Published in final edited form as:

Biochim Biophys Acta. 2009 July ; 1791(7): 584–593. doi:10.1016/j.bbalip.2009.01.007.

Emerging New Paradigms for ABCG Transporters

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Abstract

Every cell is separated from its external environment by a lipid membrane. Survival depends on the regulated and selective transport of nutrients, waste products and regulatory molecules across these membranes, a process that is often mediated by integral membrane proteins. The largest and most diverse of these membrane transport systems is the ATP binding cassette (ABC) family of membrane transport proteins. The ABC family is a large evolutionary conserved family of transmembrane proteins (> 250 members) present in all phyla, from bacteria to *Homo sapiens*, which require energy in the form of ATP hydrolysis to transport substrates against concentration gradients. In prokaryotes the majority of ABC transporters are involved in the transport of nutrients and other macromolecules into the cell. In eukaryotes, with the exception of the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7), ABC transporters mobilize substrates from the cytoplasm out of the cell or into specific intracellular organelles. This review focuses on the members of the ABCG subfamily of transporters, which are conserved through evolution in multiple taxa. As discussed below, these proteins participate in multiple cellular homeostatic processes, and functional mutations in some of them have clinical relevance in humans.

Keywords

ABC transporters; ABCG; Sterol transporters; Multidrug resistance; Sitosterolemia; Atherosclerosis

1. Introduction

ABC transporters are transmembrane proteins that facilitate the transport of specific substrates across the membrane in an ATP-dependent manner. Eukaryotic ABC transporters have been subdivided into either “full” or “half” transporters and into 7 subgroups, A–G, based on sequence similarity and domain organization [1,2]. Full transporters contain two ABC domains

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and two six-transmembrane helices, referred to as the “transmembrane domain” (TMD) on a single polypeptide. Some full transporters have additional transmembrane helices at the amino terminus [1,2]. As their name suggests, half transporters contain one ABC domain and one TMD on a single polypeptide. Half transporters are dependent upon the formation of hetero- or homo-dimers; thus the functional transporter still contains two ABCs and two TMDs. Each ATP-binding cassette spans approximately 125 amino acids that contain a number of small conserved domains including a Walker A and Walker B motifs that are found in all ATP binding proteins, and a C-loop or signature motif that is unique to members of the ABC family [1,2].

Although the first mammalian ABC transporter (MDR1/ABCB1) was identified over 30 years ago, the molecular mechanism involved in substrate recognition and transport across membranes remains largely an enigma. To date, there have been no reports describing a high-resolution structure of a eukaryotic ABC transporter. In contrast, recent crystallography studies of Sav1866, a bacterial multidrug half transporter from *Staphylococcus aureus*, have provided important insights into the structure and likely mechanism of action, which may well be highly relevant to the mammalian family members [3]. These authors reported that the high-resolution structure of the homodimer of Sav1866 shows the canonical 12 transmembrane helices forming a core that exhibit an “outward-facing conformation with a single substrate translocation pathway exposed to the extracellular environment”. Examination of the 3.0 Å crystal structure also shows that the two ABC motifs have a shared interface and are arranged in a head-to-tail conformation [3]. The authors suggested that in this conformation the substrate might escape into the outer lipid leaflet of the membrane or into the extracellular space. They predict that hydrolysis of the bound ATP will result in a return of the helices to form an inward facing direction so as to allow association of new substrate with the transporter. Nevertheless, exactly how each ABC transporter interacts with their substrate(s), especially when they often transport a broad array of structurally unrelated compounds, is currently poorly understood. Interestingly, genetic defects in 17 ABC transporters have been identified in humans and linked to a wide array of diseases [4], which in turn has been instrumental in identifying their physiological substrates. However, in general, identification of the physiological substrates for most ABC transporters has proven to be particularly difficult.

The current review is limited to members of the ABCG subfamily. These are unique half transporters in which the ABC domain is localized to the amino terminal side of the transmembrane domain (ABC-TMD) (Fig. 1). The ABCG subfamily is present in mammals (5 members), *Drosophila*, (15 members, including *white*, the founding member of this family), *Caenorhabditis elegans* (9 members) and *Aribidopsis thaliana* (24 members). Yeast and bacteria lack members of this family. Four of the five mammalian ABCG members, namely the homodimers ABCG1:ABCG1 and ABCG4:ABCG4 and the heterodimer ABCG5:ABCG8, have been shown to have a role in transporting sterols across membranes. Since the two TMDs function to facilitate substrate specificity and transport, it is perhaps surprising that no conserved sequence within the TMDs of these four transporters has been identified that might correspond to a sterol-binding domain. As discussed below, altered expression and/or activity of ABCG5:ABCG8 or ABCG2 are clinically relevant, resulting in sitosterolemia and abnormal sterol homeostasis, and resistance to chemotherapy, respectively.

2. ABCG1

2.1. Gene regulation and expression

Murine and human ABCG1 cDNAs were originally identified in 1996 and 1997 [5–7], and shown to encode proteins of 74 kDa that had 30% amino acid identity (51% similarity) with the *Drosophila* transporter *white* (see model organisms below).

Although different mRNAs have been reported that differ at the 5' end and encode proteins with different amino-termini, current evidence suggests that there is one major ABCG1 transcript/protein in mice and humans (reviewed in [8]). Early studies showed that ABCG1 mRNA levels were highly induced when macrophages were converted to lipid-loaded "foam" cells following incubation with modified low-density lipoproteins (LDL) or specific oxysterols, or following the induction of the nuclear receptor liver-X-receptor (LXR) [9–13]. Consistent with these observations, multiple functional LXR response elements (LXREs) have been identified in both the murine and human genes [12,14,15]. Many studies have focused on the role of ABCG1 and ABCA1 in macrophages, particularly lipid-loaded macrophage foam cells, since both genes are induced by activated LXR, and both proteins are thought to enhance the efflux of cholesterol/sterols out of cells.

