MINI-REVIEW

Molecular Pharmacology of ABCG2 and its Role in Chemoresistance

Alexandra E. Stacy, Patric J. Jansson, Des R. Richardson

Molecular Pharmacology and Pathology Program, Department of Pathology and Bosch Institute, University of Sydney, Sydney, NSW 2006, Australia (A. E. S., P. J. J., D. R. R)
Running title: ABCG2 in Chemoresistance

Authors for correspondence:

Patric J. Jansson, Phone:+61-2-9036-67120; Fax:+61-2-9351-3429; Email: patric.jansson@sydney.edu.au;

Des R. Richardson, Phone:+61-2-9036-6548; Fax:+61-2-9036-6549; Email: d.richardson@sydney.edu.au

Text pages: 47

Tables: 1

Figures: 9

References: 168

Word count:

Abstract: 218

Article: 7,328

Abbreviations:

ABC – ATP-binding cassette
ABCG2 – ABC transporter, sub-family G, isoform 2
Akt – γ-akt murine thymoma viral oncogene homolog
ALL – acute lymphocytic leukemia
AML – acute myeloid leukemia
BBB – blood brain barrier
BCRP – breast cancer resistance protein
ER – endoplasmic reticulum
HIF-1α – hypoxia inducible factor 1α
MDR – multidrug resistance
MRP1 – multidrug resistance protein 1
NFκB – nuclear factor light chain enhancer of activated B cells
Pgp – P glycoprotein
PI3K – Phosphatidylinositol 3 kinase
QSAR – quantitative structure activity relationships
SAR – structure activity relationships
SNP – small nucleotide polymorphisms
Abstract
The ATP-binding cassette, sub-family G, isoform 2 protein (ABCG2) is an important member of the ABC transporter superfamily which has been suggested to be involved in multidrug resistance (MDR) in cancer. Its diverse range of substrates includes many common chemotherapeutics such as imatinib, doxorubicin and mitoxantrone. Physiologically, ABCG2 is highly expressed in areas such as the blood brain barrier and gastrointestinal tract, where it is thought to play a role in protection against xenobiotic exposure. High ABCG2 expression has also been found in a variety of solid tumors and in hematologic malignancies, and has been correlated with poorer clinical outcomes. Furthermore, ABCG2 expression is a characteristic feature of cancer stem cells which are able to self-renew and differentiate. These cancer stem cells have been postulated to play an important role in MDR, where their inherent ABCG2 expression may allow them to survive chemotherapy and re-populate the tumor after exposure to chemotherapeutics. This observation raises the exciting possibility that by inhibiting ABCG2, cancer stem cells and other cancers may be targeted and eradicated, at which point conventional chemotherapeutics would be sufficient to eliminate the remaining tumor cells. Inhibitors of ABCG2 such as tyrosine kinase inhibitors, phosphodiesterase-5 inhibitors and the fumitremorgin-type indolyl diketopiperazine, Ko143, could potentially be used for this purpose. However, these agents are still awaiting comprehensive clinical assessment.
1. Introduction- The ABC Transporters

The successful treatment of cancer can be impeded by the concurrent development of resistance to multiple chemotherapeutics which are both structurally and mechanistically unrelated (Robey et al., 2009). This multi-drug resistance (MDR) phenotype can be associated with an increase in the expression of ATP-binding cassette (ABC) transporters, which efflux substrates from the cell in an energy-dependent manner, driven by ATP hydrolysis (Robey et al., 2009). Of the 48 ABC transporters currently recognized, there are three which are most commonly associated with MDR: P-glycoprotein (Pgp; also known as MDR1 or ABCB1), MDR associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) or ABC sub-family G, isoform 2 protein (ABCG2; (Robey et al., 2009).

It is well known that Pgp can transport a range of hydrophobic chemotherapeutics such as anthracyclines, taxanes, etoposide and mitoxantrone (Gottesman et al., 2002; Juliano and Ling, 1976), and was originally thought to be solely responsible for the MDR phenotype (Gottesman et al., 2002; Juliano and Ling, 1976). However, it soon became clear that other transporters could also confer resistance to chemotherapeutics. The gene encoding MRP1 (also known as ABCC1) was cloned by Cole et al in 1992, and similarly to Pgp, its over-expression conferred resistance to a range of chemotherapeutics (Cole et al., 1992). The discovery that Pgp and MRP1 alone could not account for the efflux of chemotherapeutic drugs from a subset of leukemic cells subsequently lead to the identification of BCRP from the breast cancer cell line, MCF-7/AdrVp (Doyle et al., 1998). The Human Genome Organization Nomenclature Committee has since formally designated BCRP as ABCG2, as it belongs to the ABC transporter superfamily and is the second member of the G sub-family.
While their substrate specificities differ, there is considerable overlap between ABCG2, Pgp and MRP1 in terms of their ability to transport many structural classes of agents (Fig. 1). This raises the question of the physiological rationale for this apparent redundancy. All three transporters appear to be involved in protecting the body from toxicity associated with xenobiotic exposure, and the answer may lie in where the transporter is expressed in a cell or organ. In the case of the gastrointestinal tract, Pgp and ABCG2 are apically localized (Maliepaard et al., 2001a; Thiebaut et al., 1987), while MRP1 is found at the basolateral surface of crypt cells (Peng et al., 1999). A potential explanation for this differential expression is that in the gut, Pgp/ABCG2 may function to modulate the absorption of xenobiotics, while MRP1 may protect these highly proliferating cells from xenobiotic damage (Leslie et al., 2005).

2. Chromosomal Localization and Regulation of Expression

The human ABCG2 gene is located on band 4q22, between the markers D4S2462 and D4S1557 (Knutsen et al., 2000). To date, no other ABC transporter has been assigned to chromosome 4. Cytogenetic studies using the ABCG2 over-expressing cell lines, MCF-7/AdrVp 3000 and MCF-7/MX, showed gene amplification exclusively between 4q21-4q22, suggesting that ABCG2 homo-dimerizes to create an active transporter (Knutsen et al., 2000). The ABCG2 gene consists of 16 exons and 15 introns, spanning 66 kb (Bailey-Dell et al., 2001). The 5’ untranslated region is found mainly in exon 1, while the translational start site is found in the second exon (Bailey-Dell et al., 2001). The Walker A and B motifs which are crucial for nucleotide binding have been identified in exon 3 and 6, respectively, while the ABC motif (ALSGGQ) is found in exon 6 (Bailey-Dell et al., 2001).
Like other ABC transporters, the promoter region of \textit{ABCG2} (Fig. 2) is located downstream of a putative GpC island, is TATA-less and contains several putative Sp1, AP1 and AP2 binding sites, a common feature of promoters lacking a TATA box (Bailey-Dell et al., 2001). Additionally, several transcription factors and their \textit{cis} and \textit{trans} response elements have been shown to regulate ABCG2 expression (Ee et al., 2004; Krishnamurthy et al., 2004; Pradhan et al., 2010; Wang et al., 2008). The presence of a hypoxia response element may contribute to MDR in some tumors, as HIF-1\textalpha is highly active within the hypoxic interior of solid tumors and thus can increase the expression of ABCG2 \textit{via} the hypoxia response element (Krishnamurthy et al., 2004). Likewise, the hormones progesterone and estrogen have response elements located within the \textit{ABCG2} promoter, and have been shown to increase ABCG2 expression (Ee et al., 2004; Pradhan et al., 2010; Wang et al., 2008). The estrogen response element is located adjacent to a NFkB response element, and binding of the estrogen receptor and p65 (the active NFkB subunit) at these response elements leads to a pronounced increase in \textit{ABCG2} mRNA expression (Pradhan et al., 2010). The ability of hormones to regulate ABCG2 expression may be important during pregnancy, as high ABCG2 expression is found in the placenta where ABCG2 protects the fetus from exposure to harmful xenotoxins (Jonker et al., 2000).

