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ABCG2: determining its relevance in clinical drug resistance

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Abstract Multidrug resistance is a major obstacle to successful cancer treatment. One mechanism by which cells can become resistant to chemotherapy is the expression of ABC transporters that use the energy of ATP hydrolysis to transport a wide variety of substrates across the cell membrane. There are three human ABC transporters primarily associated with the multidrug resistance phenomenon, namely Pgp, MRP1, and ABCG2. All three have broad and, to a certain extent, overlapping substrate specificities, transporting the major drugs currently used in cancer chemotherapy. ABCG2 is the most recently described of the three major multidrug-resistance pumps, and its substrates include mitoxantrone, topotecan, irinotecan, flavopiridol, and methotrexate. Despite several studies reporting ABCG2 expression in normal and malignant tissues, no trials have thus far addressed the role of ABCG2 in clinical drug resistance. This gives us an opportunity to critically review the disappointing results of past clinical trials targeting Pgp and to propose strategies for ABCG2. We need to know in which tumor types ABCG2 contributes to the resistance phenotype. We also need to develop standardized assays to detect ABCG2 expression in vivo and to carefully select the chemotherapeutic agents and clinical trial designs. This review focuses on our current knowledge about normal tissue distrubution, tumor expression profiles, and substrates and inhibitors of ABCG2, together with lessons learned from clinical trials with Pgp inhibitors. Implications of SNPs in the *ABCG2* gene affecting the pharmacokinetics of substrate drugs, including many non-chemotherapy agents and ABCG2 expression in the SP population of stem cells are also discussed.

Keywords ABCG2 · Multidrug resistance · ABC transporters

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1 Introduction

Clinical drug resistance remains a significant impediment to the successful treatment of cancer. The multidrug resistance phenotype is often associated with increased expression of ATP-binding cassette (ABC) transporters that mediate energy-dependent transport of substrate drugs out of the cell against a concentration gradient [1]. The discovery of the *MDR1* (*ABCB1*) gene 20 years ago [2, 3] ignited a fervent study of drug resistance in cancer. As a result, the protein encoded by *MDR1*, P-glycoprotein (Pgp), is by far the most intensely studied ABC transporter. Pgp is known to transport a wide range of chemotherapeutic agents including the anthracyclines, vincas, taxanes, etoposide, and mitoxantrone [1]. Despite strong evidence linking Pgp expression to poor prognosis in diseases such as leukemia, early clinical trials aimed at inhibiting Pgp were poorly designed and unsuccessful [4], leading many to abandon the idea of reversing Pgp-mediated resistance. Most later trials utilizing "second generation" inhibitors were equally unsuccessful. Several explanations for the difficulty in confirming the MDR hypothesis at the level of the clinical trial can be invoked [4].

In 1993, Cole and colleagues cloned a gene encoding another energy-dependent transporter, the multidrug resistance-associated protein, or *MRP1* (*ABCC1*) [5]. Conferring resistance to a somewhat narrower range of chemotherapy agents, namely the anthracyclines, vincas, etoposide and

teniposide, MRP1 renewed interest in ABC transporters, especially since MRP1 expression is frequently observed in non-small cell lung cancer [6, 7]. However, few MRP1-specific inhibitors were developed and the lack of success of clinical trials attempting to reverse Pgp-mediated drug resistance left many hesitant to attempt studies to inhibit MRP1 clinically.

In the early 1990s, several groups began reporting non-Pgp, non-MRP1-mediated drug resistance in a variety of drug-selected cell lines [8–12]. The resistant cell lines exhibited resistance to doxorubicin and etoposide in addition to high mitoxantrone resistance, but lacked resistance to vinblastine or cisplatin. In one drug-selected cell line, high levels of cross-resistance to the camptothecin analogs topotecan, 9-aminocamptothecin, CPT-11, and SN-38, the active metabolite of CPT-11, were also found [13].

The gene responsible for the novel cross-resistance phenotype was first cloned by Doyle and colleagues from the MCF-7 AdrVp breast cancer cell line developed in the Fojo laboratory [14] and was called *BCRP* for breast cancer resistance protein [15]. Shortly thereafter, Allikmets and colleagues reported a nearly identical gene termed *ABCP* for ATP-binding cassette transporter expressed in placenta [16]; and our laboratory reported a cDNA cloned from the mitoxantrone-selected colon carcinoma cell line S1-M1-80 and termed the gene *MXR*, or mitoxantrone resistance gene [17]. When the sequences were eventually compared, they were recognized as essentially identical and belonging to a subfamily of ABC transporters not previously associated with drug resistance in humans.

Subsequent to the cloning of *BCRP*/*ABCP*/*MXR*, the Human Genome Nomenclature Committee assigned the gene the name ABCG2, making it the second gene in the G subfamily of ABC transporters that is made up of only half-transporters. This terminology will be used throughout this review. The G subfamily of transporters contains five other members in addition to ABCG2 [18]: two involved in cholesterol transport, ABCG4 and ABCG1, the human homologue of the *Drosophila* white protein [19]; one currently found only in rodents, Abcg3, which appears to have an aberrant ATP-binding domain [20]; and two that heterodimerize with each other to form a functional sterol transporter, ABCG5 and ABCG8 [21].

Eight years after the discovery of ABCG2, we still have no clear idea what role ABCG2 plays in clinical drug resistance. Determining this role will be critical in planning future clinical trials. In this review, we will characterize the resistant phenotype conferred by ABCG2, present reports of normal tissue distribution and discuss studies directed at determining the contribution of ABCG2 to drug resistance in cancer. A summary of the current methods used to measure the levels of ABCG2 in patient samples will be provided and we will make recommendations on how to improve the reliability of data generated in future studies.

2 Chromosomal localization and mechanisms controlling expression

The *ABCG2* gene spans over 66 kb and is made up 16 exons and 15 introns; the resulting protein is 655 amino acids long and runs as a 72 kDa protein on an SDS gel under reducing conditions [22]. Fluorescence in situ hybridization studies with a bacterial artificial chromosome probe containing *ABCG2* localized the gene to 4q21-4q22 in cells with a normal chromosome 4 [23].

Few data are available regarding molecular mechanisms controlling *ABCG2* expression, but recent studies suggest that, in different systems, expression may be controlled at the promoter level by sex hormones, hypoxia and methylation status. Conflicting data exist with regard to the effect of the sex hormones estrogen, progesterone, and testosterone on ABCG2 expression [24–27], but all reports note some effect. Additionally, ABCG2 expression is upregulated in the mammary gland during lactation [28]. Krishnamurthy and colleagues were the first to demonstrate that hypoxia regulates ABCG2 expression [29]. They posit that stem cells or tumor cells in hypoxic environments may be protected from chemotherapeutic agents due to the increased levels of ABCG2 induced by hypoxia [29]. In a multiple myeloma system as well as a renal carcinoma system, ABCG2 promoter hypermethylation was linked to a decrease in ABCG2 expression [30, 31]. Further research is still needed to accurately characterize the mechanisms controlling ABCG2 expression.