Abcg1 is highly expressed in multiple tissues including the lung, brain, kidney, and spleen [16]. Specific cell types that express ABCG1 were identified by staining sections from *Abcg1*^{-/-}*LacZ* knock-in mice for β -galactosidase activity; cells that express high levels of ABCG1 included macrophages, lymphocytes, epithelial and endothelial cells and neurons [16]. In contrast, expression of *Abcg1* is low/undetectable in hepatocytes and enterocytes, suggesting that, unlike ABCA1, ABCG1 has no role in lipid absorption or lipoprotein secretion (see below) [16]. Support for this proposal also came from the finding that plasma lipid/lipoprotein levels are unchanged in *Abcg1*^{-/-}*LacZ* mice [16].

2.2. ABCG1 and intracellular sterol homeostasis

The cellular localization of ABCG1 remains controversial, since overexpression of epitope-tagged ABCG1 in cells is reported to result in intracellular as well as some cell surface expression [13,17–22]. Some authors argued that LXR activation results in the translocation of a carboxyl-terminus epitope-tagged ABCG1 from intracellular compartments to the cell surface [23], although others have disagreed [24]. Nevertheless, initial studies demonstrated that transient overexpression of ABCG1 increased the efflux of cellular cholesterol to specific extracellular lipid acceptors that included high-density lipoprotein (HDL), LDL, phosphatidylcholine (PC) vesicles and apoA1/PC complexes, but not lipid-free apoA1 [12, 17,19,25,26]. However, ABCG1 overexpression did increase cholesterol efflux to lipidated apoA1 present in conditioned media obtained from ABCA1-expressing cells [19,27] leading to the proposal that ABCA1 and ABCG1 may function sequentially to promote lipid efflux [19,27]. Conversely, efflux of cellular cholesterol to HDL was decreased when cells were derived from *Abcg1*^{-/-} mice [16] or after partial knock-down of ABCG1 [25]. Together, these studies identified a role for ABCG1 in maintaining cellular sterol homeostasis. It remains unclear whether ABCA1 and ABCG1 work in concert to control cellular sterol homeostasis, with ABCA1 initially promoting the efflux of phospholipid and cholesterol to extracellular lipid-poor apo-proteins, and ABCG1 subsequently promoting the efflux of additional cellular cholesterol to the pre-formed phospholipids-cholesterol-apoA1 complex [19,27].

The physiological importance of ABCG1 was revealed upon analysis of *Abcg1*^{-/-} *LacZ* knock-in mice [16]. Surprisingly, the major phenotype involved massive lipid accumulation in the lungs of these mice, particularly in alveolar macrophages and, to a lesser extent, the surfactant-secreting type 2 cells [16,28]. The deposition of cholesterol crystals, cholesterol esters and phospholipids was age dependent, accelerated by a high fat diet, and so severe that the lungs turned white [16,28]. Evidence has been presented that this is a result in part of the impaired ability of *Abcg1*^{-/-} pulmonary macrophages to efflux surfactant-derived cholesterol [28]. As might be expected, such sterol accumulation results in activation of LXR and repression of SREBP-2 target genes [28]. Subsequently, it was reported that either deficiency or overexpression of ABCG1 in the brain also results in altered expression of *Srebp-2* and *Srebp-2* targets [22,29]. In addition, Terasaka *et al.* [30] recently demonstrated that expression

of active eNOS in endothelial cells is also dependent upon ABCG1-dependent efflux of cholesterol/7-ketocholesterol (see below). Finally, Vance and colleagues also defined a key role for ABCG1 in the lipidation of apoE-containing lipoproteins in glial cells in the brain, a process that was independent of ABCA1 [31]. Together, the data points to a critical role for ABCG1 in controlling intracellular sterol homeostasis in a variety of cells including macrophages, endothelial cells and astrocytes/neurons (see below).

Studies with *Abcg1*^{-/-}*Abca1*^{-/-} mice demonstrated that loss of both sterol transporters resulted in an even more striking lipid-accumulation phenotype in macrophages and specific tissues than in single knock-out mice [32–34]. Thus, both transporters are important for sterol homeostasis and one cannot fully compensate for loss of the other. Using alternative approaches, that include *in vitro* cholesterol efflux assays and *in vivo* reverse cholesterol transport using normal and cholesterol-loaded primary peritoneal macrophages from *Abca1*^{-/-}, *Abcg1*^{-/-}, or *Sr-B1*^{-/-} mice, the relative contributions of these transmembrane proteins to the removal of intracellular cholesterol were recently reported [35,36]. These latter studies showed that ~20% of the intracellular cholesterol is effectively mobilized/effluxed by ABCG1, ~35% by ABCA1, ~10% by SR-B1 and ~50% by aqueous diffusion [35,36]. These multiple mechanisms point to the importance of maintaining normal cellular cholesterol levels for cell viability.

2.3. ABCG1 at the interface of sterol metabolism, inflammation and apoptosis

2.3.1. Inflammation—The lungs of old, chow fed *Abcg1*^{-/-} mice develop not only lipodosis, but also a chronic pulmonary inflammation characterized by macrophage accumulation, lymphocytic infiltration, and elevated levels of cytokines, cytokine receptors and matrix remodeling enzymes [37,38]. Current evidence suggests that specific sterols that accumulate in *Abcg1*^{-/-} pulmonary cells trigger this inflammatory response. The finding that small increases in cytokine expression were also observed in the lungs of wild-type mice fed a western diet, although there was no change in histology and no apparent lipid deposition [37], suggests that even small increases in intracellular sterol levels might be sufficient to induce inflammatory mediators.

