIX). By investigation of Ter-119+ erythrocytes from various species, Zhou *et al*. (2005) found that Abcg2 expression was induced during erythrocyte maturation in parallel with the active biosynthesis of haem and haemoglobin. Evidence of direct transport of PPIX suggests that ABCG2 may be important for homeostasis of endogenous porphyrins, particularly when levels are elevated during environmental stress such as iron toxicity, and as such, Zhou *et al*. (2005) proposed that altered ABCG2 activity might exacerbate the consequences of genetic or drug-induced protoporphyrias.

Ifergan *et al*. (2004) identified a potential role for ABCG2 in folate homeostasis. Mammalian cells are unable to support *de novo* biosynthesis of folic acid, so uptake from an exogenous source is essential, and retention is aided by the addition of glutamate residues by the folylpoly-γ-glutamate synthase (FPGS) enzyme. These polyglutamate conjugates can, however, be exported directly by ABCG2 (Chen *et al*., 2003). Ifergan *et al*. (2004) found that gradual folate deprivation led to upregulation of FPGS activity but near-complete loss of ABCG2 expression, consistent with the need to increase cellular retention of folates. Furthermore, this group highlighted that tissues with high ABCG2 expression, such as the intestine, also exhibit increased FPGS activity to ensure sufficient intracellular retention of long-chain (>3 glutamate residues) folate polyglutamates (Turner *et al*., 1999), thus implying a dynamic balance between these proteins to maintain folate homeostasis. As noted by Ifergan *et al*. (2004), the antifolate action of MTX is often a first line approach in breast cancer treatment, but resistance can be acquired by reduced folate carrier-mediated uptake of drug and folates. Such selected cells would therefore experience folate deficiency and may downregulate ABCG2, leading to a higher sensitivity to other cytotoxic drugs.

Role in cellular hypoxic responses

The adaptive cellular response to hypoxia involves upregulation of glucose transporters, glycolytic enzymes and haem biosynthetic enzymes such as 5 aminolevulinate synthetase (ALA-S). Krishnamurthy *et al*. (2004) used stem cells from *Abcg2* (−/−) mice and their (+/+) littermates to show that Abcg2 expression conferred a survival advantage during hypoxia, which was sensitive to the ABCG2 inhibitor reserpine, and additionally, that hypoxia upregulated Abcg2 expression, presumably via the identified hypoxia response element in the 5′ region of the *Abcg2* gene. As upregulation of ALA-S increases cell propensity to accumulate haem, which can become toxic because of mitochondrial dysfunction and elevation of iron and reactive oxygen species, ABCG2 might promote cell survival by direct export of the toxic porphyrins. Similar upregulation of ABCG2 in the hypoxic environment of solid tumours may increase innate drug resistance.

Role in regulating access to body compartments

In normal tissues, high expression of ABCG2 is found in stem cells (Zhou *et al*., 2001), epithelial cells of small and large intestines, ducts and lobules of the breast, endothelial cells of veins and capillaries (Maliepaard *et al*., 2001; Cooray *et al*., 2002) and synchitiotrophoblastic cells in the placenta (Litman *et al*., 2002). The localization of ABCG2 suggests that it could have a potential role in protection against toxins as the protein is ideally located to regulate the access of substances to specific body compartments (Leslie *et al*., 2005; Mao and Unadkat, 2005; Fetsch *et al*., 2006). This is supported by the upregulation of ABCG2 expression in apical mammary ductal epithelia during late pregnancy and lactation (Jonker *et al*., 2005; Van Herwaarden and Schinkel, 2006). It is also supported by studies on ABCG2 in placenta. The placenta is a site of oestrogen production, and the apical surface of the chorionic villus is predicted to experience high concentrations of oestrogens of up to 150 nM (Ee *et al*., 2004b). ABCG2 expression might augment foetal protection from these high concentrations by directly transporting oestrogens to the maternal circulation. Abcg2-associated, foetal protection from xenobiotics has also been demonstrated by Jonker *et al*. (2000). Administration of intravenous [^{14}C] topotecan to pregnant *Abcb1a/1b* (−/−) rats resulted in 3.2-fold higher foetal plasma levels of topotecan when rats received pre-treatment with ABCG2 inhibitor GF120918. Further work found a twofold higher ratio of foetal to maternal topotecan concentration in homozygous *Abcg2* (−/−) foetuses compared with wild types, and an intermediate level of accumulation in heterozygotes.

