Regulation of the function of the human ABCG2 multidrug transporter by cholesterol and bile acids: effects of mutations in potential substrate- and steroid-binding sites

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Running title: Sterol modulation of ABCG2 function

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Number of text pages: 29
number of tables: 1
number of figures: 7
number of references: 32
Number of words in the
Abstract: 250
Introduction: 750
Discussion: 1771

ABSTRACT

ABCG2 is a plasma membrane glycoprotein that actively extrudes xenobiotics and endobiotics from the cells and causes multidrug resistance in cancer. In the liver, ABCG2 is expressed in the canalicular membrane of hepatocytes and excretes its substrates into the bile. ABCG2 is known to require high membrane cholesterol content for maximal activity and, by examining purified ABCG2 reconstituted in proteoliposomes, we have recently shown that cholesterol is an essential activator, while bile acids significantly modify the activity of this protein. In the present work, by using isolated insect cell membrane preparations expressing the human ABCG2 and its mutant variants, we have analyzed whether certain regions in this protein are involved in the sterol recognition. We found that replacing ABCG2-R482 with large amino acids does not affect cholesterol-dependence, while changes to small amino acids cause altered cholesterol sensitivity. When leucines in the potential steroid-binding element (SBE, aa 555-558) of ABCG2 were replaced by alanines, cholesterol-dependence of ABCG2 activity was strongly reduced, although the L558A mutant variant, when purified and reconstituted, still required cholesterol for full activity. Regarding the effects of bile acids in isolated membranes, we found that these compounds decreased ABCG2-ATPase in the absence of drug substrates, while did not significantly affect substrate-stimulated ATPase activity. The above ABCG2 mutant variants also altered bile acid sensitivity, although cholic acid and glycocholate are not transported by the protein. We suggest that aforementioned two regions in ABCG2 are important for sterol sensing and may represent potential targets for pharmacological modulation of ABCG2 function.
**INTRODUCTION**

Human ABCG2 is a plasma membrane glycoprotein expressed in many tissues, especially in those with barrier or detoxifying functions (brain endothelium, placenta, gut, liver) (Robey et al., 2009). This protein is an active transporter, working as a homodimer, utilizing the energy of ATP hydrolysis for the extrusion of various compounds across the plasma membrane. ABCG2 is a promiscuous transporter and its physiological role is the protection of the body and fetus against exo- or endogenous toxic compounds. Among ABCG2 substrates are various anti-cancer agents, therefore the protein is one of the key players causing the so called multidrug resistant phenotype of cancer cells, resulting in the failure of cancer chemotherapy.

ABCG2 was documented to be located in cholesterol rich micro-domains, the so called rafts, and its direct interaction with caveolin-1, a marker of lipid rafts was also demonstrated (Storch et al., 2007). In accordance with this finding, high membrane cholesterol levels were found to significantly improve the function of ABCG2. We and others found that the enrichment of insect membranes with cholesterol greatly increases ATPase and transport function of ABCG2 (Pal et al., 2007; Telbisz et al., 2007). A dramatic, but reversible inhibition of ABCG2 function in mammalian cells upon depletion of cholesterol was also shown (Storch et al., 2007; Telbisz et al., 2007). It is not known whether cholesterol is only a modulator of ABCG2 function or it is directly transported by the protein. Other members of the ABCG subfamily (ABCG1, ABCG4 and ABCG5/ABCG8) are involved in cholesterol or sitosterol transport (Wang et al., 2008; Kerr et al., 2011).

We and others have recently shown that Arg 482 is important in cholesterol sensing of human ABCG2, as the function of ABCG2 mutants having Gly or Thr at position 482 was only slightly modified by cholesterol. We found that these mutants are fully active in Sf9 membranes with low cholesterol content, or in mammalian cells partially depleted from cholesterol. In contrast, the ATPase and transport function of the wild-type ABCG2 greatly increased in insect membranes enriched with cholesterol. Still, in isolated ABCG2 preparations even the R482G mutant required low levels of cholesterol for full function (Telbisz et al, 2013).