3 ABCG2 expression in normal tissues

The discovery of ABCG2 led to several subsequent reports investigating expression in normal tissues. The initial report of Doyle and colleagues noted the highest level of ABCG2 expression in the placenta, with lower levels in the brain, prostate, small intestine, testis, ovary, colon and liver, as determined with a cDNA probe [15]. The development of antibodies to ABCG2 enabled the detection of ABCG2 in formalin-fixed, paraffin-embedded tissues. Maliepaard et al examined ABCG2 expression in normal tissues and cancer cell lines using the BXP-21 and BXP-34 monoclonal antibodies. ABCG2 expression was found in the placenta, particularly in the synctiotrophoblastic cells, as well as in the colon, small intestine, biliary canaliculi, breast tissue, venous endothelium, and in capillaries [32]. Our laboratory subsequently developed a polyclonal anti-ABCG2 antibody (termed 87405 or 405) [33] and observed high ABCG2

expression in the alveolar pneumocytes, sebaceous glands, small and large intestine, islet and acinar cells of the pancreas, zona reticularis of the adrenal gland, hepatocytes, cortical tubules of the kidney, and prostate epithelium in addition to previously reported sites [34]. These tissue localization studies suggested a protective role for ABCG2, and for some tissues, further work has supported this theory.

3.1 Placenta

Since high levels of ABCG2 have been observed in synctiotrophoblasts at the chorionic villus [32, 34], it has been postulated that ABCG2 forms part of the maternal–fetal barrier, serving to protect the fetus from endogenous and exogenous toxins. Jonker and colleagues demonstrated that, when the dual Pgp and ABCG2 inhibitor elacridar (GF120918) was administered with topotecan *to Abcb1/2*-deficient pregnant mice, fetal plasma topotecan levels were twice those of mice treated with topotecan alone, supporting a protective role for ABCG2 in the placenta [35]. The use of *Abcb1/2*-deficient, thus Pgp-deficient, mice in these studies allowed the authors to isolate the ABCG2 transporter for study; as topotecan is a substrate for both Pgp and ABCG2, and elacridar is an inhibitor of Pgp as well as ABCG2. In placenta perfusion studies, Staud et al demonstrated transport of the ABCG2 substrate cimetidine from the fetal to the maternal space against a concentration gradient [36]. They suggest that ABCG2 in the placenta serves to reduce passage of substrates from mother to fetus as well as to reduce substrates in the fetal circulation [36].

3.2 Mammary gland

Surprisingly, ABCG2 expression in the mammary gland has been found to concentrate substrates, including toxins, into breast milk. Jonker et al found ABCG2 expression was induced in the lactating mammary glands of mice, cows and humans, and reported higher levels of topotecan and the carcinogen 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP) in the milk of lactating wild-type mice compared to Abcg2-deficient mice [28]. ABCG2 has also been shown to concentrate the carcinogen aflatoxin B1 [37] as well as antibiotics [38] into breast milk. The reason why ABCG2 is induced in the lactating mammary gland has yet to be elucidated.

3.3 Testis

We have reported high levels of ABCG2 in the interstitial cells of the normal testis as well as in Sertoli/Leydig cells [34]. Bart and colleagues have also reported high ABCG2 expression by myoid cells and cells of the luminal capillary endothelial wall of the normal testis [39]. Lasalle and colleagues have reported ABCG2 expression in germinal stem cells, again suggesting a role in protection against genotoxic mutagens [40].

3.4 Blood-brain barrier

ABCG2 appears to form part of the blood-brain barrier along with Pgp. Cooray et al found high ABCG2 expression at the luminal surface of the microvessel endothelium, suggesting a protective role, much like Pgp [41]. Brain vessels extracted from nonmalignant human brain tissue have been shown to have higher expression of ABCG2 compared to Pgp or MRP1 [42]. Similarly, Cisternino et al reported 700-fold higher Abcg2 expression in the mouse brain microvasculature compared to the cortex in wild-type mice [43]. Additionally, they found brain microvasculature Abcg2 expression to be 3-fold higher in Abcb1-deficient mice compared to wild-type, suggesting that ABCG2 upregulation may occur as a compensatory mechanism [43]. Preliminary studies have shown that inhibition of ABCG2 in addition to Pgp may improve brain penetration of some drugs [44]. However, the multifaceted, complex nature of the blood-brain barrier will probably limit the uptake of most, if not all, substrates [45] and the CNS will remain a sanctuary site. This is a strategy desperately in need of development, as a number of important substrates are limited in their CNS uptake. These include some HIV protease inhibitors and numerous anticancer agents known to be substrates for both Pgp and ABCG2 [45].

3.5 Gastrointestinal tract

Initial studies reporting the presence of ABCG2 in the small intestine and subsequent immunohistochemical studies localizing ABCG2 to the epithelium of the small intestine implied a potential role for ABCG2 in substrate absorption [15, 32]. Jonker and colleagues were the first to confirm a role for ABCG2 in the oral absorption of substrate drugs. They administered oral topotecan in the presence or absence of elacridar and found significantly higher plasma drug levels in mice receiving elacridar [35]. Additionally, elacridar decreased plasma drug clearance, decreased hepatobiliary excretion and increased re-uptake in the small intestine [35]. Again, Abcb1/2-deficient mice were used in the study. A subsequent clinical trial confirmed the ability of elacridar to increase oral bioavailability of topotecan in humans [46]. Inhibition of ABCG2 by coadministration of gefitinib, known to inhibit ABCG2, with irinotecan resulted in a 63% increase in the oral bioavailability of irinotecan in mice [47]. The area under the concentration curve for the orally administered ABCG2 substrate PhIP was found to be nearly 3-fold higher in Abcg2-deficient mice compared to

wild-type mice, suggesting that ABCG2 limits exposure to this dietary carcinogen [48].

Taken together, these studies suggest a major role for ABCG2 in limiting the oral absorption of drugs. In fact, the expression of Pgp in the gut has long been appreciated and studies have attempted to discover the impact of inhibiting Pgp on the oral bioavailability of drugs; however, expression of other transporters or detoxifying enzymes has sometimes led investigators to question the effect of Pgp on bioavailability [49, 50]. Further, studies correlating drug absorption with single nucleotide polymorphisms thought to alter Pgp expression levels in the gut have not yielded consistent results [51]. If the data emerging from ABCG2 studies do not reflect early publication bias, but rather an actual clinical impact of ABCG2 on drug absorption, the results are remarkable.