Cooray *et al*. (2002) observed the localization of ABCG2 in the human brain microvessel endothelium, and proposed a role for ABCG2 at the blood brain barrier. These authors suggested that as certain clinically administered corticosteroids appeared to be ABCG2 substrates, ABCG2 might limit the extent of brain penetration and suppression of the hypothalamic pituitary axis that is a common side effect of prolonged corticosteroid treatment. Conversely, drugs such as antiepileptics or chemotherapeutics for glioblastoma do require brain penetration for action. Breedveld *et al*. (2005) studied the potential involvement of Abcg2 in limiting the distribution of the anticancer drug imatinib mesylate. *In vitro* studies showed imatanib mesylate was directly transported by MDCK-II-Bcrp1 cells, and *in vivo* studies confirmed that *Abcg2* (−/−) mice had a 2.5-fold increase in brain penetration 2 h subsequent to intravenously administered imatanib mesylate.

Role in regulating systemic access

Intestinal expression of ABCG2 can potentially regulate the substrate uptake from the gut lumen by back-transport of absorbed substances. This has clinical relevance to the oral bioavailability of drugs. Despite the convenience of oral administration in chemotherapy, it is often precluded by narrow therapeutic drug indices that will not tolerate the highly variable systemic exposure that can result from inter-patient variation in bioavailability. As discussed later, there is much interest in potential ABCG2 modulating agents that may be co-administered with chemotherapeutic drugs to improve pharmacokinetic profiles. For example, Jonker *et al*. (2000) found that exposure of *Abcba/1b* (−/−) mice to the ABCB1 inhibitor GF120918 resulted in a sixfold increase in the area under the curve following oral topotecan administration. This observation was attributed to increased intestinal uptake and reduced hepatobiliary secretion. ABCG2 may also be important for limiting uptake of dietary toxins. For example, benzo(a)pyrene is a highly potent carcinogenic polycyclic aromatic hydrocarbon. Ebert *et al*. (2005) demonstrated direct transport of benzo(a)pyrene metabolites by ABCG2. Aryl hydrocarbon receptor (AhR) agonists, such as polycyclic aromatic hydrocarbon compounds, also upregulated ABCG2 expression with positive correlation to AhR agonist potency; upregulation was inhibited by AhR antagonist PD98059. These changes in the expression level of ABCG2 might reflect a protective adaptation, but does imply that diet can have unexpected effects on the outcome of administered drug therapy. Additionally, Jonker *et al*. (2002) discovered that *Abcg2* (−/−) mice were susceptible to severe phototoxicity associated with a 100-fold increased sensitivity to pheophorbide-a, the porphyrin catabolite of chlorophyll. As *in vitro* experiments showed Abcg2-expressing cells to have a reduced accumulation of pheophorbide-a, which was reversed by the ABCG2 inhibitor Ko143, it was concluded that ABCG2 reduced the bioavailability of dietary pheophorbide-a through increased efflux, back into the gut lumen.