Structural analysis of steroid hormone (progesterone, androgen, estrogen or glucocorticoid) receptors and other steroid binding proteins, e.g. oxysterol binding protein-related proteins, revealed a complex interaction between the ligand binding domains and the steroid substrates. A conserved motif of these ligand binding domains is an L/MxxLxxL
sequence or a shorter LxxL, in which the leucines form Van der Waals bounds with the rings of steroid hormones (Williams and Sigler, 1998; Im et al., 2005). In the ABCG8 protein, involved in sterol transport by forming a heterodimer with ABCG5, the fifth transmembrane helix contains an MxxLxxL motif, and a Gly to Arg mutation at the beginning of this element causes loss of activity and sitosterolaemia. Mutations in the LxxL motif in the homologous region of ABCG2, as a putative SBE, have been shown to cause failure in progesterone and estradiol recognition of ABCG2, expressed in *Lactococcus* (Velamakanni et al., 2008). Surprisingly, these mutants were similarly activated by cholesterol as the non-mutant protein.

Bile acids are abundantly formed from cholesterol in hepatocytes, and bile acids and their derivatives are present in all tissue fluids. Due to their entero-hepatic circulation, bile acid concentrations are especially high in the intestinal epithelial cells and in the hepatocytes. The interaction between ABCG2 and bile acids has been extensively investigated, but these studies yielded controversial data. Two groups reported that there was no interaction with ABCG2 or direct transport of taurocholate, tauroolithocholate sulfate and tauroursodeoxycholic acid by this protein (Suzuki et al., 2003; Vaidya and Gerk, 2006). In contrast, two other laboratories described direct interaction between ABCG2 and bile acids (Imai et al., 2003; Janvilisri et al., 2005). ABCG2-specific extrusion of a fluorescein conjugated bile acid derivative cholyglycylamido-fluorescein, and also a low level transport of cholic acid, glycocholic acid, taurocholic acid and tauroolithocholic acid-3-sulphate has been suggested (Blazquez et al., 2012).

In the present study we provide a detailed mutational analysis of the cholesterol sensing capability of different ABCG2 R482 mutants, as well as mutants carrying the L555A, L558A or L555A/L558A point mutations. In addition, we present a comprehensive characterization of the interaction between ABCG2 and bile acids. We demonstrate the modulatory effects of these mutations on ABCG2 function and their contribution to cholesterol/bile acid sensing capability of the protein. We also show, that as compared to “professional” bile acid transporters, ABCG2 has no significant bile acid transport capacity.
MATERIALS AND METHODS

Materials – If not stated otherwise, all materials were purchased from Sigma Aldrich Ltd. (Budapest, Hungary). Cholesterol-RAMEB was kindly provided by CycloLab Ltd. (Budapest, Hungary). The BXP-21 monoclonal antibody (Maliepaard et al., 2001) and Ko143 (Allen et al., 2002) were kind gifts from Drs. George Scheffer and Rik Schepers, and from Dr. G. J. Koomen, respectively.

Expression vectors - Generation of baculovirus transfer vector (pAcUW21-L) harboring the cDNA for wtABCG2 or the R482 and K86M mutants was described earlier (Ozvegy et al., 2002; Ozvegy-Laczka et al., 2005). The steroid-binding element mutants were created by site directed PCR mutagenesis using the following complementary primer pairs: L555A: 5’ T TCA GGT CTC GCG GTC AAT CT and 5’ AG ATT GAC CGC GAG ACC TGA A, L558A 5’ GT CTG TTG GTG AAT GCC ACA ACC ATT and 5’ AAT GGT TGT GGC ATT CAC CAA CAG AC, L555/558A: 5’ GT CTC GCG GTG AAT GCC ACA ACC ATT and 5’ AAT GGT TGT GGC ATT CAC CAA CAG AC. PCR fragments containing the mutant cDNAs were cloned between the NcoI-SacI sites of the pAcUW21-L/wtABCG2 vector. Base order of the constructs was confirmed by sequencing of the appropriate fragments. The R482G/ L555/558A triple mutant was created by replacing DNA fragment between the PstI-NcoI sites of the pAcUW21-L/R482G with that of derived from the pAcUW21-L/L555/558A vector.

Generation of His6-tagged ABCG2 variants- His6-ABCG2 has been generated as described in (Telbisz et al., 2013). The His6-tagged L558A and L555/558A mutants were created by cloning the PstI-SacI site from pAcUW21-L/ABCG2-L558A or L555/558A into the pAcUW21-L/His6-ABCG2.

Cell culturing, generation of recombinant baculoviruses and membrane preparation – Sf9 cells were cultured and recombinant baculoviruses were generated as described previously (Bakos et al., 2000; Ozvegy et al., 2001).