3. Single Nucleotide Polymorphisms

Screening for single-nucleotide polymorphisms (SNPs) in ethnically diverse subjects has identified over 80 synonymous and non-synonymous SNPs in the \textit{ABCG2} gene to date (Sharom, 2008). The two most frequent polymorphisms identified were the G34A (resulting in V12M) and C421A (resulting in a Q141K substitution) transitions (Fig. 3), found in 18\% and 35.5\% of the studied population, respectively (Kobayashi et al., 2005).
The functional impact of these SNPs on drug transport remains contentious as the results are not consistent. No significant change in irinotecan pharmacokinetics was observed in relation to the ABCG2 C421A genotype in American Caucasians, African Americans, Africans, Han Chinese and European Caucasian patients treated with irinotecan (de Jong et al., 2004). This was not corroborated by Morisaki et al. (2005) who found that cells with the Q141K variant of ABCG2 had IC\textsubscript{50} values 1.2- to 5-fold lower than cells transfected with wild-type (Arg482) ABCG2, suggesting that the Q141K SNP affects drug transport (Morisaki et al., 2005). Recently, studies aimed at evaluating the clinical relevance of the Q141K SNP in patients undergoing chemotherapy have also been reported. In these studies, elevated plasma concentrations of gefitinib (Li et al., 2007) and diflomotecan (Sparreboom et al., 2004) and increased bioavailability of oral topotecan (Sparreboom et al., 2005) were found. The Q141 mutation has also been associated with increased adverse effects in response to gefitinib treatment (Cusatis et al., 2006). Additionally, the Q141K mutation has been demonstrated to decrease ATPase activity by 1.3-fold compared to wild-type ABCG2 (Mizuarai et al., 2003). Furthermore, kinetic analysis of ATPase activity showed that the \(K_m\) value in Q141K cells was 1.4-fold higher than that of wild-type ABCG2 (Mizuarai et al., 2003). These results suggest that this SNP may result in altered transport functions of ABCG2 transporter which has important implications for the pharmacokinetics and drug-resistance profiles of chemotherapeutics.

4. Structure

The 655 amino acid ABCG2 protein is in a reversed configuration to most other ABC transporters, as the ATP-binding domain is at the N-terminal and the 6 putative transmembrane domains are at the C-terminal (Polgar et al., 2008) (Fig. 3). The large extracellular loop between transmembrane domains 5 and 6 contains the only proven \(N\)-linked
glycosylation site, Asp596 (Diop and Hrycyna, 2005). Additionally, ABCG2 is unusual amongst ABC transporters in that it is a “half transporter” (Doyle et al., 1998). In fact, while most ABC transporters contain two repeated halves, each containing a membrane-spanning domain and a nucleotide-binding domain, ABCG2 contains only one membrane-spanning domain and one nucleotide-binding domain (Allikmets et al., 1998). As it is widely accepted that functional ABC transporters require two transmembrane domains and two nucleotide-binding domains to form a central substrate translocation pathway, it is believed that ABCG2 must at least dimerize to become functional (Ni et al., 2010a). Similar to other related members of the ABC family which are known heterodimers (Hillebrand et al., 2007), ABCG2 probably dimerizes in the endoplasmic reticulum (ER) (Graf et al., 2003). Then ABCG2 may exit through the ER to the Golgi apparatus for post-translational processing before it is trafficked to the apical membrane of the cell (Graf et al., 2003).

4.1 Homo-Dimer or Higher Order Homo-Oligomer?

High resolution structural analysis of bacterial ABCG2 has shown that nucleotides interact with elements of two separate nucleotide-binding domains when bound, suggesting that ABCG2 is a homo-dimer (Reyes and Chang, 2005). Furthermore, under conditions of reducing SDS-PAGE, ABCG2 migrates as a 70 kDa band, but in the absence of reducing agents it migrates as a 140 kDa complex, which implies that it is a homo-dimer linked by inter-molecular disulfide bridges (Kage et al., 2001). This is supported by studies using chemical cross-linking agents which theoretically link the monomers and cause a shift in molecular mass from 72 to 180 kDa (Litman et al., 2002).

While it is apparent that ABCG2 could be capable of forming homo-dimers, it has been shown in several studies that it can also form higher oligomeric states, including
tetramers to even a dodecamer (Xie et al., 2008; Xu et al., 2007). Additionally, electron microscopy has shown an octameric complex organized as a tetramer of dimers in the mutant R482G isoform of ABCG2 (McDevitt et al., 2006). This same isoform was demonstrated to form higher order oligomers of 150 and 290 kDa (Velamakanni et al., 2008). Recently, human recombinant ABCG2 purified from *Pichia pastoris* has led to structural analysis at 5 Å resolution and this has shown ABCG2 assembled as a tetramer (Rosenberg et al., 2010). The conflicting results found between laboratories are likely due to several factors, such as the type of detergents used and the extraction conditions. Hence, whether ABCG2 forms a dimer or a higher order oligomer under physiological conditions remains unclear.

### 4.2 Sequences Involved in Dimerization

Mutational analysis has indicated that there are two regions of ABCG2 which may be involved in dimerization (Polgar et al., 2010; Polgar et al., 2006; Wakabayashi et al., 2005). The GXXXG sequence has been linked to dimerization in other proteins such as glycophorin a (Langosch et al., 1996), and this motif is also located in transmembrane domain 1 of ABCG2 (Fig. 3) (Polgar et al., 2010). Mutation of the glycine residues to leucines resulted in impaired drug transport of several ABCG2 substrates, suggesting that this motif plays a role in dimerization, although it also may be important in the formation of higher order complexes (Polgar et al., 2004).

Mutational analysis of Thr402 which is located near the GXXXG motif (TXXGXXXG) in combination with mutations of the GXXXG motif (T402L or T402R, and G406L/G410L; Fig. 3) resulted in a reduction in protein expression and drug efflux, alterations in glycosylation, and retention of ABCG2 in the ER (Polgar et al., 2010). While the mutants could still be chemically cross-linked, this may be simply an indication of their
close proximity within the ER (they must be physically close in order to dimerize) (Polgar et al., 2010). It does not necessarily reflect their ability to form a fully-functional transporter since functions such as drug efflux were shown to be affected by the mutations (Polgar et al., 2010).