Renal and hepatic expression of ABCG2 may reflect physiological secretory function. Consistent with this notion, Janvilisri *et al*. (2005) demonstrated ABCG2-mediated transport of hepatic primary bile acids such as cholate and deoxycholate in *L. lactis*. However, renal and hepatobiliary excretion can also impact on drug pharmacokinetics. Mizuno *et al*. (2004) found urinary excretion of benzothiazole sulphates was 2.4-fold lower in *Abcg2* (−/−) mice when compared with wild-type animals. Unexpected drug toxicity can arise through pharmacokinetic interactions with other drugs or endogenous substances, and this is well documented for MTX. Reduced clearance has been reported with co-administration of non-steroidal anti-inflammatory drugs (Kremer and Hamilton, 1995) and omeprazole (Reid *et al*., 1993). Breedveld *et al*. (2004) demonstrated competitive interactions between benzimidazoles and MTX for transport by ABCG2. Clinically relevant concentrations up to 10 μM pantoprazole or omeprazole resulted in 46 and 25% inhibition of 1 μM MTX transport. *In vivo* studies showed that MTX clearance was reduced 1.8-fold by co-administration of pantoprazole. Theoretically, this may be used advantageously, as suggested by Breedveld *et al*. (2004); MTX bioavailability can be as low as 20% with doses in excess of 80 mg/m^2, and given that pantoprazole is already clinically administered in high doses for peptic ulcer treatment, co-administration may reduce some inter-patient variation in systemic MTX exposure by increasing its bioavailability.

Clinically important single-nucleotide polymorphisms in the *ABCG2* gene

Fundamental genotypic variation may underlie a degree of susceptibility to toxicity. Although the gene sequence of

ABCG2 is highly conserved, to date more than 40 non-synonymous and synonymous single-nucleotide polymorphisms (SNPs) have been revealed in promoter and both in exon and intron sequences (Staud and Pavek, 2005; Yanese *et al*., 2005). Imai *et al*. (2002a) sequenced cDNA from 11 human tumours and identified SNPs G34A (V12M) and C421A (Q141K), a splice variant 944–949 deletion (A315-T316), and later, an additional C376T (G126Stop) polymorphism. The same group studied C421A allelic frequency in the normal Japanese population and found that 46% were carriers and 7% were homozygous, but that the frequency of variant alleles differed significantly between different ethnic populations. For example, 1% in sub-Saharan Africans, 34% in the Han-Chinese, whereas Caucasians averaged around 10%. Imai *et al*. (2002a) and a later study by Kondo *et al*. (2004) both found that cells expressing the C421A polymorphism had reduced ABCG2 expression levels when compared with wild type, and were 2–3 times more sensitive to the anticancer chemotherapeutic compounds SN-38 and MX. Although this polymorphism does occur in the functionally important ATP binding region, and Mizuarai *et al*. (2004) subsequently reported 1.3-fold reduced ATPase activity, Imai *et al*. (2002a) attributed the increased drug sensitivity to a reduced expression level of ABCG2. They suggested that the glutamine to lysine substitution at position 141 might have produced a different tertiary structure that was more susceptible to degradation. Sparreboom *et al*. (2004) conducted a phase 1 study on the impact of the C421 allele, and reported consequential variations in diflomotecan pharmacokinetics. Genotype may therefore influence chemotherapeutic outcome in the absence of epigenetic factors. In a recent study by Kobayashi *et al*. (2005), it was observed that the expression level of ABCG2 in placenta was significantly lower in homozygotes for the A421 allele than those for the C421 allele, and heterozygotes had an intermediate level. *Cis*-acting SNPs have been reported to be a causative factor for mRNA expression imbalance. If the *cis*-acting SNPs alter the expression of the gene transcript from the chromosome carrying it, these SNPs may cause an imbalance of expression between the paternal and maternal allele. Subsequent analysis of the effects of C421A allele on ABCG2 expression levels in placenta suggested that the predominant expression pattern for ABCG2 is biallelic, and that C421A is not a *cis*-acting SNP but might affect translation efficiency (Kobayashi *et al*., 2005). A potentially larger clinical impact is expected from the C376T polymorphism, as this introduces a stop codon instead of glycine 126, and thus no active ABCG2 will be expressed from this allele.

Modulation of ABCG2 activity

Inhibitors of ABCG2 hold potential not only as chemosensitizers and for improving pharmacokinetics by co-administration, but they are also much needed tools for functional analyses of ABCG2. In some situations, a combined inhibition of more than one transporter may be of therapeutic benefit, but alternatively a more selective inhibition may be required. ABCB1 inhibitors remain the most extensively studied, but Table 2 shows substances that can modulate ABCG2 activity. Several of these are promising for further development and clinical use.