Membrane preparation was also performed as described earlier (Ozvegy et al., 2001) Cholesterol loading of the membrane preparations was achieved by incubation with 2 mM cholesterol-RAMEB on ice for 30 minutes, as published previously (Telbisz et al., 2007).

Immunodetection of ABCG2 – Sf9 membranes were suspended in a Laemmlli sample buffer containing 2% of the reducing agent β-mercaptoethanol. Western blot analysis was performed as described earlier (Ozvegy et al., 2002), by using the BXP-21 monoclonal antibody in a 2,000x dilution, and a goat anti-mouse HRP-conjugated secondary antibody (10,000x dilution, Jackson Immunoresearch).
Solubilization, purification and reconstitution into proteoliposomes—Solubilization of His6-tagged wild-type ABCG2 and its SBE mutant variants was performed with 1% (w/v) DDM (dodecyl maltoside), 0.4% *Escherichia coli* lipid extract (Avanti Polar Lipids, 100500), as described earlier (Telbisz et al., 2013). Purification and reconstitution were performed as described in our recent paper (Telbisz et al., 2013). Briefly, Ni-NTA (Ni2+-nitrilotriacetate; Sigma His-select, H-0537) was used for purification and the purified ABCG2 protein (wild-type or its SBE mutants) was reconstituted in *E.coli* lipid extract supplemented with 0-0.4 mM cholesterol.

**ATPase Activity Measurements** — ATPase activity was measured on isolated membranes of Sf9 cells expressing wtABCG2 or its mutant variants by colorimetric detection of inorganic phosphate liberation, as previously described (Ozvegy et al., 2002). Figures represent the mean values of at least two independent experiments with four parallels, measured in two different membrane preparations for each type of ABCG2. ATPase activity of ABCG2 in proteoliposomes was determined as described previously (Telbisz et al., 2013).

**Vesicular uptake measurements** - Sf9 membrane vesicles were incubated in the presence or absence of 4 mM MgATP (or 4 mM MgATP + 1 μM Ko143) in a buffer containing 40 mM 3-(N-morpholino) propanesulfonic acid–Tris (pH 7.0), 56 mM KCl, 6 mM MgCl2 and 2 mM dithiothreitol, in a final volume of 140 μl, at 37°C for 5 min. The measurement was started by the addition of 100 μM [3H]methotrexate (Moraveck Biochemicals), 25 or 50 μM [3H]estradiol-glucuronide (Perkin Elmer), 100 μM [3H]glycocholate (Perkin Elmer) or 100 μM [3H]cholic acid (American Radiolabeled Chemicals, Inc.). The experiments were carried out as described earlier (Ozvegy-Laczka et al., 2005).

**Cellular dye uptake and calculation of ABCG2 transport activity** - Measurement of Pheophorbide A and rhodamine 123 extrusion by ABCG2 from intact insect cells was described earlier (Ozvegy-Laczka et al., 2008). Geo mean fluorescence values measured in the absence (M0) and in the presence of inhibitor (M_i) were determined, activity factor was calculated as follows: (M_i - M0)/ M0 *100.

**Measurement of Hoechst 33342 transport activity**- Hoechst 33342 transport was determined as described earlier in (Ozvegy-Laczka et al., 2004). Transport activity was calculated as ((F100-F0)/F100)*100, where F100: fluorescence in the presence of 1 μM Ko143, F0: fluorescence in the absence of the inhibitor.
RESULTS

I. Effect of cholesterol on the function of ABCG2 R482 mutant variants

I.a. Effect of cholesterol on the ATPase activity of the ABCG2 R482 mutants

Cholesterol dependence of human ABCG2 in Sf9 cell membranes has been characterized in detail earlier (Pal et al., 2007; Telbisz et al., 2007). Briefly, human ABCG2, when expressed in cholesterol „poor” insect cells (5-8 μg cholesterol/mg membrane protein) has a well measurable ATPase and transport activity. However, increasing the cholesterol content of the insect membranes by cholesterol-loaded random methylated beta cyclodextrin (cholesterol-RAMEB) up to 40-60 μg cholesterol/mg membrane protein, results in 4-20-fold activation of the transport function of ABCG2. Accordingly, substrate stimulation of the ATPase activity becomes more pronounced in cholesterol-enriched Sf9 membranes (Pal et al., 2007; Telbisz et al., 2007). Moreover in our recent study we have demonstrated that cholesterol is essential for ABCG2 function, i.e. purified ABCG2 is only active when the proteoliposomes contain 20-30 mol % cholesterol (Telbisz et al., 2013). Despite the crucial role of cholesterol in ABCG2 function, the cholesterol sensing sites in ABCG2 has not yet been defined.