3.6 Hematopoietic stem cells

Hematopoietic stem cells had previously been characterized by transport of the fluorescent compound Hoechst 33342, recognized as the "side population" or SP when isolated by flow cytometry [52]. Zhou et al were the first to report Abcg2 to be responsible for the transport of Hoechst dye in murine hematopoietic stem cells [53] and Scharenberg and colleagues showed ABCG2 to be responsible in human cells [54]. Interestingly, ABCG2 is not required for normal hematopoiesis, as Abcg2-deficient mice are viable and have no hematologic abnormalities; however, Abcg2-deficient mice no longer display a SP phenotype [55, 56]. Zhou and colleagues have shown that Abcg2-expression does serve to protect the bone marrow from toxins using a competitive repopulation assay [56].

Subsequent to studies in the bone marrow, a side-population has been described for many normal tissues [57–61], although its role in these tissues has yet to be elucidated. Side population cells have also been identified in several cancer cell lines and primary tumor samples and have been shown to be resistant to ABCG2 substrates [62–65]. This finding has led to the idea of an intrinsically resistant cancer stem cell (see below) that has been postulated to account for the resistance of cancer to chemotherapy.

4 Substrates of ABCG2

Since ABCG2 was first described in drug-resistant cell lines, a number of chemotherapeutic agents have been shown to be transported by the protein. Resistance to mitoxantrone is the hallmark of the phenotype conferred by ABCG2 expression as is resistance to the camptothecin derivatives 9-aminocamptothecin, topotecan, irinotecan, and SN-38 (the active metabolite of irinotecan) [66, 67]. Selection with mitoxantrone [68], topotecan [69, 70] or SN-38 [71] results in ABCG2 overexpression as does selection with flavopiridol [72]. Even selection with DX-8951f or BNP-1350, camptothecins that are relatively poor ABCG2 substrates, results in ABCG2 upregulation [73, 74]. Indolocarbazole topoisomerase I inhibitors J-107088 and NB-506 [75] have been shown to be transported by ABCG2, as have the tyrosine kinase inhibitors CI1033 [76], gefitinib [77], and imatinib [78]. Overexpression of ABCG2 has also been shown to confer resistance to methotrexate and, to a lesser extent, methotrexate di- and triglutamate [79, 80]. The antifolates Tomudex and GW1843 are also substrates of ABCG2 [81]. A summary of chemotherapeutic agents transported by ABCG2 is provided in Table 1.

One early mystery surrounding the cross-resistance profile conferred by ABCG2 overexpression concerned the transport of anthracyclines and the fluorescent compound rhodamine 123. In addition to high levels of cross-resistance to mitoxantrone, the drug-selected cell lines MCF-7 AdVp3000 and S1-M1-3.2 exhibited high levels of resistance to doxorubicin and transport of rhodamine was readily observed [82, 83]. However, MCF-7 MX cells did not display equally high levels of cross-resistance to doxorubicin despite very high levels of resistance to mitoxantrone and high levels of ABCG2 protein expression [84]. In addition, we found that the MCF-7 MX cell line did not transport rhodamine 123 [84]. Upon sequencing the *ABCG2* gene in a series of parental and ABCG2-overexpressing cell lines, we found that cells with a glycine or threonine at amino acid 482 readily transported doxorubicin and rhodamine 123 in addition to mitoxantrone while cells that expressed wild-type ABCG2 with an arginine at position 482 only transported mitoxantrone [85]. Allen and colleagues reported mutation of the arginine at position 482 in mouse Abcg2 to a methionine or serine, suggesting it to be a "hot spot" for mutation [86]. Substitution of the various amino acids by site-directed mutagenesis at position 482 has shown that mutation of the wild-type arginine to almost any other amino acid results in this gain-of-function phenotype [87–89]. Mutations at amino acid 482 have also been shown to affect the potency of ABCG2 inhibitors [90, 91] as shown in Fig. 1. While it is possible that tumors harboring mutations in ABCG2 may turn out to be cross-resistant to a wider variety of chemotherapeutic agents than tumors that express wild-type ABCG2, amino acid 482 mutations have yet to be found in clinical samples, suggesting that this observation has limited clinical relevance.

Table 1 Selected substrates and inhibitors of ABCG2

Substrates	Inhibitors
Mitoxantrone [66]	Fumitremorgin C [83, 102]
Daunorubicin[a] [14, 86, 90, 169]	Ko143 [103]
Doxorubicin[a] [14, 83, 86, 90, 169]	Cyclosporin A [82, 187, 188]
Epirubicin[a] [90]	Tacrolimus [187]
Bisantrene[a] [83, 90]	Sirolimus [187]
Flavopiridol [72, 170]	Gefitinib [110, 189–191]
Etoposide [10, 11, 14, 171]	Imatinib [191, 192]
Teniposide [171]	Elacridar (GF120918) [106, 175]
9-aminocamptothecin [13, 172–175]	Tariquidar (XR9576) [107]
Topotecan [13, 69, 70, 173, 175–177]	Biricodar[a] (VX-710) [91]
Irinotecan [13, 175, 178, 179]	Chrysin [113]
SN-38 [13, 71, 172, 175, 180, 181]	6-prenylchrysin[a] [115]
Diflomotecan[a] [182]	Tectochrysin[a] [115]
Homocamptothecin[a] [182]	Naringenin [96]
DX-8951f [73, 178]	Quercetin[111, 181]
BNP1350 [74]	Acacetin [96]
J-107088 [75]	Silymarin [111]
NB-506 [75]	Genistein [96]
UCN-01 [107]	17{beta}-estradiol [193]
Methotrexate[a], methotrexate di[a]- and triglutamate[a] [79–81, 100, 183, 184]	Estrone [193]
	Tamoxifen [194]
GW1843[a] [81]	Ortataxel [165]
Tomudex[a] [81]	Novobiocin[a] [108, 109]
Imatinib [78]	Reserpine [53]
Gefitinib [77, 110]	WK-X-34 [195]
CI1033 [76]	Curcumin [196, 197]
Pheophorbide a [107, 185]	Dipyridamole [198]
Pyropheophorbide a methyl ester [186]	Nicardipene [116, 117, 198]
Chlorin e6 [186]	Nitrendipene [116, 117, 198]
Protoporphyrin IX [29, 186]	Nimodipene [198]

[a] Designates compounds known to be affected by the amino acid at position 482.

ABCG2 has also been shown to play a role in the transport of non-chemotherapeutic agents. It has been observed to be a factor in the biliary excretion of HMG-CoA reductase inhibitors such as rosuvastatin [92], pitavastatin [93, 94], pravastatin [95] and cerivastatin [95]. The flavonoids genestein and quercetin have been shown to be transported by ABCG2 [96–98] as have several antibiotics [38, 99, 100]. Anthelmintic benzimidazoles have also been demonstrated to be ABCG2 substrates [101]. The expanding list of ABCG2 substrates highlights the fact that ABCG2 may play a significant role in pharmacology and points to a need for systematic studies aimed at identifying drugs that have ABCG2 as a principal modulator of oral absorption. The identification of SNPs, as outlined below, that reduce ABCG2 function makes this area of study a high priority in the effort to determine sources of interpatient variability in drug disposition.