Additionally, the results of the mutation of Gly553 found in transmembrane domain 5 to Leu553 mimicked those found for the TXXGXXXG motif (Fig. 3) (Polgar et al., 2006). Although the monomers of the mutant forms of ABCG2 can be cross-linked and form disulfide bonds, the fact that they display impaired glycosylation and trafficking is consistent with, but does not prove, the involvement of the TXXGXXXG motif and Gly553 in homodimerization and formation of a fully-functional ABCG2 transporter (Polgar et al., 2006).

Mutagenesis studies targeting cysteine residues (Cys592, Cys603 and Cys608) suggest that they are likely to be involved in the formation of intra-molecular disulfide bonds (Fig. 3) (Wakabayashi et al., 2007). While the majority of evidence demonstrates that Cys603, which is highly conserved amongst ABCG2 orthologues, plays a key role in the formation of disulfide bonds, the disulfide bonds themselves do not appear to be essential for protein function (Shigeta et al., 2010). This observation suggests that non-covalent protein-protein interactions could also contribute to dimerization (Henriksen et al., 2005; Ni et al., 2010b). Notably, Cys592 and Cys608 also appear to be important residues, as substituting these residues for Gly results in reduced expression and aberrant localization to the intracellular compartments as opposed to the plasma membrane (Wakabayashi et al., 2005).

Taken together, these results suggest that correct disulfide bond formation involves these residues and impacts on intracellular sorting and function of the protein (Wakabayashi et al., 2007).
4.3 Homology Modeling

In order to further elucidate the structure of ABCG2, homology models have been
generated. Notably, models based only on computer predictions have been found to be
inconsistent with experimental topology structures (Ni et al., 2010a). Due to this problem,
three homology models of ABCG2 have been deduced which are based on experimental
topology structures, and represent different conformational states. The templates used were
MsbA (an *Eschericia coli* ABC transporter) for the substrate-unbound, nucleotide-free,
inward-facing apo (open) conformation (Ward et al., 2007); parts of murine Pgp to model the
substrate-bound, nucleotide-free, inward-facing holo (closed) conformation (Aller et al.,
2009); and Sav1866 (a *Staphylococcus aureus* ABC transporter) to model the nucleotide-
bound outward-facing conformation (Dawson and Locher, 2006). The nucleotide-binding
domains were modeled using the nucleotide-binding domains of the *E. coli* maltose
transporter MalK, (Chen et al., 2003a). The results for homology modeling of MsbA suggest
that in the presence of a substrate, ABCG2 displays a more closed conformation (Rosenberg
et al., 2010; Ward et al., 2007).

The models suggest that the large extracellular loop that connects transmembrane
domain 1 and transmembrane domain 2 may form contacts with the extracellular loop
connecting transmembrane domain 5 and transmembrane domain 6, and this could act to
stabilize the dimeric structure (Figs. 3 and 4) (Rosenberg et al., 2010). The large extracellular
loop which connects transmembrane domain 5 and transmembrane domain 6 might also play
a critical role in modulating substrate-binding (Özvég-Laczka et al., 2008). This is
corroborated by evidence from Xu *et al*, who demonstrated that the transmembrane domain
5-loop- transmembrane domain 6 fragment plays a critical role in ABCG2 oligomerization
(Xu et al., 2007).
Interestingly, the coupling helix 1 that is involved in the interactions between the nucleotide-binding domain and the transmembrane domain was not predicted by the homology models for ABCG2, although it is present in the templates and in all other ABC efflux transporter structures (Rosenberg et al., 2010). However, there is a putative linker region which connects the nucleotide-binding domain to the transmembrane domain (Fig. 4), and this is much longer in ABCG2 than in other ABC transporters (Ni et al., 2010a). This potential linker region may perform the same function as coupling helix 1, although it could not be modeled due to a lack of appropriate templates (Rosenberg et al., 2010). A second coupling helix is also present in the model and associates with the nucleotide-binding domain of the opposite monomer (Rosenberg et al., 2010). This motif has been found in the intracellular loop which connects transmembrane domain 4 and transmembrane domain 5 (Fig. 4), and may enable the flexibility of the transporter which in turn aids substrate interactions (Rosenberg et al., 2010). On substrate-binding, the homology models predict dramatic conformational changes. In fact, the intracellular entry of ABCG2 closes, which alters the shape of the substrate-binding cavity to form the ‘ATP sandwich’ necessary for ATP hydrolysis to occur (Rosenberg et al., 2010). A large V-shaped gap then forms on the extracellular side of the transporter, and may release the substrates after ATP hydrolysis (Rosenberg et al., 2010; Ward et al., 2007).

These homology models are in agreement with existing data. For example, Arg482 has been found in the homology models to be located in the central cavity, with the side chain pointing towards the drug translocation pathway (Fig. 4) (Cai et al., 2010). This residue has been the subject of many site-directed mutagenesis studies which point to its importance in determining substrate specificity and transport activity (Miwa et al., 2003; Özvegy-Laczka et al., 2005; Robey et al., 2003). This observation could explain why resistance to methotrexate
which interacts with Arg482) is decreased by the R482G and R482T mutations (Chen et al., 2003b), while efflux of prazosin (which binds to a separate and distinct binding site) was not affected (Giri et al., 2009). It would therefore appear that homology models of ABCG2 do provide relatively accurate and useful information, and may be valuable tools in the development of ABCG2 substrates and inhibitors.

5. ABCG2 Transport

5.1 Substrates

ABCG2 transports a structurally diverse array of substrates (Fig. 5), the list of which is constantly expanding (Polgar et al., 2008). Initially, many of the ABCG2 substrates were reported to be chemotherapeutics such as mitoxantrone (Doyle et al., 1998), which can be used to select for increased ABCG2 expression (Ross et al., 1999). Substrates of the wild-type ABCG2 transporter include tyrosine kinase inhibitors such as imatinib and gefitinib (Elkind et al., 2005), flavopiridol (Allen et al., 2002a; Elkind et al., 2005; Robey et al., 2001) and the camptothecins: topotecan, irinotecan and its metabolite, SN-38 (Ma et al., 1998; Maliepaard et al., 2001b; Yang et al., 1995). Some drug-selected cell lines that express mutant forms of ABCG2, R482G and R482T (previously discussed in Section 4.3), are considered to be gain-of-function mutants, as their altered substrate specificity increases resistance to anthracyclines (doxorubicin, daunorubicin) and rhodamine 123 (Fig. 6) (Allen et al., 2002a; Chen et al., 1990; Honjo et al., 2001). The residue at this position therefore appears to play a key role in substrate recognition (Chen et al., 2003b).

Other drugs also act as substrates of ABCG2 and include: cimetidine (Pavek et al., 2005), prazosin (Litman et al., 2000), statins (Hirano et al., 2005; Huang et al., 2006) and
zidovudine (Wang et al., 2004). Additionally, ABCG2 can also transport biological substrates like estrone, 17β estradiol, porphyrins such as heme (Krishnamurthy et al., 2004), protoporphyrin IX (Robey et al., 2004); and the dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (Pavek et al., 2005). It should also be noted that only sulfated steroids have been found to be transported by ABCG2 in mammalian cells (Imai et al., 2003; Suzuki et al., 2003), while unconjugated estradiol can be transported by ABCG2 identified in Lactococcus lactis (Janvilisri et al., 2003).