The Arg482 residue, residing in or near the third transmembrane helix of ABCG2 has been documented to significantly alter the substrate interactions of the transporter. Several of the R482 variants gain a rhodamine 123 transport function, while all of them lose MTX transport capacity (Ozvegy et al., 2002; Ozvegy-Laczka et al., 2005). We have shown earlier that Arg482 also has a critical role in the cholesterol sensitivity of human ABCG2. In contrast to the wild-type protein, in Sf9 cell membranes increasing membrane cholesterol levels did not significantly influence the activity of the R482G and R482T mutants (Telbisz et al., 2007), although experiments on purified ABCG2 reconstituted in proteoliposomes revealed that cholesterol is also essential for the function of the R482G variant (Telbisz et al., 2013).

In order to examine how the characteristics of amino acid 482 influence the cholesterol sensing capability of ABCG2, we have analyzed seven additional R482 mutants (R482D, I, M, N, S, Y and K). These ABCG2-R482 variants were expressed in Sf9 insect cells at similar level to the wild-type protein (data not shown) and, as documented earlier, all these mutants were active, showing a measurable basal ATPase activity that was sensitive to Ko143, a specific inhibitor of ABCG2 (Ozvegy-Laczka et al., 2005). In the present study the ATPase activity of the mutant variants was measured in “control” (native, cholesterol-poor) and cholesterol-enriched Sf9 membranes. In cholesterol-loaded membranes the basal ATPase
activity of most of these variants increased (see Supplemental Table I), still, the increase in membrane cholesterol levels did not change the relative substrate stimulation of the R482D, G, N, S, and T variants (Figure 1A). In contrast, in the case of the R482I, K, M, and Y variants, similarly to the wild-type ABCG2, cholesterol enrichment significantly improved the ratio of substrate stimulation, as examined after the addition of prazosin (Fig. 1A). We have also observed a similar effect of other substrates (e.g. quercetin) on the ATPase activity of these mutants (data are summarized in Supplemental Table I).

1.b. Effect of cholesterol on the transport activity of the ABCG2 R482 mutants

In order to find out whether membrane cholesterol has a direct effect on the transport activity of the R482 mutants, we measured [3H]methotrexate uptake in Sf9 inside-out membrane vesicles containing different R482 variants, either in the presence or absence of excess cholesterol. As shown (Supplemental Figure 1A), in contrast to wild-type ABCG2, none of the R482 mutants exhibited significant methotrexate transport, either in the control or in the cholesterol-loaded membrane vesicles. These findings suggest that cholesterol enrichment of the membranes did not alter this particular substrate interaction of the mutant ABCG2 variants.

Intact Sf9 cells, transiently expressing ABC transporters, are suitable for fluorescent dye accumulation measurements in which the transport activity of the human ABCG2 can be determined (Ozvegy et al., 2002). In the following experiments intact Sf9 cells were harvested 24 – 40 h after transfection, when approximately 60-70% of the cells are viable, and accumulation of different ABCG2 substrates was measured in control or cholesterol-enriched Sf9 cells.

When the Sf9 cells were loaded with cholesterol by incubating them with 2 mM cholesterol-RAMEB, we found that this treatment did not influence cell viability, at least during the period of the transport experiments. Cellular fluorescence of Hoechst 33342 (Hst) and rhodamine 123 (R123) was analyzed by fluorometry and flow cytometry, respectively. The activity factors for the different ABCG2 variants were calculated based on the intracellular fluorescence detected in the presence or absence of the specific inhibitor Ko143. The actual expression levels of the ABCG2 variants were examined by Western blotting, showing similar expression levels of the transporter (data not shown).

Similarly to that found earlier, there was a well measurable, Ko143-sensitive Hst dye transport both in the cells expressing the wild-type ABCG2, as well as in those expressing most R482 mutants, with only very low activity in the case of the R482K and R482Y variants (Figure 1B). When the cells were loaded with cholesterol, this Hst dye uptake was
significantly improved in the wt, and the R482I and M variants. Moreover, a significant Hst transport activity occurred in the case of the R482K and Y variants. In contrast, we did not observe a significant effect of cholesterol on the Hst transport by the R482D, G, N, S and T variants. Note, that these cholesterol-mediated alterations in the Hst transport capacity of ABCG2 correspond to the effects of cholesterol on the ABCG2-ATPase activity.