5 Inhibitors of ABCG2

Interestingly, the first reported inhibitor of ABCG2, fumitremorgin C (FTC), was described before the gene had been cloned [83]. FTC, a diketopiperazine isolated from the fermentation broth of *Aspergillus fumigatis*, was first shown by Rabindran and colleagues to inhibit resistance in the mitoxantrone-selected S1-M1-3.2 colon cancer cell line [83]. Subsequently, FTC was shown to inhibit ABCG2-mediated transport of antineoplastics in stably-transfected MCF-7 cells [102]. The neurotoxicity of FTC prevented its clinical use, thus prompting the discovery of the FTC analogue Ko143 [103]. Other diketopiperazine inhibitors, including the indolyl diketopiperazines [104] and tryprostatin a [105], have also been described.

Several Pgp inhibitors have also been reported to inhibit ABCG2. We demonstrated that elacridar (GF120918) acts as an ABCG2 inhibitor [106], as does the potent Pgp inhibitor tariquidar (XR9576) [107]. As noted earlier, the ability of elacridar to inhibit ABCG2 in vivo was independently confirmed in studies with topotecan in Abcb1/2-deficient mice [35]. Reserpine has also been shown to inhibit ABCG2-mediated Hoechst 33342 transport in SP cells [53]. Minderman and colleagues have observed that biricodar (VX-710) is able to inhibit Pgp-, MRP1- and ABCG2-mediated transport [91].

The list of reported ABCG2 inhibitors has been growing rapidly. Novobiocin was identified early on as an ABCG2 inhibitor [108, 109]. Tyrosine kinase inhibitors have been shown to inhibit ABCG2, most likely as competitive inhibitors, since ABCG2 has been shown to directly transport or confer resistance to CI1033, gefitinib and imatinib [76–78, 110]. The flavonoids silymarin, hesperetin, quercetin and daidzein, as well as the stilbene resveratrol, were shown to increase intracellular accumulation of mitoxantrone and BODIPY-prazosin in ABCG2-expressing cells [111]. Chrysin and biochanin a have also been shown to inhibit ABCG2 [112], in addition to [113] genestein, naringenin, acacetin and kaempferol [96]. Structure activity studies have also identified 6-prenylchrysin and tectochrysin as ABCG2 inhibitors [114, 115]. Dihydropyridines and pyridines have been shown by several groups to interact with ABCG2 [116, 117]. A summary of selected ABCG2 inhibitors is provided in Table 1. Despite the explosion of publications identifying ABCG2 inhibitors, none have been used in the clinical setting.

6 Single nucleotide polymorphisms (SNPs)

Given the putative role of ABCG2 in pharmacology, SNPs and their impact on protein expression and function may have a direct impact on drug dosing and may play a role in response to treatment. While a number of SNPs have been reported, a nonsynonymous SNP at amino acid 141 resulting in a glutamine to lysine amino acid change has been studied extensively [118]. Various researchers have found that this SNP can lead to lower plasma membrane expression [119–122], reduced drug efflux [123, 124], and/or reduced ATPase activity [122, 123]. Expression of the Q141K SNP in cell lines has been shown to lead to significantly lower IC50 values for ABCG2 substrates, including mitoxantrone, irinotecan, and SN-38 [119, 122]. The Q141K SNP has been shown to influence the pharmacokinetics of orally administered drugs, including topotecan [125], diflomotecan [126] and 9-aminocamptothecin [127]. As noted earlier, the higher plasma drug levels due to the Q141K SNP may result in exquisite sensitivity to certain orally administered chemotherapy drugs. While this could result in tumors being exposed to higher drug levels, eliciting a greater response to treatment, it could also cause increased toxicity. Which outcome prevails depends upon individual drug pharmacodynamics.

7 Expression of ABCG2 in clinical samples

In view of the fact that chemotherapeutic agents are readily transported by ABCG2, determining its relevance in drug resistance has become an important goal. Since leukemia samples are ideal for flow cytometric as well as PCR-based studies, several reports have surfaced that examine expression of ABCG2 in leukemic blasts. Ross and colleagues were the first to report on ABCG2 expression in a series of leukemia samples. They examined 20 acute myelogenous leukemia (AML) samples and one acute lymphocytic