Previous studies documented increased expression of specific cytokines following treatment of macrophages with acetylated or oxidized LDL [39–41]. More recently it was shown that cytokine expression is significantly increased when ox-LDL-challenged cells are derived from *Abcg1*^{-/-} or *Abcg1*^{-/-}*Abca1*^{-/-} as compared to wild type mice [34,37]. The changes in cytokine expression are generally far more pronounced in cells lacking ABCG1, suggesting again that ABCA1 cannot compensate for loss of ABCG1. Tall and colleagues have also reported that loss of ABCG1 results in increased signaling through TLR-4/Myd88/TRIF following stimulation with LPS [42]. This latter study, together with the work of Parks and colleagues [43], suggest that alteration of the sterol content in lipid rafts or other specific microdomains of cellular membranes by ABCG1 and, to a lesser extent, by ABCA1 might lead to altered sensitivity to LPS or other stimuli via TLR receptors. Interestingly, disruption of lipid raft integrity has been hypothesized to affect not only TLR, but also a plethora of signaling cascades related to immunological responses, especially in T-cells (reviewed in [44]). Future studies should provide insight into how the disruption of intracellular sterol mobilization impacts lipid raft composition/stability, signaling cascades and the production and secretion of pro-inflammatory mediators.

2.3.2. T cell proliferation—Bensinger *et al.* recently reported that ABCG1, together with LXR and the sulfotransferase enzyme SULT2B1, modulate the proliferation of T cells following an antigenic challenge [45]. These authors propose that specific, yet undetermined sterols in the endoplasmic reticulum constitute a critical metabolic checkpoint that defines

whether or not the cell can undergo division. According to this model, induction of LXR-ABCG1 or SULT2B1 results in depleted levels of these intracellular signaling sterols, preventing mitosis. Conversely, deletion of ABCG1 increases the levels of the signaling sterols and promotes cell division [45]. The identity of the signaling sterol remains elusive, although the authors speculate that it could be cholesterol itself [45]. The finding that cell division, in response to T cell activation, was not abrogated in *Abca1*^{-/-} cells supports again the proposal that ABCA1 and ABCG1 perform distinctive functions in the cell and control different pathways *in vivo*. The authors speculate that, although this mechanism was identified and characterized in T cells, it is likely to be conserved in other cell lineages.

2.3.3. Apoptosis—It is now well documented that macrophages lacking ABCG1 are more susceptible to apoptosis as compared to wild type cells [34,38,46,47]. Increased numbers of apoptotic macrophages have been observed in the lungs of *Abcg1*^{-/-} mice [38], following incubation of *Abcg1*^{-/-} peritoneal macrophages with oxidized LDL [46] [47], and also in atherosclerotic lesions of *Ldlr*^{-/-} mice transplanted with *Abcg1*^{-/-} bone marrow [46] or in myocardium of *Ldlr*^{-/-} mice transplanted with *Abca1*^{-/-}*Abcg1*^{-/-} bone marrow [34]. In contrast, overexpression of ABCG1 protected HeLa cells from ⁷β-hydroxycholesterol-induced apoptosis [21]. Consistent with these observations, it has been suggested that ABCG1 normally effluxes cholesterol and 7-ketocholesterol from cells and that intracellular accumulation of 7-ketocholesterol in *Abcg1*^{-/-} cells triggers apoptosis [47].

Interestingly, increased levels of unesterified cholesterol in the endoplasmic reticulum had been shown to result in activation of the unfolded protein response (UPR) pathway, which also ultimately results in apoptosis [48]. However, loss of ABCG1 does not lead to activation of CHOP or ATF-4 (critical effectors in UPR-mediated cell death) ([42] and our unpublished observations). Consequently, the exact intracellular events that lead to compromised cell viability following disruption of ABCG1 activity remain to be established.

2.4. ABCG1 and atherosclerosis

Abcg1^{-/-} mice are not hyperlipidemic and, consequently, do not develop spontaneous atherosclerosis [16]. Three independent studies were published simultaneously in which the progression of atherosclerotic lesions was measured following transplantation of bone marrow cells from wild-type or *Abcg1*^{-/-} mice into hypercholesterolemic *Ldlr*^{-/-} atherosclerosis-prone mice [46,49,50] (reviewed in [51]). In one study, Out *et al.* reported a “moderately significant” increase in lesion size in mice that received *Abcg1*^{-/-} cells [49]. In contrast, the other two studies reported significant *decreases* (20–50%) in lesion size in mice transplanted with *Abcg1*^{-/-} cells [46,50]. These latter paradoxical results were attributed to increased susceptibility of the macrophages to apoptosis [46] or to an increase in ABCA1 expression and secretion of apoE [50] from *Abcg1*^{-/-} macrophages. Data obtained with transgenic mice were also unexpected since overexpression of human ABCG1 in either *Ldlr*^{-/-} or *ApoE*^{-/-} hyperlipidemic mice did not attenuate atherosclerosis development [52,53].

The generation of *Abca1*^{-/-}*Abcg1*^{-/-} DKO mice has led to a broader understanding of the physiological importance of these two transporters [32–34]. As expected, loss of both transporters resulted in impaired cellular cholesterol efflux to serum, apoA1 and HDL [32–34]. Unexpectedly, the phenotypes of the DKO mice generated in two laboratories were not identical, possibly because the genetic backgrounds of the mice differed. Nonetheless, the DKO mice presented massive neutral lipid deposition in macrophages in several tissues [32–34]. Thus, deletion of both transporters resulted in a far more extensive phenotype than in single knock-out mice. However, atherosclerotic lesions did not develop in the *Abca1*^{-/-}*Abcg1*^{-/-} mice, likely because they are not hyperlipidemic. Consequently, bone marrow transplants were performed using *Abca1*^{-/-}*Abcg1*^{-/-} donor cells and recipient *Ldlr*^{-/-} mice [33,34].