Table 2 Some compounds that modulate ABCG2 activity[a]

Endogenous modulators	*Dietary modulators*	*Exogenous modulators*
Oestrone	Apigenin	FTC
17 β-Oestradiol	Biochanin A	Ko143, Ko132 and Ko134
	Chrysin	GF120918
	Genistein	Novobiocin
	Hesperetin	Tamoxifen
	Kaempferol	Toremifene
	Naringenin	TAG 139, TAG 11
	Silymarin	Gefitinib
	Folate	Imatinib mesylate
		Flavopiridol

Abbreviation: FTC, fumitremorgin C.
[a]See main text for further details.

Existing modulators

GF120918 is an acridine carboxamide derivative, first identified as a third generation ABCB1 inhibitor. In 1999, De Bruin *et al*. showed that 1 μM GF120918 could increase MX sensitivity of S1-M1-80 cells by 1850-fold. Following the preclinical murine studies of Jonker *et al*. (2000), Kruijtzer *et al*. (2002) showed that a single 1000 mg dose of GF120918 to human patients receiving 1.0 mg/m^2 oral topotecan increased apparent bioavailability from 40 to 97.1%, and inter-patient variability was reduced from 17 to 11% relative to controls. Rabindran *et al*. (1998, 2000) were the first to identify the mycotoxin fumitremorgin C (FTC) as a potent agent to reverse the MX, doxorubicin and topotecan resistance of ABCG2 overexpressing cell lines. Effects were ABCG2 specific and might result from competitive inhibition because of a common planar multiring structure. Clinical use is, however, precluded by neurotoxicity, and thus, Van Loevezijn *et al*. (1998) screened a combinatorial panel of 42 FTC analogues, and identified Ko132 and Ko134 for further study. Allen *et al*. (2002b) performed studies on Ko143, which appeared to have twice the potency of GF120918. Cytotoxicity experiments suggested that the IC_{50} (concentration inhibiting cell proliferation by 50%) of these compounds was 50–1000 times greater than their EC_{90} (effective concentration reversing 90% of drug resistance), and no toxicity was evident in mice receiving oral doses as high as 50 mg/kg. Several currently prescribed drugs can influence ABCG2 function in addition to their therapeutic applications. Examples include kinase inhibitors such as gefitinib. Yanese *et al*. (2004) showed that *in vivo* gefitinib administration in multiple tumour models increased the antitumour activity of irinotecan, a topoisomerase I inhibitor, which is activated by hydrolysis to SN-38. Stewart *et al*. (2004) demonstrated increased bioavailability of irinotecan in the presence of gefitinib. These effects of gefitinib are likely due to alterations in ABCG2 activity, as Nakamura *et al*. (2005) obtained evidence for a direct inhibition of the ABCG2-mediated transport of topotecan by gefitinib. Hence,

the combination of gefitinib and topoisomerase I inhibitors could be clinically effective in cancers expressing ABCG2.

Potential use of polyphenols

Plant polyphenols including flavonoids, stilbenes, phenolic acids and lignans are present in fruits, vegetables, tea and red wine, and are increasingly consumed as dietary supplements because of the proposed health benefits such as anti-oxidant, anticarcinogenic and anti-inflammatory properties (Havsteen, 2002). Cooray *et al*. (2004) investigated ABCG2 interactions with a range of polyphenols, and found that all compounds tested increased accumulation of MX and bodipy-FL-prazosin in ABCG2-expressing MCF-7/MR and K562/ABCG2 cell lines. Similarly, all compounds stimulated ABCG2-associated ATPase activity in *L. lactis*. Hesperetin was the most potent compound, with 30 μM concentrations being similar to the effects produced with 10 μM Ko143. The authors highlighted the potential significance of direct transport by ABCG2, not only in terms of chemosensitization prospects but also because these compounds are thought to have important neuroprotective effects; brain penetration may be limited if ABCG2 proves significant in blood brain barrier function. In pursuit of potential ABCG2 modulators, Zhang *et al*. (2004) evaluated 20 naturally occurring flavonoids with ABCG2 overexpressing MCF-7/MX100 cells. Apigenin, biochanin A, chrysin, genistein, hesperetin, kaempferol, naringenin and silymarin were found to be the most potent in MX accumulation experiments, and chrysin and biochanin A showed significant inhibition of ABCG2 activity at 0.5 or 1.0 μM concentrations. Zhang *et al*. (2005) studied structure–activity relationships for flavonoid-mediated inhibition of ABCG2, and identified specific structural properties important for potent flavonoid–ABCG2 interaction. These structural requirements are similar but not identical to those yielding potent flavonoid–NBD interactions in ABCB1 (Di Pietro *et al*., 2002) and ABCC1 (Leslie *et al*., 2001) indicating that inhibition of ABCG2 by flavonoids involves, in part, the binding of flavonoids to the NBD of the protein. In view of these findings, it might be possible to generate ABCG2-specific derivatives of flavonoids with low toxicity. However, the modulation of ABCG2 by low concentrations of flavonoids also implies that diet might increasingly influence drug or toxin bioavailability.