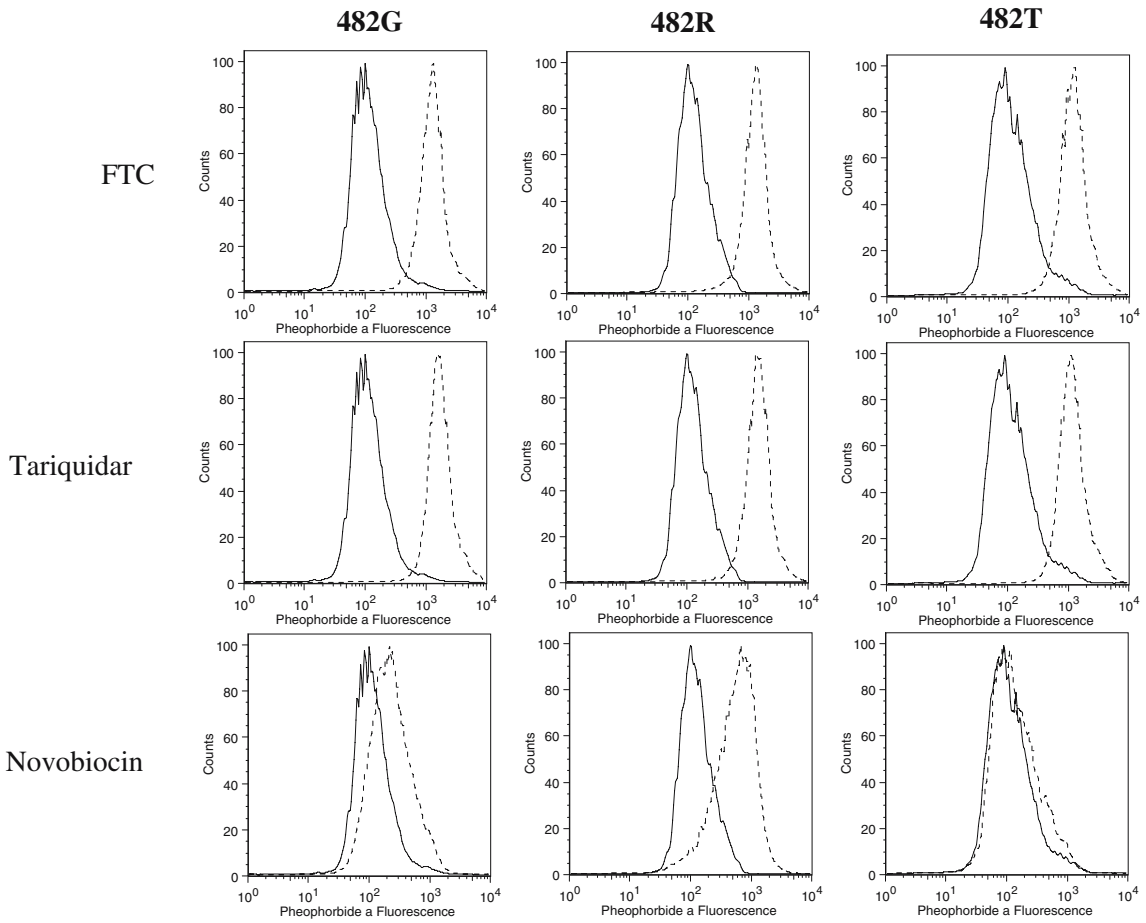

Fig. 1 Mutations at amino acid 482 of ABCG2 affect inhibitor efficacy. Human embryonic kidney cells (HEK-293) stably expressing wild-type (482R) or mutant (482G, 482T) were incubated with 1 μM PhA in the presence of absence of 10 μM FTC, 10 μM tariquidar or 50 μM novobiocin for 30 min. Cells were then washed then allowed to incubate for 1 h in PhA-free medium continuing with (*dashed line*) or without (*solid line*) inhibitor. Note that, while FTC and tariquidar completely inhibited all forms of the protein, denoted by the difference between the solid and dashed histograms, novobiocin was only able to completely inhibit wild-type (482R) protein

leukemia (ALL) sample, finding relatively high expression in seven samples and a 1,000-fold variation in expression [128]. This early report seemed to suggest that ABCG2 expression might be a significant prognostic factor in leukemia. Since this initial report; however, results have varied widely, with relatively high ABCG2 expression in AML reported by some groups [129] and low levels reported by others [130–132]. Similarly, some groups reported ABCG2 expression was associated with response [133, 134] while others found no correlation [131]. In the largest study reported to date, with 149 patient samples, Benderra and colleagues reported that ABCG2 expression was predictive of complete remission 4-year disease-free survival, and 4-year overall survival in patients receiving daunorubicin or mitoxantrone [133].

It has also been suggested that ABCG2 might play a role in drug resistance in acute lymphoblastic leukemia (ALL). Steinbach et al reported a correlation between ABCG2 expression and prognosis [136]. Similarly, Stam et al reported ABCG2 expression correlated with resistance to Ara-C despite the fact that the drug is not an ABCG2 substrate [137]. In contrast, Sauerbrey and colleagues did not find a correlation between ABCG2 expression and response in childhood ALL [138].

To clearly delineate the role of ABCG2 in drug resistance in leukemia, larger studies of ABCG2 expression in clinical samples are necessary. Results available to date for studies in leukemic samples are reported in Table 2. Such studies have repeatedly shown over the past two decades that Pgp expression is important in leukemia [4]. While the precise clinical endpoint was not always the same, the conclusion was—Pgp impacted negatively on treatment outcome in AML. Fewer data exist for ALL. It would be a mistake to carry out further studies of ABCG2 in AML and ALL without also assessing Pgp expression. Methods for evaluating the impact of each transporter alone and together are needed. A recent cDNA array analysis of AML samples identified six subsets of AML based on their gene expression profile [139]. One of these subsets had notably higher Pgp and ABCG2 expression and was associated with the highest rate of resistant disease [139].

Reports of ABCG2 expression in solid tumors have begun to appear in the literature and Table 3 presents a summary of the clinical findings. The report of Diestra et al, examining ABCG2 expression in 150 paraffin-embedded, untreated tumor samples from various origins is by far the most extensive. They found frequent expression of ABCG2, especially in tumors from the digestive tract, endometrium, lung and melanoma [140]. Before a definitive answer can be reached regarding the contribution of ABCG2 to cancer drug resistance, larger studies such as these will be needed.

In breast cancer, the most widely studied tumor type, ABCG2 expression was relatively low and did not appear to correlate with clinical outcome in the studies of Kanzak et al [141] or Faneyte et al [142], but was correlated with response in patients treated with anthracyclines in the study by Burger et al [143]. This is a somewhat surprising result, as anthracyclines do not appear to be transported by ABCG2 [90]. Yoh et al reported that ABCG2 expression in non-small cell lung cancer was predictive of a lower response rate in patients receiving platinum-based chemotherapy [144], but, again, this is an unexpected result, since platinum compounds are not transported by ABCG2. Friedrich et al found that increased ABCG2 expression correlated with loss of differentiation and shorter survival in oral squamous cell carcinoma [145]. In studies of testicular tumors, Zurita et al and Diestra et al found no correlation between ABCG2 expression and response despite positive immunohistochemical staining in some samples [146, 147].

The finding that ABCG2 expression could correlate with clinical outcome when a non-ABCG2 substrate is being used is intriguing. At first glance, this might appear to be publication bias, wherein those of us interested in clinical correlates only follow up results that are statistically significant. Such early correlates often prove incorrect in followup studies. However, alternate explanations can be posed. For one, ABCG2 may be transporting a substrate that is necessary for cell death, thus mediating resistance without actually transporting the drug with which the tumor is treated. Alternatively, whatever the treating drug, the most sensitive cells in the tumor are eliminated leaving behind drug resistant cells. Tumors with higher levels of drug resistant cells will be more refractory, resulting in poor treatment outcome. What cells would be both ABCG2 expressing and drug resistant?

8 Cancer stem cells

The cancer stem cell theory is based on the idea that only a small fraction of tumor cells is capable of repopulating a tumor, much like hematopoietic stem cells found in bone marrow from which all blood cells differentiate. These cells are capable of long-term self renewal, divide slowly, are drug resistant, and express ABCG2 [148, 149]. The “side population” or “SP” phenotype has been used to identify bone marrow stem cells based on their ability to transport the fluorescent compound Hoechst 33342 [52]. As mentioned earlier, SP cells express high levels of ABCG2 and they thus appear dimmer than the rest of the cell population due to transport of Hoechst 33342 out of the cell [53]. Using Hoechst 33342 as a probe, SP cells have been found in normal breast, lung, and liver tissue [58, 59, 150] as well

Table 2 Summary of clinical reports examining ABCG2 expression in leukemia studies