Remarkably, very different results were obtained; in one study the atherosclerotic lesions in mice receiving DKO cells were larger as compared to mice receiving bone marrow from single knock-out or wild type mice [34]. In contrast, in the second study, the lesion size of the mice receiving DKO cells was smaller than those receiving *Abca1*^{-/-} cells, but similar to the lesions noted with *Abcg1*^{-/-} or wild type donor cells [33]. The reasons behind these contradictory results remain obscure although it has been proposed that these differences may be result of different levels of hyperlipidemia [54].

2.5. A new role for ABCG1 in vasoconstriction/relaxation

ABCG1 is highly expressed in endothelial cells [16,55], where it was shown to mediate cholesterol efflux to exogenous HDL *in vitro* [55]. More recently, ABCG1 was also reported to play an essential role in balancing vasoconstriction/vasorelaxation. [30]. Thus, functional levels of endothelial nitric oxide synthase (eNOS) were significantly reduced in endothelial cells from cholesterol-fed *Abcg1*^{-/-} mice, compared to wild-type controls [30]. Accordingly, myographic recordings showed an attenuated/delayed relaxation in arteries from *Abcg1*^{-/-} animals after treatment with a vasoconstrictive agent, such as acetylcholine [30]. Moreover, *Abcg1*^{-/-} endothelial cells accumulated increased levels of 7-ketocholesterol, and treatment of human endothelial cells with this oxysterol reduced the amount of functional eNOS [30]. The authors postulate that ABCG1-dependent sterol efflux to exogenous HDL improves endothelial function by removing harmful oxysterols (i.e. 7-ketocholesterol) and thus increasing the production of NO [30].

2.6. ABCG1 and obesity/diabetes

Mauldin *et al.* reported that *Abcg1* levels are repressed in macrophages derived from *db/db* or *KK^{ay}* diabetic mice, compared to C57Bl6 mice and that chronic high glucose levels down-regulated ABCG1 expression in macrophages isolated from diabetic mice [56]. Monocyte-derived macrophages obtained from patients with type 2 diabetes and cultured in autologous serum also expressed very low levels of ABCG1 and had increased contents of cholesterol esters, compared to cells from healthy donors [57–59]. These changes in ABCG1 were associated with a compromised ability to promote cholesterol efflux to HDL, but not to apoA1 [57,58]. Additional studies will hopefully provide the link between the observed changes in ABCG1 and diabetes.

To our knowledge, the *Abcg1*^{-/-} mice used in all studies cited above were on a C57BL/6 background and obtained from Deltagen. Recently, Buchmann *et al.* generated their own *Abcg1*^{-/-} mice on a mixed genetic background [60]. In contrast to the “Deltagen” KO mouse, these latter mice exhibit decreased food intake, increased energy expenditure, reduced body weight and adipose mass, resistance to diet-induced obesity, and increased insulin sensitivity, compared to wild-type controls [60]. Identification of the loci that result in these different phenotypes, as compared to the “Deltagen” mice, may provide insight into the genes that interact with ABCG1.

2.7. Clinical perspective

To date no functional mutations in ABCG1 have been linked to any human disease. Since *Abcg1* heterozygous mice do not develop pulmonary lipidosis or inflammation, it is conceivable that individuals with residual ABCG1 activity are healthy and only those with a severe or complete loss of function of the transporter present symptoms of lung disease. Interestingly, Thomassen and collaborators recently identified four patients with pulmonary alveolar proteinosis who had decreased levels of ABCG1 mRNA and protein in alveolar macrophages recovered by bronchoalveolar lavage [61]. Whether decreased ABCG1 expression in these patients is the cause or a secondary consequence of the lung disease, and

whether functional mutations are present in the cDNA and/or regulatory regions of the ABCG1 promoter in these patients remain to be established.

3. ABCG2

Cancer cells that develop resistance to the cytotoxic effects of multiple drugs administered as part of normal chemotherapeutic treatments have limited the clinical efficacy of these approaches. This resistance is often the result of increased expression in malignant cells of specific members of the ABC family of transporters that function to actively export cytotoxic drugs out of the cell, thus preventing cell death. Such transporters include P-glycoprotein/MDR-1/ABCB1 and ABCG2. ABCG2, also known as breast cancer related protein (BCRP), mitoxantrone resistant protein (MXR), or placenta-specific ABC transporter (ABC-P), was first identified in the breast cancer cell line, MCF-7/AdrVp, where the expression was associated with resistance to a number of drugs including doxorubicin, methotrexate, mitoxantrone, bisantrene and topotecan [62]. Subsequently ABCG2 has been shown to be overexpressed in many other tumor cells (reviewed in [63]). ABCG2 is also highly expressed in normal epithelial cells of the placenta, kidney, and intestine, where it has been suggested that it may have a role in regulating the absorption, circulation and metabolism of xenobiotics [64,65]. Additionally, ABCG2 has been reported to interact and in some cases transport sterol-based compounds, polyphenols, porphyrins and other dietary substances (summarized in [63]).