For those studying ABCG2, the discovery of fluorescent substrates has been extremely useful. Fluorescent substrates include rhodamine 123 (Litman et al., 2000) and pheophorbide a (Robey et al., 2004). The ABCG2-specific substrate, pheophorbide a, is a dietary chlorophyll breakdown product, that was discovered serendipitously in ABCG2⁻/⁻ mice, which were extremely sensitive to the compound and developed lethal photo-toxic lesions on light-exposed skin (Jonker et al., 2002). Lysotracker®, prazosin-BODIPY and Hoechst 33342 have also been reported to be ABCG2 substrates (Litman et al., 2000).

Although there is considerable substrate overlap between ABCG2, Pgp and MRP1 (Fig. 1), there are some characteristics of this transporter which set ABCG2 apart (Leslie et al., 2005). ABCG2 is able to transport hydrophilic conjugated anions, especially if they have been sulfated, in addition to hydrophobic substrates which Pgp can also transport (Kodaira et al., 2010). This substrate overlap is thought exert a synergistic effect to limit penetration of drugs across the blood brain barrier (BBB) and other areas (Kodaira et al., 2010), and may affect the pharmacokinetics of substrate drugs. It is still unknown what features determine whether a compound will be a substrate of ABCG2, as to date there has only been one group of compounds (camptothecin analogues) where there is an example of a clear structure-
activity relationship (SAR) (Yoshikawa et al., 2004). For instance, in the camptothecin analogue, SN-38 (the active metabolite of irinotecan), the presence of hydroxyl or amino groups at carbons 10 and 11 increases polarity and facilitates its recognition by ABCG2 (Fig. 7) (Yoshikawa et al., 2004).

5.2 ABCG2 Inhibitors

The list of inhibitors of ABCG2 is similarly large and diverse (Fig. 5). The first inhibitor to be identified was fumitremorgin C, isolated from Aspergillus fumigates (Rabindran et al., 2000). Fumitremorgin C was reported to inhibit mitoxantrone resistance in the S1-M1-3.2 cell line, before ABCG2 had been cloned (Rabindran et al., 1998). Due to the neurotoxicity of fumitremorgin C, several analogues were produced, of which Ko143 was found to be the most potent and selective inhibitor of ABCG2 (Allen et al., 2002b). Unlike fumitremorgin C and Ko143, many inhibitors of ABCG2 are not selective and inhibit other ABC transporters as well, such as elacridar (de Bruin et al., 1999), tariquidar (Robey et al., 2004), sildenafil (commercially marketed as Viagra™; (Shi et al., 2011) and cyclosporine A (Gupta et al., 2006). Also of note, some inhibitors of ABCG2 are also substrates of this molecule, suggesting that they are competitive inhibitors. For instance, the tyrosine kinase inhibitors imatinib and gefitinib fall into this category (Houghton et al., 2004; Özvegy-Laczka et al., 2004).

Several SAR and quantitative SAR (QSAR) studies have been carried out for ABCG2 inhibitors, allowing the prediction of some of the structural requirements for an inhibitor. Lipophilicity has been shown to be a predictor of inhibition in both flavonoids (Zhang et al., 2005) and fumitremorgin C analogues (van Loevezijn et al., 2001). However, this was not shown to be the case for either tariquidar analogues (Pick et al., 2008) or propafenone
analogue (Cramer et al., 2007). Planar structure has also been suggested to increase the ABCG2-inhibitory properties of a drug, as evidenced by results from SARs of different structural series of compounds. For example, the two cyclin-dependent kinase inhibitors, purvalanol A and WHI-P180, have a planar structure and are also the two strongest ABCG2 inhibitors of this class (An et al., 2009). A planar structure including a 2,3-double bond in ring C increases the inhibition potency for flavonoids (Zhang et al., 2005), and the same effect is seen for rotenoid derivatives (Fig. 7) (Ahmed-Belkacem et al., 2007). Although QSAR analysis has provided some useful information for the development of ABCG2 inhibitors, their efficacy will remain limited until a high resolution 3D structure has been solved.

### 6. Localization and Physiological Function

Many studies have investigated the tissue distribution of ABCG2 expression, providing clues as to the physiological function of the transporter (Robey et al., 2009). High ABCG2 expression has been found in the placenta, brain, prostate, small intestine, testes, ovaries, liver, adrenal gland, uterus and central nervous system (Doyle and Ross, 2003). Several physiological roles have been posited for ABCG2, and in general it seems to protect cells and organs from harmful xenobiotics (Robey et al., 2007). As a result of this function, ABCG2 has been shown in many instances to affect the pharmacokinetics of its substrates, making its expression relevant in a clinical setting (Fig. 8).

#### 6.1 Placenta

High ABCG2 expression has been found on the apical membrane of syncytiotrophoblasts of the chorionic villi, leading to the assertion that ABCG2 protects the fetus from exposure to toxins and also assists in removing toxins from the fetus (Fig. 8).
Importantly, \textit{ABCB1a/1b}^{-/} (Pgp knockout) mice were often used to avoid the confounding effects of having Pgp present, as Pgp and ABCG2 have overlapping substrate specificities (Fig. 1). The administration of oral topotecan and elacridar to \textit{ABCB1a/1b}^{-/} mice resulted in fetal plasma topotecan levels twice as high as the maternal plasma levels (Jonker et al., 2000). Other groups have also similarly demonstrated that i.v. administration of the antibiotic, nitrofurantoin, to pregnant mice leads to a 5-fold higher fetal concentration in \textit{ABCG2}^{-/} mice \textit{versus} wild-type mice (Zhang et al., 2007). In humans, an \textit{ex vivo} study employing human placental vesicles demonstrated that fetal-to-maternal concentration ratios of the gestational diabetes drug, glyburide, were increased 2-fold by co-administration of the ABCG2 inhibitor, novobiocin (Gedeon et al., 2008).

There is also evidence demonstrating the transport of toxins which are ABCG2 substrates from the fetal to the maternal space (Myllynen et al., 2008; Staud et al., 2006). In rats, the H2 histamine receptor antagonist, cimetidine, was shown to be transported against the concentration gradient from the fetal space to the maternal space (Staud et al., 2006), and analogous results have been obtained in perfused human placenta using the dietary carcinogen, \textsuperscript{14}C-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (Myllynen et al., 2008).

6.2 Blood Brain Barrier

Generally, the influx and efflux of drugs through the BBB has been thought to depend, at least in part, on the physiochemical properties of the drug such as lipophilicity and molecular weight (Pardridge, 2005). However, many drugs have lower BBB penetration than would be predicted based on their physiochemical properties and this is largely due to the expression of efflux transporters, such as the ABC proteins (Pardridge, 2005). This poor penetration of some drugs affects the treatment of diseases such as Alzheimer’s and
Parkinson’s disease, as well as cancers such as gliomas because concentrations high enough to penetrate the BBB often cause systemic toxicity (Pardridge, 2005). The BBB also poses several problems for drug development as it places significant restrictions on the size, lipophilicity and transporter affinity of drugs (Pardridge, 2005). Of the three ABC transporters in the BBB, ABCG2 is thought to be the most highly expressed, followed by Pgp and then MRP1, and all are localized to the luminal side of endothelial cells lining BBB capillaries (Fig. 8) (Eisenblätter et al., 2003).