Study	Methods	Samples	Findings
Acute Myelogenous Leukemia (AML)			
Sargent et al [199]	Immunohistochemistry	20 blast samples	ABCG2 positivity in six out of 22 (27%) samples
van der Kolk [130]	Flow cytometry	20 paired samples: pre treatment and relapse/refractory	ABCG2 expression low in AML. No increase in expression at relapse.
Abbott et al [131]	RT-PCR	40 de novo samples	High ABCG2 levels in AML may be due to small subset to cells. ABCG2 expression did not correlate with response.
van Den Huevel-Eibrink et al [157]	RT-PCR	20 paired de novo and relapsed/refractory AML samples	Higher ABCG2 expression in relapse samples compared to samples at diagnosis. ABCG2 expression associated with ABCB1 expression.
van der Pol et al [132]	Flow cytometry	45 paired samples	No detectable ABCG2 function in almost all of the samples. No changes in expression post therapy.
Galimberti et al [129]	RT-PCR	51 AML samples	ABCG2 expression in 56% of samples, intermediate levels in 48.2%. ABCG2 expression associated with ABCB1 expression.
Suvannasankha et al [200]	RT-PCR Flow cytometry	31 pretreatment blast samples	ABCG2 expression between 8226 and 8226/MR20 levels. ABCG2 expression and function in subsets of cells. No correlation between function, protein or gene expression in AML samples.
Benderra et al [133]	RT-PCR	149 de novo samples	Complete remission, 4-year DFS and 4-year overall survival associated with ABCG2 expression. Predictive in patients receiving daunorubicin, and mitoxantrone but not idarubicin.
Uggla et al [134]	RT-PCR	40 AML samples	Patients responding to therapy with higher ABCG2 expression had shorter overall survival. No difference in expression between responders and non-responders.
Acute Lymphocytic Leukemia (ALL)			
Sauerbrey et al [138]	RT-PCR	47 de novo samples 20 relapsed samples	Lower ABCG2 expression in T-cell lineages. No correlation between expression and response.
Steinbach et al [136]	RT-PCR	59 untreated AML Nine relapse cases	Median ABCG2 levels 10-fold higher in patients who did not achieve remission compared to responders. ABCG2 levels higher in first relapse than at diagnosis. Expression related to prognosis.
Plasschaert et al [201]	Flow cytometry	46 de novo samples	BXP-34 positivity in B-lineage 2.4-fold higher than T-lineage. Higher gene expression correlated with higher function. No aa482 mutations.
Stam et al [137]	RT-PCR	13 samples from infants 13 samples from non-infants	ABCG2 expression 2.4-fold less in samples from infants. Expression correlated with Ara-C resistance, but drug is not a substrate.
Suvannasankha et al [135]	RT-PCR Flow cytometry	30 pretreatment samples	BXP-21 positive cells in 43% of cases. Mitoxantrone transport in 70% of cases. BXP-21 staining predictive of shorter DFS.

Table 3 Summary of clinical reports examining ABCG2 expression in solid tumors

Study	Cancer	Method	Samples	Findings
Scheffer et al [202]	Various	Immunohistochemistry	34 paraffin-embedded, untreated tumor samples from various cancer types	Positive staining in only one small-intestine adenocarcinoma sample.
Diestra et al [140]	Various	Immunohistochemistry	150 paraffin-embedded, untreated tumor samples from various cancer types	Frequent expression of ABCG2. Highest levels in tumors from the digesting tract, endometrium, lung and melanoma. Both cytoplasmic and membrane staining.
Kanzaki et al [141]	Breast	RT-PCR	43 untreated breast carcinoma samples	Low ABCG2 expression. No correlation with ABCB1, ABCC1 or LRP expression. No correlation with clinical outcome.
Faneyte et al [142]	Breast	RT-PCR	25 primary breast carcinoma 27 tumor samples from preop anthracycline therapy	Wide variation of expression by PCR, no detectable expression by immunohistochemistry. No difference between untreated and treated tumor samples. No correlation with survival.
Burger et al [143]	Breast	RT-PCR	59 primary breast tumor samples	Expression of ABCG2 and ABCB1 correlated with response in anthracycline-treated patients. Expression not related to PFS.
Kawabata et al [164]	NSCLC	RT-PCR Flow cytometry	23 untreated non-small cell lung cancer tumors	Five samples (23%) expressed ABCG2 at levels higher than topotecan-resistant NCI-H441 cells.
Yoh et al [144]	NSCLC	Immunohistochemistry	72 formalin-fixed samples	Lower response rate to platinum-based therapy and shorter PFS in ABCG2 positive samples
Zurita et al [146]	Testicular germ cell cancer	Immunohistochemistry	56 paraffin-embedded, de novo samples	Strong and intermediate ABCG2 expression in 86 and 7% of samples, respectively. No correlation with response.
Bart et al [39]	Testicular tumors	Immunohistochemistry	Ten non-seminoma, ten seminoma and nine testicular lymphoma cases	ABCG2 expression in seminomas and non-seminomas but not lymphoma. Newly formed vessels in all tumors expressed ABCG2.
Diestra et al [147]	Bladder Cancer	Immunohistochemistry	83 locally advanced bladder tumors	ABCG2 positivity in 28% of samples. No prognostic impact.
Friedrich et al [145]	Oral squamous cell carcinoma	RT-PCR Immunohistochemistry	45 primary tumor samples	2.4-fold increase in ABCG2 expression between stage III and IV disease. Increased ABCG2 expression associated with loss of differentiation and shorter survival. Moderate association with ABCB1 expression.
Aust et al [203]	Gallbladder carcinoma	Immunohistochemistry	41 untreated gallbladder carcinoma samples	Strong ABCG2 staining in 11 of 41 samples.
Glasgow et al [155]	Colorectal cancer	RT-PCR	21 mucinous colorectal tumors 30 non-mucinous colorectal tumors	No difference in ABCG2 expression between mucinous and non-mucinous tumor types.
Konig et al [156]	Pancreatic carcinoma	RT-PCR	31 pancreatic carcinoma samples	No difference in ABCG2 expression between normal or cancerous tissue.
Deichmann et al [204]	Melanoma	RT-PCR Immunohostochemistry	50 primary cutaneous melanoma 16 melanoma metastases Ten acquired melanocytic nevi 29 neuroendocrine skin carcinomas Ten dermatofibrosarcoma samples	Moderate BXP-21 positivity in three of 29 neuroendocrine tumors in single clusters of cells.

as in the corneal stroma [61]. SP cells have been found in a number of established tumor cell lines as well as tumor samples and have been shown to have stem cell-like qualities, overexpress ABCG2, and possess inherent drug-resistance [62–65].