3.1. Structure and localization of ABCG2

ABCG2 forms a homodimer that localizes to the plasma membrane [8]. Interestingly, a fusion protein comprised of two wild-type ABCG2 proteins is active as a transporter, whereas mutations in the Walker B region of the first unit of the fusion protein result in a dominant negative phenotype on the fusion protein [66]. Although human ABCG2 has also been reported to form tetramers [67], the physiological importance of this finding remains to be clarified. Single nucleotide polymorphisms (SNPs) in the human ABCG2 gene have been linked to altered substrate specificity and to the efficacy of substrate-transporter interactions [68,69]. Additional studies will hopefully identify the physiological importance of such linkage.

3.2. Putative physiological roles of ABCG2

3.2.1. Cellular homeostasis—The normal physiological substrates and function of ABCG2 are less well defined. As mentioned above, ABCG2 may provide protection against cytotoxic substances by exporting these harmful molecules out of the cell. It has also been proposed that ABCG2 has an important role in stem cells from both haematopoietic and non-haematopoietic origin. Although *Abcg2*^{-/-} mice display normal haematopoiesis, over expression of ABCG2 caused expansion of the side population cells (characterized by faint Hoechst 33342 staining) [70–73]. It should be noted that it has also been suggested that other members of the ABC family can mediate this phenotype [73,74].

To date, no major phenotype has been identified in *Abcg2*^{-/-} mice. However, these mice do have increased levels of erythrocyte protoporphyrin IX (PPIX) in erythroid cells, suggesting ABCG2 may be important in maintaining endogenous porphyrin homeostasis [70,75]. On the other hand, ABCG2 seems to also play a role in mobilizing glutamate conjugates out of the cell [76]. Proper control of glutamate levels is essential and retention of intracellular folic acid (which mammalian cells cannot synthesize *de novo*) is facilitated by conjugation to glutamate, in a reaction catalyzed by folypoly-g-glutamate synthases (FGPS) [77]. Hence, gradual restriction of folate in MCF-7 cells has been shown to lead to increased expression of FGPS and repression of ABCG2, consistent with a feedback mechanism aimed at maintaining cellular folate levels [78]. The physiological importance of this finding remains to be elucidated.

3.2.2. Hypoxia—During hypoxia the stimulation of both glycolysis and heme biosynthesis allows cells to switch to anaerobic metabolism and increase oxygen supply. Expression of ABCG2 confers increased survival to hypoxia in progenitor cells [79]. The intracellular accumulation of heme, together with the generation of reactive oxygen species, can be toxic. It has been proposed that ABCG2 may have a role in promoting cell survival in these situations by exporting toxins, and thus limiting their intracellular accumulation. It is also possible that protection against chemotherapeutic drugs in cancerous cells may be the result of hypoxia-induced expression of ABCG2 [79].

3.3. ABCG2 and clinical multidrug resistance

There is substantial evidence for an important role of ABCG2 in determining the efficacy of chemotherapeutic treatments of tumors [80]. Some cases of innate tumor resistance, for example treatment of acute myeloid leukemia (AML) with methotrexate and topotecan, have been associated with increased expression of ABCG2 [81]. Indeed, elevated ABCG2 expression was reported in a third of the patients with AML [81].

3.4. Modulators of ABCG2

Inhibitors of ABCG2 function could function as chemo sensitizers and thus improve drug pharmacokinetics. In certain clinical situations, combinatory inhibition of more than one ABC transporter may be the most appropriate therapeutic action. In other circumstances, more selective and specific inhibition may be more advantageous. Although molecules that inhibit ABCB1 have been studied more comprehensively, there are several substances, including 17- β -estradiol, folate, hesperetin, fumitremorgin C, Tamoxifen and Ko143, that have been shown to affect ABCG2 activity (reviewed in [63]).

In summary, our current knowledge about ABCG2 is relatively limited. For example, the normal physiological function of ABCG2 remains unclear and it is not known exactly what conditions drive ABCG2 expression in malignant cells, or whether such overexpression is favored by certain cell types. In the future, the development of specific inhibitors of ABC transporters, such as ABCG2 and MDR1, might be expected to improve the efficacy of certain chemotherapies that depend on the accumulation of toxins within malignant cells.

4. ABCG4

ABCG4 was originally discovered based on its high sequence homology with ABCG1 [82, 83]. Since these two proteins exhibit 82% amino acid identity it is not surprising that they exhibit functional similarities; overexpression of either protein in cultured cells facilitates the efflux of cellular cholesterol to HDL, but not to lipid-poor apoA-I [25,27]. The major difference between ABCG4 and ABCG1 is the response of the two genes to the nuclear receptor LXR; unlike ABCG1, which is highly induced following activation of LXR, ABCG4 is unresponsive to LXR activation [22]. Another significant difference is the tissue expression of the two proteins. In contrast to ABCG1, which is expressed in numerous cell types and tissues, ABCG4 expression is highly restricted. Initial reports identified high ABCG4 expression in the brain and in the neural layer of the retina, with lower expression in a number of other tissues [82, 83]. More recent studies, that involved staining tissues of *Abcg4*^{-/-}*LacZ* knock-in adult mice for β -galactosidase activity, demonstrated that ABCG4 expression is restricted to astrocytes and neurons of the CNS [22,84]. Parallel studies with *Abcg1*^{-/-}*LacZ* mice indicate that although ABCG1 is highly expressed in these same cells of the CNS, it is also expressed in many other cell types including microglia [22,84]. Co-localization of the mRNAs encoding ABCG4 and ABCG1 in the brain was also noted following *in situ* hybridization studies [85]. In contrast, based on a polyclonal antibody that recognizes a protein of 63–90 kDa, it has been reported that ABCG4 is expressed in the testes>>brain (specifically cortex and medulla)>spleen

and heart [86]. The development of additional specific antibodies will hopefully address these apparent discrepancies. Nevertheless, given the close similarity between ABCG1 and ABCG4, it was proposed that these two half-transporters might heterodimerize in those cell types in which they are co-expressed. Indeed, Cserepes *et al.* reported that overexpression in Sf9 cells of an ABCG4 Walker A mutant abolished the ATPase activity of ABCG1, suggesting that both transporters heterodimerize [87]. Whether these *in vitro* interactions also occur *in vivo* in astrocytes or neurons remains to be established.