New opportunities for steroid-based compounds?

ABCG2 is known to interact with a variety of steroids and steroid drugs. Following reports by Imai *et al*. (2003) and Suzuki *et al*. (2003) on the ABCG2-mediated transport of sulphated oestrogens, Janvilisri *et al*. (2003) found ABCG2-mediated transport of free oestrogens using the bacterial model *L. lactis*, which is devoid of mammalian steroids and might therefore be more sensitive to detect additional interactions compared to mammalian cells. Investigations on phyto-oestrogens by Imai *et al*. (2004) showed that 3 μM genistein or naringenin was effective in reversal of MX resistance, and that even at 10 μM, inhibition was specific to ABCG2 rather than ABCB1 or ABCC1. Recently, ABCG2 was also shown to interact with other phyto-steroids, such as ginsenosides (Jin *et al*., 2006). In addition, Pavek *et al*. (2005) and Cooray *et al*. (2006) observed inhibition of ABCG2-mediated drug transport by glucocorticoid drugs, such as beclomethasone, 6α-methylprednisolone, dexamethasone, mometasone and triamcinolone.

A body of evidence suggests that steroids also regulate the expression of ABCG2. In initial studies, several anticancer drugs including doxorubicin (Dobbs *et al*., 1995) and topotecan (Gallo *et al*., 2000) were reported to display sex-dependent pharmacokinetics in human patients. Tanaka *et al*. (2005) investigated sex-dependent tissue distribution of Abcg2 in the rat and mouse. In rats, male kidneys showed higher expression levels than females, and through a combination of experiments involving castration, hypophysectomy, ovariectomy or hormone replacements, this was attributed to a suppressive effect of female sex hormones. In mice, the male liver showed highest expression because of inductive effects of male sex hormones. Merino *et al*. (2005) then investigated the pharmacokinetic consequences of these observations. Whereas female wild-type mice had a twofold higher area under the curve following oral administration of the ABCG2 substrate nitrofurantoin because of the lower Abcg2 expression levels in the kidney and liver, no sex differences were seen between nitrofurantoin levels in *Abcg2* (−/−) mice. The higher expression in the male liver was correlated with increased biliary excretion, and sex differences only became apparent at 5 weeks of age, corresponding to the murine puberty. This may be directly relevant to human medicine, as investigations of human livers also found male samples to have a consistently higher level of ABCG2 expression. Ee *et al*. (2004b) were the first group to identify an oestrogen response element in the 5′ flanking region of the *ABCG2*. This followed the observation in T47D-A18 breast cells that physiological (nanomolar) concentrations of 17β-oestradiol could induce ABCG2 mRNA at the transcriptional level, but that this was blocked by addition of the antioestrogen, ICI 182780. The induction of ABCG2 expression may be a physiological adaptation to augment foetal protection from the high oestrogen levels at the placenta, but equally a reservoir of oestrogens within a tumour could induce expression and increase innate resistance of cells, so antioestrogen therapy might prove beneficial. This is countered, however, as Imai *et al*. (2005) found a dose-dependent downregulation of ABCG2 levels with physiological concentrations of oestrone, 17β-oestradiol or diethylstilbestrol in T-47D and MCF-7 breast cancer cells. These effects were dependent on the expression of oestrogen receptor (ER) α, as ERα knockdown by small interference RNA (siRNA) or the addition of the ERα antagonist tamoxifen prevented downregulation of ABCG2 expression. The results from pulse-chase labelling experiments in this study suggest that decreased protein biosynthesis and maturation, but not alterations in protein turnover, might underlie the steroid-mediated ABCG2 downregulation. The downregulation of ABCG2 expression by 17β-oestradiol in an ER-dependent manner was also observed by Wang *et al*. (2005), who used human placental BeWo cells. Interestingly, progesterone stimulated ABCG2 expression, and this effect was enhanced by 17β-oestradiol