The considerable substrate overlap between ABCG2 and Pgp (Fig. 1) makes it difficult to determine the pharmacological and potentially the physiological roles of ABCG2 alone. This conclusion is exemplified by a study by Breedveld et al (2005) who examined the uptake of imatinib in ABCG2−/− and ABCB1a/b−/− (i.e., Pgp−/−) mice. In this investigation, ABCB1a/b−/− mice displayed a 3.6-fold higher brain penetration of imatinib compared to wild-type animals, and ABCG2−/− a 2.5-fold higher concentration, while inhibition with elacridar caused a 4.2-fold increase in imatinib penetration in wild-type mice (Breedveld et al., 2005). This suggests that while ABCG2 is highly expressed at the BBB, Pgp is perhaps more clinically relevant. This is corroborated by another study which found that the blood-to-plasma ratios of imatinib were increased by >10 fold in ABCB1a/b/ABCG2−/− mice, while in ABCB1a/b−/− mice the increase was only approximately 2-fold, and no difference was observed for ABCG2−/− mice (Oostendorp et al., 2009). Again, this observation suggests that while Pgp may play a more important role in limiting the penetration of substrates at the BBB, ABCG2 is also of importance and the combination of the two transporters may even be synergistic (Fig. 8).
6.3 Mammary Gland

ABCG2 has been found to be up-regulated in the lactating breast of mice, cows and humans, where it may function to concentrate vitamins and minerals into milk for breast-feeding young (Fig. 8) (Hogue et al., 1999). One such vitamin is riboflavin, which is necessary for fat metabolism and many redox reactions (Van Herwaarden et al., 2007). Unfortunately, ABCG2 may also play a role in the concentration in milk of toxins and drugs such as the dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (Jonker et al., 2005), and the antibiotic, ciprofloxacin (Merino et al., 2006). Since offspring of ABCG2-/− mice do not appear to suffer any abnormalities (Foraker et al., 2003), the exact function of ABCG2 in the mammary gland remains unclear. However, considering the substrate specificity overlap between ABCG2 and other commonly expressed transporters (e.g., P-gp and MRP1; Fig. 1), this latter observation may be due to compensatory response of these transporters. Such metabolic compensation has been observed for other targeted knockouts e.g., the compensatory response observed after myoglobin deletion in the heart (Gödecke et al., 1999).

6.4 Blood-Testis Barrier

High levels of ABCG2 expression have been detected in the apical membranes of myoid cells surrounding the seminiferous tubules in humans (Fig. 8) (Bart et al., 2004). On the basis of studies using ABCG2+/− mice which found that xenobiotic penetration into the testis and epididymis was greatly increased, it has been suggested that ABCG2 may protect developing spermatocytes (Enokizono et al., 2007). This protective effect may be particularly relevant in respect to phytoestrogens, several of which (i.e., genisteine, daidzen and coumestrol) are ABCG2 substrates and have been shown to impair male reproductive function (Adeoya-Osiguwa et al., 2003).
6.5 Gastrointestinal Tract

ABCG2 expression is found to decrease along the length of the gastrointestinal tract, with the highest expression found in the duodenum and lowest in the rectum (Gutmann et al., 2005). Within the gut, ABCG2 has been observed to localize to the apical membrane, which supports the theory that in the gastrointestinal tract ABCG’s role is to limit the oral bioavailability of its substrates (Fig. 8) (Maliepaard et al., 2001a). These substrates include therapeutic agents, and a potential means of increasing the absorption of orally administered drugs/substrates could involve ABCG2 inhibition. For instance, a study by Allen et al (2002b) elegantly illustrates this principle, in which plasma topotecan levels were increased 4-6-fold in ABCB1a/1b−/− mice in the presence of orally administered Ko143 (a specific ABCG2 inhibitor; (Allen et al., 2002b). Another potential method to increase the oral bioavailability of drugs is to adjust their structure so that they are no longer ABCG2 substrates. Notably, it is possible that the increased bioavailability of newer camptothecin analogues is due to the fact that they are poor ABCG2 substrates (Yoshikawa et al., 2004).

6.6 Kidneys and Liver

Hepatic and renal excretion are major mechanisms by which most drugs are cleared from the body (Seldin, 2004; Wilkinson and Shand, 1975). As such, these processes should involve ABCG2 if its main physiological function is to protect the body from exposure to harmful xenobiotics. Consistent with this, ABCG2 expression is found in the canalicular membrane of hepatocytes (Maliepaard et al., 2001a), in the bile ducts (Vander Borght et al., 2006), the gall bladder (Aust et al., 2004), and in the apical membrane of proximal kidney tubule epithelial cells (Huls et al., 2007) (Fig. 8). Notably, ABCG2−/− mice have also been shown to have impaired renal or hepatic excretion of several ABCG2 substrates (Mizuno et al., 2004).
6.7 *Subcellular Localization*

ABCG2 is predominantly located in the plasma membrane of drug-selected and *ABCG2*-transfected cell lines, as has been demonstrated by a number of immunohistochemical studies and confocal microscopy drug localization assays (Maliepaard et al., 2001a; Scheffer et al., 2000). This is somewhat unusual, as almost all other known half-transporters are localized to intracellular membranes such as those of the mitochondrion or the ER. Examples of such transporters include the ABC family members, ABC7 and M-ABC1, which are localized in the mitochondria (Hogue et al., 1999), and ABCB2 and ABCB3 that are found in the ER (Kleijmeer et al., 1992). Only one other half transporter has been found to be at least partially localized to the plasma membrane, namely the human homologue of the Drosophila white gene, ABCG1 (Vaughan and Oram, 2005). The localization of ABCG2 may in part be regulated by cell signaling pathways such as the phosphatidyl inositol 3-kinase (PI3K)/γ-Akt murine thymoma viral oncogene homolog (Akt) pathway. In fact, inhibition of both PI3K (Misra et al., 1998) and Akt (Chu et al., 2008) has been shown to cause ABCG2 translocation from the plasma membrane to an intracellular compartment. Moreover, ABCG2 may also be present in the membranes of acidic vesicles (*e.g.* lysosomes), as co-localization studies with Lysotracker® demonstrated sequestration of mitoxantrone in acidic vesicles of both S1 cells and the S1-M1-80 subline that expresses high ABCG2 levels (Litman et al., 2000).

7. *Significance of ABCG2 in Cancer*

ABCG2 is over-expressed in several MDR cancer cell lines, which is indicative of its importance in the MDR phenotype of cancer cells (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). Given that a large number of commonly used chemotherapeutics are ABCG2 substrates, the clinical impact of ABCG2 on MDR in cancer is being actively...
investigated (Robey et al., 2007). To date, studies which have attempted to link ABCG2 expression to clinical outcomes have been inconclusive and often contradictory (Doyle and Ross, 2003). However, the possibility remains that modulation of ABCG2 may increase the oral bioavailability of chemotherapeutics or overcome MDR.