Recent studies utilizing *in situ* hybridization and immunostaining of tissues, reported that ABCG4 levels were elevated in microglial cells that were adjacent to senile plaques in the brains of patients with Alzheimer's disease (AD) [88]. These authors suggested that upregulated ABCG4 may accelerate the lipidation of apoE in the AD brain in order to attenuate the toxicity of apoE and suppress the development or progression of AD. However, to date, there is no evidence that ABCG1 levels are altered in the brains of AD patients or in mouse models for this disease. Although detailed studies of the brains of *Abcg4*^{-/-} mice (<1 year old) did not identify any pathological changes (our unpublished data), it is possible that crosses between *Abcg4*^{-/-} or *Abcg1*^{-/-}*Abcg4*^{-/-} mice and mouse models of AD may be valuable in elucidating the role of this ABC transporter in the brain. Nevertheless, further studies will be necessary to fully characterize the role of ABCG4 in the biogenesis of apoE in the CNS.

The cellular localization of both ABCG4 and ABCG1 remains to be resolved. Overexpression of ABCG4 or ABCG1 tagged with a small epitope at the carboxy terminus reported that both proteins co-localize to intracellular vesicles [22]. It was proposed that both proteins may be involved in the transfer of endogenous sterols away from the endoplasmic reticulum [22]. This hypothesis was based on the observation that overexpression of either ABCG1 or ABCG4 in astrocytes resulted in increased processing of the SREBP precursor to form mature SREBP-2, that in turn induced the expression of cholesterologenic genes and elevated cholesterol synthesis [22]. It is tempting to speculate that intracellular ABCG1 and/or ABCG4 activity leads to the depletion of a regulatory pool of sterols in the endoplasmic reticulum, which in turn modulates SREBP maturation and activity. Likely, as the ER becomes depleted of sterols, other membranous compartments close to the plasma membrane might become enriched, thus explaining the facilitation of cellular sterol removal by these transporters. However, Koshiba *et al.* reported that ABCG4 was expressed in the plasma membranes of insect Sf9 cells following infection with recombinant baculovirus [86]. Whether these differences are a result of overexpression, or antibody specificity is unknown.

To better understand the functional relationship between ABCG4 and ABCG1, and to overcome the possibility that either transporter could functionally compensate for the loss of the other, Wang *et al.* recently generated *Abcg1*^{-/-}*Abcg4*^{-/-} mice; the brains of these mice contain elevated levels of several intermediates of the cholesterol biosynthetic pathway, including desmosterol, lathosterol and lanosterol, and the cholesterol metabolite 27-hydroxycholesterol [84]. Additionally, astrocytes from *Abcg1*^{-/-}*Abcg4*^{-/-} mice exhibited reduced efflux of desmosterol and cholesterol to HDL that led to accumulation of these sterols in primary astrocytes [84]. It was suggested that ABCG4 and ABCG1 could act synergistically in astrocytes by stimulating the efflux of cellular cholesterol and desmosterol to HDL-like particles in the CNS. Despite these observations, the precise role of ABCG4 and/or ABCG1 in lipid homeostasis in the CNS and its potential role in neurological disease remains to be determined.

5. ABCG5 and ABCG8

The transcriptional start sites of the genes encoding ABCG5 and ABCG8 lie on opposite strands of the DNA and are separated by only a few hundred base pairs [89]. These two half-transporters

form obligate heterodimers that are expressed on the apical membranes of both enterocytes and hepatocytes [90]. They function to limit the absorption of plant sterols and cholesterol from the diet by effluxing these sterols from the enterocyte back into the intestinal lumen, and by facilitating efficient secretion of plant sterols and cholesterol from hepatocytes into the bile [89,91]. Genetic mutations that inactivate either half-transporter result in a rare autosomal recessive disorder, sitosterolemia, characterized by the accumulation of cholesterol and plant sterols, eventually leading to premature coronary atherosclerosis.

Both ABCG5 and ABCG8 are co-ordinately upregulated upon LXR activation [89]. Lack of expression of either half-transporter results in the accumulation of the other in the endoplasmic reticulum compartment as a result of impaired translocation to the plasma membrane [90]. Recent studies have shown that, similarly to the TAP1:TAP2 (ABCB2:ABCB3) heterodimer [92], both ABC motifs in ABCG5:ABCG8 are not functionally equivalent [93]. According to these studies, the active site required for ATP binding and hydrolysis is comprised of the Walker A and B domains from ABCG5 and the Signature motif from ABCG8 [93].