presumably via a non-classical progesterone receptor (PR)-mediated and/or 17β-oestradiol-mediated synthesis of PRβ.

Steroids have an interesting potential as modulators of ABCG2 by directly acting on protein activity. Following a report by Imai *et al*. (2002b) that 10 μM oestrone was sufficient for 3.6- and 7.5-fold increase in toxicity of SN-38 and MX in ABCG2-transduced K562 cells, respectively, Sugimoto *et al*. (2003) showed increased topotecan accumulation in these cells with diethylstilbestrol, oestrone, toremifene and tamoxifen. A chemical library of tamoxifen derivatives was screened by Sugimoto *et al*. (2003) in a search for modulators of ABCG2 activity, and the assessment of SN-38 cytotoxicity with the lead compounds revealed the tamoxifen derivative TAG 139 to be five times more active than oestrone, closely followed by tamoxifen derivative TAG 11. Further evaluation found that these new derivatives did not have antioestrogenic effects; it may therefore be possible in rational drug design to develop compounds without additional biological effects. Furthermore, as TAG 139 also showed enhancement of doxorubicin and vincristine cytotoxicity in K562/ABCG2 cells, this may be a lead compound for development of multi-action third generation modulators of ABCG2. As tamoxifen and MTX are already widely used in breast cancer treatments, it may be worthwhile considering a co-administration strategy, but similarly, the use of antioestrogen therapies could have unintentional side effects on co-administered drugs due to pharmacokinetic interactions.

Additional ways to modulate of ABCG2 expression

Hammerhead ribozymes are oligonucleotides that possess an intrinsic enzymatic endoribonucleolytic activity. They reduce expression of target genes by specific cleavage of mRNA at a defined position in trans, provided that the target has a consensus NUX motif (N is any nucleotide, and X is adenosine, cytosine or uracil). Kowalski *et al*. (2002) developed RzB1, a highly active ribozyme directed against ABCG2 mRNA. RzB1 could reverse the MX resistance phenotype of an ABCG2-overexpressing cell line by over 80%, with coincident reduction in ABCG2 expression. In contrast, a ribonucleolytic-inactive control ribozyme did not show any alteration in ABCG2 mRNA or protein expression when compared with the drug-resistant cell line, indicating that no unspecific side effects occurred because of transfection procedures. Hence, this methodology might be a potential gene therapeutic approach to ABCG2-dependent MDR, especially if the ribozyme can be introduced via an adenoviral-based construct and made tumour cell specific. This could then leave healthy cells with a relatively increased resistance to toxicity. In an alternative strategy, antisense oligonucleotides (ASOs) could be used to block expression of specific genes by forming a DNA–mRNA heteroduplex. There may be a steric hindrance to prevent normal association with the ribosome for protein synthesis, or alternatively, recruitment of RNAse H can cleave the mRNA while leaving the ASO intact for further cycles. Potential disadvantages include nonspecific hybridization, and requirements for relatively high concentrations of ASOs that must then be maintained for a sufficient length of time to allow protein degradation. A review by Fojo and Bates (2003) noted the use of phosphorothioates in the synthesis of ASOs, as this circumvents the easy degradation by circulating DNAse. A very similar concept involves chemical synthesis of siRNA, but Li *et al*. (2005) have also shown successful expression of siRNA in mammalian cells from plasmid DNA under the control of an RNA polymerase III promoter. Ee *et al*. (2004a) synthesized siRNA molecules using T7 RNA polymerase and showed that the transfection of these siRNAs into the cell markedly downregulated both exogenous and endogenous expression of ABCG2. As a functional consequence, knockdown of ABCG2 by siRNAs increased the sensitivity of human choriocarcinoma BeWo cells to MX and topotecan by 10.5- and 8.2-fold, respectively. These studies indicate that siRNAs can modulate MDR *in vitro* and that they might present a new approach to overcome ABC transporter-mediated drug resistance.