7.1 Expression in Hematologic Malignancies

ABCG2 has been found to be frequently expressed on malignant hematopoietic and lymphoid cells, and has been most thoroughly investigated in acute myelogenous leukemia (AML; Table 1) (Doyle and Ross, 2003). In AML, ABCG2 has been found to show potential as a prognostic indicator despite the fact that common first line therapies for AML are not ABCG2 substrates (Natarajan et al., 2012). To date, all AML patients examined have possessed the wild-type ABCG2 sequence (Plasschaert et al., 2003; Suvannasankha et al., 2004), and these patients should therefore not be resistant to anthracyclines as they are not substrates of wild-type ABCG2 (Fig. 6) (Honjo et al., 2001). However, it has been found that there is a correlation between ABCG2 expression and failure to achieve complete remission in AML patients after induction therapy with the cytotoxic agent, cytarabine (Steinbach et al., 2002). This is likely due to Pgp expression, as high levels of Pgp have been found in 45% of AML cases when the patient is aged over 55 (Leith et al., 1997), although this was not directly investigated. High ABCG2 levels in AML have also been correlated with a relapsed/refractory disease state, and lower complete response rate and shorter survival (Table 1) (Benderra et al., 2004; Benderra et al., 2005).

These latter results are inconsistent with those found by other investigators who found a correlation with survival in AML patients, but not the complete response rate (Uggla et al., 2005). Interestingly, ABCG2 expression has been observed in small sub-populations of AML
cells (Abbott et al., 2002; Suvannasankha et al., 2004), and seems to be most highly expressed on CD34+/CD38- “cancer stem cells” (see Section 7.3) which have an immature phenotype (de Figueiredo-Pontes et al., 2008; Raaijmakers et al., 2005). It should be noted that the detection of ABCG2 in AML cells has been afflicted by methodological issues. These problems have potentially contributed to the discordance observed between studies correlating ABCG2 expression to prognostic outcomes (Table 1).

The role of ABCG2 in acute lymphoblastic leukemia (ALL) drug resistance has also been investigated (Table 1). As in AML, high ABCG2 expression has been correlated with resistance to the cytotoxic agent, cytarabine, a non-ABCG2 substrate (Stam et al., 2003). Many common drugs used to treat ALL are not ABCG2 substrates, but this latter observation suggests that in both AML and ALL, ABCG2 expression may be a marker of resistance, as opposed to a mechanism that is directly involved in this process (Natarajan et al., 2012). Results linking ABCG2 expression to prognostic outcomes in ALL have been inconsistent, particularly between adults and children. For example, ABCG2 expression in adults has been correlated with poorer disease-free survival (Suvannasankha et al., 2004), while in children, ABCG2 expression has shown not to be related to relapse-free survival time (Table 1) (Sauerbrey et al., 2002). In contrast, low expression of ABCG2 is correlated to a higher incidence of death due to drug toxicity in children (Cortez et al., 2009). This observation may simply be due to the fact that children have better clinical outcomes than adults in ALL.

### 7.2 Expression in Solid Tumors

The expression of ABCG2 in solid tumors was initially not detected by immunohistochemical studies (Scheffer et al., 2000), although with the use of a different monoclonal antibody (BXP21 as opposed to BXP34), ABCG2 was subsequently identified
by the same group of researchers in over 40% of tumors examined (Diestra et al., 2002). These tumors represented more than 21 different types of untreated cancer, such as colon, esophageal, endometrial, breast, pancreatic, and lung cancers (Diestra et al., 2002).

Like hematological malignancies, ABCG2 expression in some types of solid tumors has been correlated with poorer outcomes (Mo and Zhang, 2012). In small cell lung cancer, high ABCG2 expression has been associated with poor clinical response and progression-free survival to platinum drug (carboplatin and cisplatin)-containing regimens (Kim et al., 2009), and lower overall survival in patients with non-small cell lung cancer (Ota et al., 2009). Unfortunately, the results of studies examining this latter tumor-type and ABCG2 expression lack consistency (Burger et al., 2003; Faneyte et al., 2002; Kanzaki et al., 2001), as do those for breast cancer (Burger et al., 2003; Kanzaki et al., 2001) and so it is impossible to draw firm conclusions on whether there is a link between ABCG2 and clinical outcomes in solid tumors (Burger et al., 2003; Faneyte et al., 2002; Kanzaki et al., 2001). The majority of recent studies have moved away from attempting to assess this link, and have instead focused on ABCG2 and its role in the cancer stem cell phenotype (see below).

### 7.3 Cancer Stem Cells and MDR

While the idea of cancer stem cells is still controversial, the theory has gained increasing traction in recent years (Visvader and Lindeman, 2008). Cancer stem cells are a subset of tumor cells so named in order to reflect their shared properties with normal stem cells as they retain the ability to self-renew and differentiate, although to a limited extent (Visvader and Lindeman, 2008). The first evidence of cancer stem cells was found in immune-deficient mice, where it was ascertained that a small subset (0.1-1%) of AML cells transplanted into these mice could induce leukemia (Lapidot et al., 1994). Cancer stem cells...
have also been separated from solid tumors, and usually express specific markers which have been useful in their isolation (Hirschmann-Jax et al., 2004). These markers include CD24 (Al-Hajj et al., 2003), CD44 (Al-Hajj et al., 2003) and CD133 (Singh et al., 2004). Notably, ABCG2 is also a marker of cancer stem cells, as the efflux of the ABCG2 substrate, Hoechst 33342, is exploited to isolate them (Zhou et al., 2001). Cancer stem cells are often referred to as a ‘side population’ because they display low Hoechst fluorescence upon flow cytometry and appear as negatively stained cells to one side of the main population (Zhou et al., 2001). Although Hoechst 33342 is also a Pgp substrate, it has been established that the side population phenotype is due to ABCG2 expression since the side population is still present in ABCB1a/1b−/− (Pgp knockout) cells (Zhou et al., 2001).

Cancer stem cells offer an alternative to the conventional model of acquired MDR, in which MDR occurs by cells gaining mutations which confer drug resistance, giving them an advantage over other cells in the tumor population by allowing them to survive chemotherapy (Fig. 9a) (Dean et al., 2005). The cancer stem cell model of MDR differs in that the resistant cancer stem cells are inherently present in the tumor cell population, and following chemotherapy these cells survive (possibly due to the presence of ABCG2 and/or Pgp), and ultimately re-populate the tumor with both cancer stem cells and more differentiated cells (Fig. 9b) (An and Ongkeko, 2009). In an additional model of cancer stem cell MDR, some cancer stem cells acquire new mutations which further increase their resistance resulting in a drug-resistant phenotype (Fig. 9c) (Dean et al., 2005). This model has been shown to occur in imatinib-resistant CML through acquisition of BCR-ABL mutations (O’Hare et al., 2007). The final model, which can be found in renal cell cancer, is one of intrinsic resistance where both the cancer stem cells and variably differentiated cells are drug-resistant, and therapies therefore have no effect on their survival (Fig. 9d) (Fojo et al., 1987). Through these varied
routes, ABCG2 expressed on cancer stem cells may contribute to the development and propagation of MDR cancer.