Overexpression of human ABCG5 and ABCG8 transgenes in mice results in a 50% decline in the fractional absorption of dietary cholesterol and a concomitant increase in the biliary excretion of sterols [94]. Conversely, disruption of both ABCG5 and ABCG8 in mice results in a 3-fold increase in dietary plant sterol fractional absorption, a 30% increase in plasma sitosterol levels, together with a reduction in biliary cholesterol levels [95]. Thus these mice displayed many characteristics similar noted in patients with sitosterolemia. Studies with *Abcg5^{-/-}Abcg8^{-/-}* mice revealed transport selectivity since cholesterol, but not bile acids or phospholipid levels, were abnormally low in the bile of these mice [95]. By controlling sterol intestinal absorption and hepatic excretion, ABCG5 and ABCG8 effectively limit circulating sterols in plasma, suggesting that modulation of the activity of these transporters might be used as a novel therapeutic intervention in the treatment of hypercholesterolemias. Indeed, *Ldlr^{-/-}* mice overexpressing both ABCG5 and ABCG8 showed a marked decrease in circulating cholesterol and reduced atherosclerotic lesions, compared to *Ldlr^{-/-}* controls [96]. Whether these results can be extrapolated to patients will need additional studies. Additionally, Kahn and colleagues reported that mice lacking the insulin receptor in the liver develop not only hepatic insulin resistance but also increased gallstone formation, partly because of increased expression of both ABCG5 and ABCG8, that presumably pump excess cholesterol into bile [97]. Conversely, incubation of rat hepatoma cells with insulin resulted in suppressed expression of both transporters in a dose-dependent manner [97]. The authors suggested that therapies leading to improve glucose sensitivity in the liver might also affect hepatic cholesterol homeostasis and bile secretion. The effect of insulin was described to be dependent of the transcription factor FOXO1 [97], but the exact mechanism remains to be elucidated.

Studies by Hobbs and Cohen and colleagues [98,99] established conclusively that sterols are the direct substrates of ABCG5 and ABCG8. Thus, ATP-dependent transfer of both cholesterol and sitosterol was confirmed using “inside-out” membrane vesicles prepared from either *Sf9* cells or liver membranes [98,99]. Interestingly transport of cholesterol was stereoselective, favoring the transfer of the natural form of cholesterol, indicating that there is a strong likelihood of a direct interaction between cholesterol and the ABC transporter. However, the precise mechanism for the sterol transfer remains to be determined.

6. The ABCG family in model organisms

As mentioned in the Introduction, the ABCG family is present in several tractable model organisms including *Drosophila melanogaster*, *Caenorhabditis elegans* and *Arabidopsis thaliana*, with 15, 9 and 24 members, respectively. These organisms provide a relatively

untapped resource for the characterization of novel functions for this group of ABC transporters.

6.1. *Drosophila*

The human ABCG1 gene was originally called “human white” as it was cloned by degenerate PCR using primers based on the genomic sequence of the *Drosophila white* gene (5). Extensive characterization of *Drosophila white* mutants has shown that *white* forms a heterodimer with either *brown* or *scarlet*, two other ABCG family members. In the fly eye, these heterodimers localize to intracellular membranes of pigment granules that are present in specialized pigment cells. These pigment granules are considered to be modified lysosomes that form a specialized intracellular compartment. The two heterodimers, (*white:brown* and *white:scarlet*), are thought to transport different pigment precursors into these lysosome-like vesicles for the synthesis of ommochromes and pteridines, which together confer the “red eye” color. As a historical note of interest, Morgan and colleagues described “white eyed flies” in 1910 [100]. Morgan’s subsequent studies on the transmission of the “white eye” phenotype would become the basis for his chromosomal theory of heredity, that linked discrete units on chromosomes he termed “genes” with the inheritance of traits in offspring, and provided a mechanism for Mendelian heredity. For his groundbreaking work Morgan received the Nobel Prize in Physiology and Medicine in 1933. Many years later the *white* gene was identified and mutations shown to result in the white-eyed phenotype [101,102]. Interestingly, in addition to the eye, *white* is also highly expressed in the malpighian tubules (that perform filtration functions similar to the mammalian kidney) and to a lesser extent in the fly brain. As predicted from the eye phenotype, the normally pigmented malpighian tubule epithelium of *white* flies is colorless, in contrast to the pigmented malpighian epithelium of wild type flies [103,104]. There is also anecdotal evidence that high expression or intracellular mislocalization of *white* in the fly brain results in altered male sexual behavior [105,106].

Coincidentally, mammalian ABCG1 is also highly expressed in the eye, the epithelial cells of the kidney and the brain (see above), it is associated with intracellular vesicles of the endocytic pathway (at least when overexpressed [22]), and it has high amino acid sequence similarity (51%) with *Drosophila white*. Taken these facts together, it was suggested that a possible overlapping function between *white* and mammalian ABCG1 might exist. Indeed, based solely on sequence similarity, *white* and ABCG1 have long been regarded as orthologs. This hypothesis was recently tested by performing complementation studies to determine whether mammalian ABCG1 or ABCG4, that themselves exhibit 82% amino acid similarity, can function to rescue a *Drosophila white* mutant (Fig. 2). cDNAs under the control of a UAS promoter and encoding either mouse *Abcg1* or *Abcg4*, or *Drosophila white* were stably incorporated into *Drosophila yw¹¹¹⁸* flies that harbored a heat-shock inducible Gal-4 transgene. From the third instar larval stage the expression of the UAS-transgene was induced by a brief heat shock at 37°C for 30 min daily until adult flies emerged. As expected, expression of *Drosophila white* restored wild-type red pigmentation in the eyes of *yw¹¹¹⁸* flies (Fig. 2). However, induction of the mouse *Abcg1* or *Abcg4* transgenes failed to restore eye pigmentation in *yw¹¹¹⁸* flies (Fig. 2). Consequently, these data demonstrate that mammalian ABCG1 or ABCG4 are *not* the functional orthologs of *Drosophila white*. However, these data do not exclude the possibility that *Drosophila* contains another ABCG gene that is the true ortholog for mammalian ABCG1. Consequently, a detailed phylogenetic tree of all 15 *Drosophila* and 5 mammalian ABCG transporters was constructed (Fig. 3).