While some reports seem to suggest ABCG2 to be a marker of stem cells, several pieces of evidence suggest that ABCG2 expression alone does not define the stem cell population. First, ABCG2 expression is not an absolute requirement for stem cells. Abcg2-deficient mice are viable and demonstrate no defect in steady state hematopoiesis, although the bone marrow of Abcg2-deficient mice does lack an SP [56, 151]. Second, the SP fraction is not known to be a pure stem-cell population and the method used for generating SP cells greatly affects the cells included in it [152]. In some cases, ABCG2 expression is part of the normal differentiated phenotype; A549 cells have been shown to express ABCG2 and transport Hoechst 33342 [54] but the A549 cell line is not considered a "stem cell" line. Finally, drug-resistant cells that overexpress ABCG2 in response to selection pressure are not necessarily believed to be stem cells and ABCG2-positive and negative tumor cells have been reported to be similarly tumorigenic [153].

If an ABCG2-positive population of stem cells is, in fact, a characteristic of tumors, then they will most likely be a drug-resistant population of cells. Haraguchi and colleagues have shown SP cells to be more resistant to chemotherapeutic agents than non-SP cells in gastrointestinal cancer cell lines [65]. Similarly, Hirschmann-Jax et al. demonstrated increased mitoxantrone transport in SP cells obtained from neuroblastoma tumors as well as an increase in the SP fraction when neuroblastoma cell lines were treated with increasing concentrations of mitoxantrone [63]. Thus, the development of potent, specific inhibitors to target ABCG2-mediated transport may hold the key to eliminating this stem cell population.

9 Avoiding mistakes of the past

Two decades of Pgp research have not yet clarified the role of Pgp in drug resistance in the clinical setting. Reasons for this include the lack of validated assay methods, reliance on the clinical trial process to define the role of Pgp, and the possibility that other ABC transporters may be present, confounding clinical trial results. A major problem in assessing the significance of Pgp expression in clinical drug resistance has been the variability in measuring Pgp expression [154]. Despite the existence of several reliable methods to detect Pgp, findings often vary across laboratories, as evidenced by the results of a Pgp detection workshop [154]. Established, validated, widely-available, and consistent methods are still needed to further confirm the contribution of Pgp to clinical drug resistance. As the idea of ABCG2 as a mediator of drug-resistance is in its infancy, it is important that the same mistakes made with Pgp are avoided with ABCG2. Validated, standard, reproducible, and sensitive methodologies for detecting ABCG2 must be developed in order to characterize the role of ABCG2 in drug resistance.

9.1 PCR

PCR assays for ABCG2 detection have been carried out by several groups; however, there is no consistent cell line used as a reference. Comparing ABCG2 expression in patient samples to that of a well-characterized, stable cell line with low but detectable ABCG2 expression becomes important when trying to understand resistance conferred by ABCG2. Otherwise, the numbers generated by PCR analyses have no real meaning. While some PCR studies have included a low level ABCG2-expressing cell line such as MCF-7 [129, 131, 134, 136, 142], many do not include a reference at all [135, 155–157]. While highly ABCG2-positive cell lines can be included in the assay as a positive control, they should not be a substitute for cells expressing low levels. We recommend that the MCF-7 cell line, at the very least, be included in the analysis and, preferably, that more than one reference cell line be used as a control.

It is tempting to use highly drug-resistant cells as positive controls for an assay, but expression is usually so high that the levels are often not clinically relevant. Faneyte et al presented an elegant study of the breast cancer resistance profile in breast cancer by PCR analysis. Although the PCR assay was standardized to a drug-resistant cell line, several unselected cell lines were included. By PCR, the drug-resistant cell line Igrov/T8 was assigned a value of 4.0 units and MCF-7 cells had a value of 0.48 units [142]. ABCG2 expression in the breast cancer samples examined averaged 0.18 units [142]. Furthermore, immunochemistry showed staining in normal duct cells, but not in breast cancer cells [142]. This result is reminiscent of the observations with Pgp in that normal cell expression exceeds that of the de-differentiated cancer cell [158]. Taken together, Faneyte concluded that ABCG2 would not play a major role in drug resistance in breast cancer [142].

Present day studies of ABC transporters in drug resistance should also examine expression of multiple ABC transporters. Several excellent examples of such an effort to detect multiple transporter proteins have been reported [143, 157]. When Burger et al examined MDR-1, BCRP, LRP, MRP1, and MRP2 expression in 59 primary breast cancer samples by PCR, they concluded that MDR-1 expression was most closely associated with poor progres-

sion-free survival [143]. These results supported the findings of a meta-analysis that concluded that Pgp was important in breast cancer outcome [159] and affirmed the results of Faneyte et al. A new marker of drug resistance apparently is not always better.

9.2 Immunohistochemistry

Several antibodies are available for detection of ABCG2 by immunohistochemistry. Maliepaard and colleagues developed the BXP-21 antibody that is able to detect ABCG2 by immunoblot and in formalin-fixed, paraffin-embedded tissue samples [70]. The monoclonal 5D3 antibody, reported by Zhou and colleagues [53], recognizes an external epitope of ABCG2 and our laboratory has successfully used it to detect ABCG2 on formalin-fixed, paraffin-embedded tissue samples [34]. The polyclonal antibody 87405 (or 405) was developed in our laboratory using an immunizing peptide [33]. The antibody detects ABCG2 by immunoblot as well as in formalin-fixed, paraffin embedded tissues [33, 34], but is unable to detect ABCG2 by flow cytometry. Additionally, 87405 can detect ABCG2 in humans as well in mice, pigs, cows, and sheep [160, 161].

As suggested for PCR studies, several cell lines that express low but detectable levels of ABCG2 should be used to assess the sensitivity of the antibodies used. The MCF-7 cell line is again an ideal choice, although levels may be too low for reproducible detection, thus rendering immunohistochemistry a rather insensitive method. Tissues known to express ABCG2, such as the placenta or skin (sebaceous glands), may be included as positive controls. It will be interesting to see whether ABCG2 parallels Pgp in being higher in differentiated tissue. Pgp is higher in adjacent normal tissue than in cancer tissue due to the dedifferentiation that occurs in cancer [158]. So the inclusion of placenta tissue as a positive control in PCR or immunoblot analyses could result in setting up an assay to be relatively insensitive [162]. Particularly for polyclonal antibodies, the peptide used to generate the antibody should be used to verify that the observed staining is not background. Until the antibodies used for immunohistochemistry are better characterized, at least two antibodies should be used to ensure internal consistency of the results. It appears BXP-34 is not as sensitive as BXP-21 in immunohistochemical studies [140].