7.4 Pharmacological Targeting of ABCG2 in Cancer

The expression of ABCG2 by cancer stem cells raises the possibility of selectively targeting cancer stem cells in chemoresistant tumors. As of yet, this remains an elusive ideal. ABCG2 is expressed on normal hematopoietic stem cells, as well as in the BBB, so any drug would need to be carefully titrated to ensure that toxicity is minimized. Serious adverse events related to altered pharmacokinetics of co-administered cytotoxics are one of the many reasons why clinical trials of Pgp inhibitors have been disbanded (Nobili et al., 2006). The first ABCG2 inhibitor, fumitremorgin C, is not used in clinical settings due to its severe neurotoxic effects, and this has led to the development of a series of structural analogues, of which Ko143 is the most potent and does not cause neurotoxicity in mice (Allen et al., 2002b). These inhibitors of ABCG2 have not been tested in clinical trials to date, but remain a promising possibility if cell-type specificity were to be achieved.

As ABCG2 inhibitors, tyrosine kinase inhibitors are an interesting option for pharmacological targeting of ABCG2. Clearly, these agents have already been in clinical trials, and thus, it may be theoretically easier to gain approval for their use, making them a more tenable possibility than untested inhibitors such as Ko143. In cell-based assays, tyrosine kinase inhibitors have been shown to be substrates of ABCG2 (possibly binding at the prazosin-binding site (Özvegy-Laczka et al., 2004) and at high concentrations can inhibit ABCG2 transport (Shi et al., 2009; Yang et al., 2005). Erlotinib, gefitinib and imatinib have all been shown to increase the accumulation, and therefore, cytotoxicity of ABCG2 substrates.
such as mitoxantrone and methotrexate in vitro (Misra et al., 1998; Özvegy-Laczka et al., 2004; Yang et al., 2005).

In addition to inhibiting ABCG2, tyrosine kinase inhibitors may also down-regulate the expression of ABCG2 via the PI3K-Akt pathway (Nakanishi et al., 2006). These results suggest that combination therapies involving tyrosine kinase inhibitors and conventional chemotherapeutics may prevent MDR and enhance the efficacy of both treatments. This intervention has yet to be trialed, although co-administration of imatinib and the photodynamic therapy, 2-(1-hexyloxethyl)-2-devinyl pyropheophorbide a, has been tested in vivo (Liu et al., 2007). Many photosensitizers are ABCG2 substrates, meaning that their intracellular accumulation is decreased in tumors that express the transporter (Robey et al., 2005). The combination of imatinib and 2-(1-hexyloxethyl)-2-devinyl pyropheophorbide a, significantly enhanced the efficacy of the photosensitizer, halving the rate of tumor growth (Liu et al., 2007). These promising results warrant further investigation in clinical trials.

The potential of phosphodiesterase-5 inhibitors as potential chemosensitizers has also been investigated (Black et al., 2008; Das et al., 2010). In fact, sildenafil has been shown to inhibit ABCG2 and Pgp at clinically achievable concentrations, and reverse the efflux of mitoxantrone, paclitaxel and methotrexate (Shi et al., 2011). There are currently no published clinical trial results examining the effect of phosphodiesterase-5 inhibitors on ABC transporters. However, studies in animal models have demonstrated that the use of sildenafil in combination with doxorubicin results in improved transport of doxorubicin across the BBB (Black et al., 2008) and improved efficacy of doxorubicin in prostate cancer (Das et al., 2010). It is possible that the increased activity of doxorubicin is due at least in part to the
inhibition of ABCG2 and Pgp by sildenafil, and this suggests that phosphodiesterase-5 inhibitors may prove to be useful chemoadjuvants in the future (Tiwari and Chen, 2013).

8. Conclusion

While a plethora of research has been conducted examining ABCG2 biochemistry and pharmacology with respect to substrates, inhibitors, protein structure, function and gene regulation, there are still vast gaps in our knowledge. For example, it is still not known what form higher order oligomers of ABCG2 take, as a high resolution crystal structure has yet to be solved. Hence, this shortcoming limits the ability to predict structural requirements for developing new substrates and inhibitors. Such information would be invaluable in exploiting ABCG2 as a therapeutic target, although this is another area which has not been thoroughly investigated. Probably the major reason for this is the failure of Pgp inhibitors in clinical trials, principally due to methodological errors such as not selecting patients based on tumor expression of Pgp (Robey et al., 2009). This failure has lead to an unwillingness to invest in targeted treatments for MDR in cancer, and to date, the importance of ABCG2 is cancer biology and treatment remains unclear.

Recent research has suggested that combination therapies involving tyrosine kinase inhibitors and phosphodiesterase-5 inhibitors with conventional chemotherapeutics have potential to treat MDR tumors, and this is currently the best prospect for exploiting ABCG2 in the clinic, providing lessons are learnt from past errors. Until this and further translational research has been completed, it will remain difficult to establish the impact of ABCG2 on chemotherapy outcomes.
Acknowledgments
The authors would like to thank Dr Darius Lane, Dr Vera Richardson and Ms Nicole Seebacher of the Molecular Pharmacology and Pathology Program (University of Sydney) for their critical appraisal of the manuscript before submission.

Authorship Contributions
Wrote or contributed to the writing of the manuscript: Stacy, Jansson, Richardson
References


MOL #88609


is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 7(9): 1028-1034.
Footnotes

This work was supported by the National Health and Medical Research Council of Australia (NHMRC) [Project Grant #1021607 and #1021601, Senior Principal Research Fellowship #571123]. A.E.S. sincerely appreciates an Australian Postgraduate Award from the Sydney Medical School, University of Sydney.

Patric J. Jansson and Des R. Richardson contributed equally as co-corresponding senior authors.

Reprint requests to be sent to Prof. Des R. Richardson (Molecular Pharmacology and Pathology Program, Department of Pathology, Blackburn Building D06, University of Sydney, NSW 2006, Australia). Email: d.richardson@med.usyd.edu.au.

The authors declare no conflict of interest.
**Figure Legends**

**Figure 1** There is considerable substrate overlap between the ABC transporters, Pgp, MRP1 and ABCG2. *Indicates substrates transported by ABCG2 mutant R482G.

**Figure 2** The *ABCG2* promoter has multiple *cis* regulatory elements, SP1, AP1 and AP2 sites, the position of which is depicted in relation to the transcription start site of the first *ABCG2* exon. Abbreviations: Hypoxia response element (HRE), estrogen response element (ERE), aryl hydrocarbon response element (AhRE), NFκB response element (NFκBRE).

**Figure 3** Schematic illustration of the membrane topology of ABCG2 and highlights of residues or mutations which affect the functioning of the transporter. Val12M and Q141K are SNPs thought to affect the pharmacokinetics of ABCG2 substrates. Notably, T402, G406 and G410 are near or part of the GXXXG motif and are thought to play a role in dimerization, as do Gly553 Cys592, 603 and 608. The R482G isoform of ABCG2 appears in drug-selected cell lines and displays altered substrate specificity to the wild-type transporter. Asp596 is the sole N-linked glycosylation site.