Sequence alignments for all 20 combined *Drosophila* and *Mus musculus* ABCG members were generated and three different phylogenetic methods (Bayesian inference of phylogeny method, maximum likelihood, and distance matrix methods) were applied to the aligned sequences to obtain tree topologies to map the evolutionary history and ancestral relationships of the ABCG

family between the members in these two species. The sequence alignments of all 20 members revealed that ABCG1 shares significant amino acid sequence identity (~44%) with two *Drosophila* proteins, *Atet* and *CG3164* (Fig. 3). Surprisingly, this phylogenetic tree indicates that the *Drosophila* eye pigmentation transporters *white*, *brown*, and *scarlet* are the most distantly related to the mammalian ABCG family (Fig. 3). This new analysis, together with the data from the complementation experiments, suggests that the *Drosophila* eye pigmentation genes have not been retained in the evolutionary transition from insects to mammals. Further analysis of the phylogenetic tree identifies an interesting bifurcation into two distinct segments; the mammalian transporters ABCG2 and ABCG5/ABCG8 cluster into a segment of the tree that is separate from ABCG1, ABCG4, and the majority of the *Drosophila* ABCG family members (Fig. 3). In this segment of the phylogenetic tree ABCG1 and ABCG4 are most closely related to *Drosophila Atet* and *CG3164*, sharing 42–44% identity (59–63% similarity) at the amino acid level (Fig. 3). This phylogenetic analysis suggests that, if functional orthologs to ABCG1 exist in *Drosophila*, *Atet* and *CG3164* are the likely candidates. To our knowledge, the function of these latter two *Drosophila* is unknown.

6.2. Plants

As mentioned above, *Arabidopsis* have 24 ABCG half-transporters [107,108]. Although the function of most of these transporters is unknown, recent studies have shown that mutations in either ABCG11 or ABCG12 result in defects in the waxy cuticle that normally forms a lipid barrier to reduce water loss and defend the plant against pathogens [109,110]. These data suggest that both ABCG11 and ABCG12 function to transport lipids out of the epidermal cells [109,110]. To date, mutants of ABCG11 and ABCG12 are the only two ABCG half transporters that have been analyzed in *Arabidopsis*. Due to the large collection of mutants available to the *Arabidopsis* community it will of particular interest, as more mutants of this family analyzed, to see how many have lipid transport defects.

Acknowledgments

The authors apologize to those investigators whose papers had to be omitted due to space limitations. We are indebted to Larry Zipursky and members of the Zipursky lab at UCLA for their invaluable help with the experiments in *Drosophila*. We also thank the members of the Edwards lab for critical reading of the manuscript. This work was supported in part by National Institutes of Health Grants NIH30568 and NIH68445 (to P.A.E.), a grant from the Laubisch Fund (to P.A.E.), and a grant from Pfizer, Inc. (to P.A.E.).

Abbreviations

ABC	ATP binding cassette
DKO	double knock-out
HDL	high-density lipoprotein
LDL	low-density lipoprotein
LXR	liver X receptor
TMD	transmembrane domain

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Figure 1.

Structure of a typical ABCG half-transporter dimer. ABCG proteins contain an amino-terminal ABC domain followed by six transmembrane helices. The functional transporter is thought to be dependent on the formation of homo- or heterodimers, in which the resultant twelve transmembrane helices configure a channel in the membrane phospholipid bilayer. The energy necessary for the translocation of the substrate is derived from the hydrolysis of two ATP molecules at the ABC domain. Studies have shown that the Walker A, Walker B and Signature motifs within the ABC domain are not functionally equivalent (see text for details).

Figure 2.

Complementation experiments in *Drosophila* suggest that neither mammalian ABCG1 nor ABCG4 are the true orthologs of *white*. **A.** P-element vectors containing a Gal4 cassette under transcriptional control of a heat shock-inducible promoter (*hs-Gal4*), or containing the gene of interest (GOI, murine *Abcg1* or *Abcg4*, CD8GFP (a negative control) or *Drosophila white*) under transcriptional control of UAS sequences were introduced into *Drosophila yw¹¹¹⁸* embryos using standard germ line transformation procedures. The F1 progeny of these crosses were heat shocked at 37°C for 30 min daily starting at the third instar larval stage. **B.** Expression of the exogenous *Abcg1* and *Abcg4* after heat shock was confirmed by real time PCR using RNA harvested from 10–15 F1 progeny of each genotype, and gene specific primers. **C.** Effectiveness of the heat shock process was also confirmed in flies carrying an inducible GFP cassette. **D.** Eye color was assessed in new emerged adult flies and pictures were taken at 5× magnification with a SPOT camera attached to a Zeiss dissecting microscope. Detailed experimental protocols have been published [111]. Only flies carrying both UAS-*white* and *hs-Gal4* exhibited red eyes following heat shock treatment (second panel).

Figure 3.

Phylogenetic analysis of *Drosophila* and mammalian ABCG genes. Amino acid sequences for the 15 *Drosophila* and 5 *Mus musculus* ABCG proteins were retrieved from the NCBI protein database. Sequences were aligned using the multiple alignment algorithms in the ClustalX program version 1.83 with the BLOSUM 62 protein weight matrix [112,113]. Pairwise identity and similarity scores were generated using the MatGAT v2.02 program [114]. Three different phylogenetic methods were applied to aligned sequences to obtain tree topologies: the Bayesian inference of phylogeny method, maximum likelihood, and distance matrix methods. The final tree presented was obtained from the Bayesian inference analysis that represented the best fit for the data generated from the MrBayes program v3.1 [115]. Detailed parameters used in the

Bayesian analysis are available [111]. The tree shows the distant relationship of *Drosophila* pigmentation genes with the other members of this family in *Drosophila* and mice. The *Drosophila* members most closely related to ABCG1 and ABCG4 are *Atet* and CG3164 and are highlighted. The posterior probabilities of the tree are indicated at each branch point.