Future perspectives

Increasing knowledge of physiological roles of ABCG2 and structure–function relationships in the protein has highlighted potential areas where intervention to modulate the protein's action may be harnessed to clinical benefit. Previous sections have presented evidence for the contribution of ABCG2 to MDR and the pharmacokinetic behaviour of drugs, and these remain the key areas of interest where modulation of ABCG2 is hoped to improve the drug efficacy:toxicity ratio. This section will discuss some of the areas that have been preliminarily investigated, along with some suggestions that may warrant attention in the future. In all cases where modulators will be used to interfere with ABCG2 activity, there is a caveat regarding potential side effects due to action on endogenous ABCG2 in healthy tissues, or potential resistance arising to the chemosensitizing agent. For example, co-administration of topotecan and the ABCG2 inhibitor GF120918 has been tested in patients, with promising results in terms of pharmacokinetics, but unanticipated toxicity such as dietary photosensitivity (Kruijtzer *et al*., 2002). One way in which the drug efficacy:toxicity ratio may be improved is by prior knowledge of the patient's ABCG2 status. A functional assay could indicate the level of ABCG2-mediated resistance within individual tumours before decisions on chemotherapeutic strategy. Alternatively, the susceptibility of healthy cell to drug toxicity may be predicted with genotypic analysis for polymorphisms such as the C421A allele that reduces endogenous *ABCG2* expression. Various strategies have *in vitro* proof of principle, but may only have clinical viability if they can be specifically targeted *in vivo* to given sub-populations of cells such as tumours. Potentially this may be achieved in several ways. Small liposomes with a diameter of less than 100 nm might be able to passively extravasate in tumour tissues to selectively deliver a drug and reduce exposure of susceptible healthy tissues. Krishna and Mayer (2000) reviewed this strategy in terms of reversal of ABCB1-mediated resistance, but the concepts could similarly be applied to delivery of ABCG2 modulators. Han and Amidon (2000) suggested several ways to target enzymes to

particular sites in order to activate prodrugs. These ideas could be adapted to potential strategies for ABCG2 modulation in terms of delivery of the chemotherapeutic agent or prodrug form of an ABCG2 inhibitor, such that healthy tissues retain native ABCG2 activity. Activating enzymes could be linked to monoclonal antibodies that are generated to tumour-selective surface markers, so that the systemic prodrug will only be activated at tumour sites. Alternatively, intracellular activation might be achieved following introduction of the enzyme using viral vector containing a tumour-selective promoter. Finally, given the fairly widespread distribution and manifold functions of ABCG2, the effects of systemic inhibitors are potentially wide-ranging and unpredictable. As such, a safer approach might be the development of drugs that circumvent the activity of ABCG2. Nakagawa *et al*. (2005) provided an example of how structure–activity relationships can be used to model drugs. Study of the molecular structure of active camptothecin analogues identified the lactone E ring as being a prerequisite for antitumour activity, but the A or B rings could be modified without detriment. ABCG2 substrates were found to have a planar structure with conjugated π-orbitals and hydroxyl or amino groups at position 10 of the A ring; modification at these regions may retain anticancer activity and avoid ABCG2 interaction. Further knowledge of the structure of ABCG2 and its mechanism of transport may therefore be harnessed in design of new chemotherapeutics, modification of existing drugs or the development of novel inhibitors with increased ABCG2 specificity.

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Conflict of interest

The authors state no conflict of interest.

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