9.3 Flow cytometry

Functional assays for ABCG2 have been developed using fluorescent ABCG2 substrates and an ABCG2 inhibitor. While assaying transporter function by flow cytometry can be quite reliable, and can in some ways be considered the "gold standard" since it measures actual transporter function, the assay has limitations, mostly due to the fact that the fluorescent compounds used are substrates for multiple transporters. Mitoxantrone and BODIPY-prazosin were among the first substrates used in flow-cytometry based assays for ABCG2 function [84, 163]. Transport of topotecan has also been shown to correlate with ABCG2 expression [164]. However, as mitoxantrone, BODIPY-prazosin, and topotecan are also substrates of Pgp [84, 165], these substrates may not be useful in samples where other transporters are expressed at high levels. The porphyrin pheophorbide a (PhA) has been identified as an ABCG2-specific substrate, as it was not found to be transported by Pgp or MRP1 [107]; however, we have not examined whether it is transported by other ABC transporters. If confirmed, pheophorbide a would be an ideal substrate for

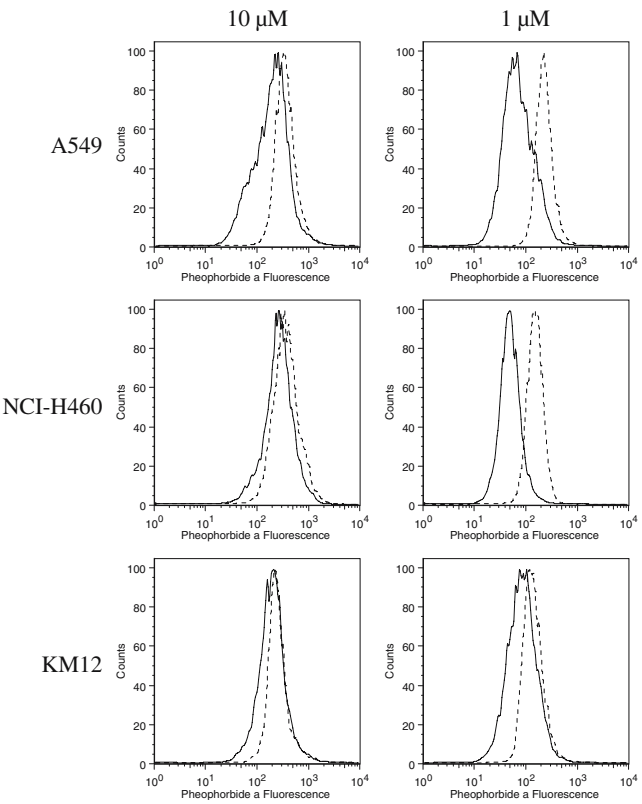

Fig. 2 Increasing sensitivity of the PhA flow assay. The A549, NCI-H460 and KM12 cell lines which are known to express low but detectable levels of ABCG2 were incubated with 1 μM (*left column*) or 10 μM (*right column*) PhA in the presence of absence of 10 μM FTC, for 30 min. Cells were then washed then allowed to incubate for 1 h in PhA-free medium continuing with (*dashed line*) or without (*solid line*) FTC. ABCG2 expression, denoted by the difference between the solid and dashed histograms, is more readily detected when cells are incubated with 1 μM PhA

studying ABCG2 function in clinical samples where multiple drug transporters may be expressed.

When determining ABCG2 function in clinical samples, we recommend that cells be incubated with pheophorbide a in the presence or absence of an ABCG2 inhibitor such as FTC for an initial 30 min period. Subsequently, cells should be washed and allowed to incubate for an additional hour in pheophorbide-free medium continuing with FTC to obtain the mean value of the FTC/Efflux histogram, or without FTC to obtain the mean value of the Efflux histogram [107]. Results can be presented as the difference in channel number between the Efflux and FTC/Efflux histograms or the Kolmogorov–Smirnov D value can be reported. In this way, one can assess the ability of the protein to prevent PhA accumulation, as well as the ability to extrude intracellular PhA. This becomes especially important when examining cells that express low levels of ABCG2. As shown in Fig. 2, using PhA at a concentration of 1 μM allows for greater sensitivity in detecting the low ABCG2 levels in MCF-7, A549, and H460 cells compared to using PhA at a concentration of 10 μM. Use of several low-level ABCG2-expressing cell lines is recommended, with MCF-7 included as a minimum.

Relatively few antibodies exist for detection of ABCG2 protein by flow cytometry. Minderman and colleagues have developed methodologies to detect ABCG2 by flow cytometry using the BXP-21 or BXP-34 antibodies [163] and the 5D3 antibody has been used extensively in flow cytometry-based assays [90, 163, 166]. Recent studies have shown that the binding of 5D3 is sensitive to conformational changes of the ABCG2 protein, with 5D3 binding being highest in the presence of the ABCG2 inhibitor Ko143 [166]. Compounds that decreased the ATPase activity of ABCG2 were found to increase 5D3 binding [166], much like what has been reported for the UIC2 antibody that recognizes Pgp [167]. Results with the 5D3 antibody, therefore, may not be entirely accurate unless conditions are carefully controlled. Again, low level controls must be included in the analysis and multiple antibodies should be used when possible. Similar to functional assays, results can be quantitated as the difference in channel number between the negative control histogram and ABCG2 antibody histograms or the Kolmogorov–Smirnov D value.

Clearly, sensitivity issues exist among the antibodies used to measure ABCG2 expression by flow cytometry. This is exemplified in the findings of Suvannasankha et al, where ABCG2 expression was measured in pretreatment blasts from 30 adult ALL patients [135]. Expression was determined by PCR analysis, immunophenotyping with the 5D3, BXP-21 or BXP-34 antibodies, or by functional assay using mitoxantrone as the substrate. While ABCG2 expression measured by the BXP-21 antibody correlated with expression measured by the BXP-34 or 5D3 antibody, expression measured by the BXP-34 antibody did not correlate with expression measured by 5D3 antibody, nor did antibody staining correlate with gene expression [135].

In contrast, van der Kolk et al reported a good correlation between ABCG2 expression measured with BXP-21 or BXP-34 and ABCG2 function assayed with mitoxantrone [130], suggesting that differing methodologies, as well as antibody sensitivities, may be the cause of discordant results reported in patient samples. Development of sensitive and specific antibodies to detect ABCG2 by flow cytometry as well as use of consistent methodologies will be crucial to determining ABCG2 expression in clinical samples which usually express low levels. Additionally, as the BXP-34 and BXP-21 antibodies require cell permeabilization, these antibodies will most likely recognize intracellular ABCG2 and it is not yet clear what the relationship of total intracellular ABCG2 expression is to drug transport capacity.

10 Conclusion

The challenges of translating ABCG2 into a target for future clinical trials are daunting. Given the lack of success of phase II and III trials with Pgp inhibitors [168], one may argue that we still have not successfully translated Pgp into a clinical target. With regard to ABCG2, we do not yet know what cancers express ABCG2 as a mechanism of resistance, we have not developed potent, selective inhibitors to target ABCG2 and we have no surrogate assay to test for ABCG2 inhibition in vivo. However, if we make a concerted effort now to avoid the mistakes made with Pgp and instead develop sensitive, reproducible methods for assessing ABCG2 expression in clinical samples, we will be able to develop effective clinical trials aimed at overcoming ABCG2-mediated drug resistance.

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