**Figure 4** Homology modeling of ABCG2 predicts that substrate-binding induces significant conformational change which brings the nucleotide-binding domains closer together and closes the substrate-binding cavity. This structural alteration possibly allows these domains to interact and results in the transport of substrates through the central translocation pathway. The transmembrane domains are numbered 1-6, and possible areas of significance to the transporter’s function are highlighted. This figure was adapted from (Rosenberg et al., 2010).

**Figure 5** The structural diversity of selected ABCG2 substrates and inhibitors.
Figure 6 Drug-selected cell lines can express the mutant form of ABCG2-R428G. This isoform has altered substrate specificities as compared to wild-type ABCG2, possibly because some substrates interact with the amino acid at position 428. This figure was adapted from (Sarkadi et al., 2004).

Figure 7 Substitution of polar groups at carbons in positions 10 and 11 (circled in red) of camptothecins analogs has been found to increase the substrate recognition by ABCG2, as demonstrated by the increase in IC\text{50} for SN-38 compared to SN-443 in the ABCG2-expressing cell line PC-6/SN2-5H2. A planar structure due to double bonds (circled in red) has been found to increase the potency of both flavonoids (between carbons 2 and 3) and rotenoids (between carbons 6 and 12). This is illustrated by the lower EC\text{60} for increasing mitoxantrone accumulation in MCF-7 MX100 ABCG2-expressing cells for flavone compared to flavanone; and the increase in maximal mitoxantrone accumulation for boeravinone G compared to boeravinone C in HEK-293/ABCG2 cells.

Figure 8 ABCG2 is highly expressed in the placenta, blood brain barrier (BBB), gastrointestinal tract, liver, kidney, testis and the lactating breast. Generally, ABCG2 it is found on the apical surface of cells where it is thought to actively transport substrates out of the tissues so that they may be excreted. Hence, this prevents exposure to harmful xenobiotics. This figure was adapted from (Vlaming et al., 2009).

Figure 9 Different models of tumor MDR. (a) In the conventional model of MDR\textsubscript{2} sporadic mutations arise which confer drug resistant clones (yellow). After chemotherapy these cells survive and re-populate the tumor. (b) The cancer stem cell model indicates that tumors
contain small populations of cancer stem cells (red) and their descendants (blue) which are committed to a particular lineage. The cancer stem cells expressing ABC transporters are able to survive chemotherapy and proliferate to form a heterogeneous tumor population of cancer stem cells and differentiated descendant cells. (c) The acquired resistance cancer stem cell model is similar to b. However, following chemotherapy, where the variably differentiated cells die, mutations arise in the surviving cancer stem cells (orange) which further enhances their drug resistance and that of their differentiated descendants (purple). (d) In the intrinsic resistance model, both the cancer stem cells (orange) and the variably differentiated cells (purple) are inherently drug resistant, so chemotherapy has no effect on their survival.
Table 1 Summary of Clinical Studies of ABCG2 Expression in Human Leukemia

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute myelogenous leukemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sargent et al</td>
<td>20 blast samples</td>
<td>27% of samples were ABCG2 positive.</td>
</tr>
<tr>
<td>van der Kolt et al</td>
<td>20 paired (pretreatment and refractory/relapse) samples</td>
<td>Low ABCG2 expression, no increase in relapse samples.</td>
</tr>
<tr>
<td>Abbott et al</td>
<td>40 de novo samples</td>
<td>78% of samples had higher ABCG2 expression than normal blood and bone marrow, 7% had levels within the range of drug resistant cell lines.</td>
</tr>
<tr>
<td>van Den Huevel-Eibrink et al</td>
<td>20 paired (de novo and refractory/relapsed) samples</td>
<td>Relapsed samples had higher ABCG2 expression, ABCG2 expression associated with Pgp expression.</td>
</tr>
<tr>
<td>van der Pol et al</td>
<td>45 paired samples</td>
<td>No ABCG2 detected.</td>
</tr>
<tr>
<td>Galimberti et al</td>
<td>51 AML samples</td>
<td>ABCG2 expression detected in 56% of samples, intermediate levels in 48.2%. ABCG2 expression associated with Pgp expression.</td>
</tr>
<tr>
<td>Suvannasankha et al</td>
<td>31 pretreatment blast samples</td>
<td>No correlation between function, protein or gene expression.</td>
</tr>
<tr>
<td>Steinbach et al</td>
<td>59 untreated AML, 9 relapse samples</td>
<td>Median ABCG more than 10 times higher in patients who did not achieve remission, compared to responders. ABCG2 expression higher at relapse than diagnosis. High ABCG2 linked to worse prognosis.</td>
</tr>
<tr>
<td>Benderra et al</td>
<td>149 de novo samples</td>
<td>ABCG2 expression and ABCG2 expression associated with Pgp function were correlated with poorer treatment outcomes: achievement of complete remission, 4-year disease-free survival and 4-year overall survival.</td>
</tr>
<tr>
<td>Ugglia et al</td>
<td>40 AML samples</td>
<td>Patients with higher ABCG2 expression who respond to therapy had shorter overall survival. No difference in expression between responders and non-responders.</td>
</tr>
<tr>
<td><strong>Acute lymphocytic leukemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sauerbrey et al</td>
<td>47 de novo, 20 relapsed samples</td>
<td>No correlation between expression and response, ABCG2 expression lower in T-cell lineages.</td>
</tr>
<tr>
<td>Plasschaert et al</td>
<td>46 de novo samples</td>
<td>BXP-34 positivity in B-cell lineage 2.4-fold higher than T-cell lineage.</td>
</tr>
<tr>
<td>Stam et al</td>
<td>13 non-infant and 13 infant samples</td>
<td>ABCG2 expression 2.4-fold less in infant samples.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expression correlated with Ara-C resistance.</td>
</tr>
<tr>
<td>Suvannasankha et al</td>
<td>30 pretreatment samples</td>
<td>43% of cases BXP-21 positive. BXP-21 staining predictive of shorter disease-free survival.</td>
</tr>
</tbody>
</table>
Figure 1
Figure 4

Substrate unbound

Arg482

Lipid Bilayer

1 2 3 4 5 6

Coupling helix

Substrate bound

Extracellular loop between transmembrane domains 1 and 2 and 5 and 6

Nucleotide binding domains

Linker region
Figure 6
**Camptothecin Analogs**

SN-443, IC$_{50}$
$1.52 \pm 0.04 \mu$M

SN-38, IC$_{50}$
$262 \pm 5.70 \mu$M

**Flavonoids**

Flavanone, EC$_{60}$
$24.61 \pm 5.21 \mu$M

Flavone, EC$_{60}$
$3.99 \pm 1.78 \mu$M

**Rotenoids**

Boeravinone C, %
maximal mitoxantrone accumulation $92 \pm 6.5$

Boeravinone G, %
maximal mitoxantrone accumulation $31 \pm 4.2$

**Figure 7**
Figure 8