In order to examine the potential effects of cholesterol on the substrate specificity of the mutant variants, we also measured ABCG2-dependent R123 extrusion both in the control and cholesterol-loaded Sf9 cells, expressing the human ABCG2 variants (Supplemental Figure 2B). As noted earlier, the wild-type ABCG2 had no R123 transport activity, while several mutant variants acquired such a transport function. Cholesterol loading significantly increased R123 extrusion in cells expressing the R482I and M variants, while there was no measurable effect in the D, G, N, S and T variants. In the case of the wt protein and the R482K and Y variants there was no detectable R123 extrusion either in the absence or presence of cholesterol.

II. Effect of cholesterol on the function of ABCG2 - SBE mutant variants

A predicted steroid–binding element (SBE, LxxL) of ABCG2 is located in the potential fifth transmembrane helix of the transporter (see Introduction). The motif was reported to alter ABCG2-mediated recognition of progesterone and estradiol, two steroid hormones closely related to cholesterol (Velamakanni et al., 2008). We have generated the Leu to Ala mutations L555A, L558A and L555A/L558A, in this motif. The mutants were expressed in Sf9 cells at equal levels to the wild-type ABCG2 (Figure 2A) and the effect of cholesterol on the ATP hydrolysis and transport activity of the mutants was investigated using membrane vesicles or transporter-expressing intact Sf9 cells.

II.a. Effect of cholesterol on the ATPase activity of the SBE mutant ABCG2

As shown in Figure 2B, the L555A, L558A and L555A/L558A mutants exhibited a well-measurable vanadate-sensitive ATPase activity. However, given the similar expression levels of the wild-type and the mutant proteins, we found that L555A and L555A/L558A had only about 1/3 basal ATPase activity as compared to wtABCG2. Moreover in the case of the L555A and L555A/L558A mutants, ATPase turnover in the presence of prazosin was also well below of that measured for the wild-type protein or the L558A mutant.

When analyzing the effect of cholesterol loading on the prazosin-stimulated ATP hydrolysis, we found no significant change in ATPase turnover in any of the three SBE mutants (see Fig. 2B).
Next we have studied the effects of numerous wild-type ABCG2 substrates on the ATPase activity of the SBE mutants in both control and cholesterol-enriched Sf9 cell membranes. Several activators of the wild-type ABCG2 ATPase (doxorubicin, flavopiridol, SN-38 and topotecan) did not stimulate ATP hydrolysis of the mutants even in cholesterol-enriched membranes (not shown). However, we found a few substrates (nilotinib, prazosin, quercetin and EKI-785) that enhanced ATP hydrolysis in these mutants as well. In order to investigate the effect of cholesterol on substrate-stimulated ATPase activity of the SBE mutants, we have measured the effects of these “activators” in membranes loaded with different amounts of cholesterol (by applying 0.025 – 2 mM cholesterol-RAMEB for cholesterol loading). We found that increasing the cholesterol content of the Sf9 cell membranes did not significantly enhance the substrate stimulation of the ATPase activity in the LxxL mutants. These effects of nilotinib and quercetin in such membrane preparations are shown in (Supplemental Figure 2) - prazosin and EKI-785 gave similar results (data not shown).

II.b. Effect of cholesterol on the transport activity of the SBE mutant ABCG2

In these experiments we have examined the effect of cholesterol on the [3H]methotrexate ([3H]MTX) and [3H]estradiol-glucuronide ([3H]ESG) transport activity of ABCG2 L555A, L558A and L555A/L558A mutant variants expressed in Sf9 insect cells. As discussed above, cholesterol enrichment of the Sf9 inside-out membrane vesicles greatly increases MTX and ESG transport by the wild-type ABCG2 (see section I.b. and (Telbisz et al., 2007)). To test whether cholesterol has any effect on the transport function of the SBE mutants, Sf9 membrane inside-out vesicles containing the SBE mutants were analyzed for their [3H]MTX and [3H]ESG transport activity.

We found that despite their comparable expression level to the wild-type ABCG2, the L555A and L555A/L558A mutants did not show any detectable vesicular transport activity for methotrexate either in control or in “cholesterol-rich” membranes. Even in the case of the L558A mutant, showing high ATPase activity, we could detect only a very low methotrexate transport activity, similar to that observed in the R482 mutants (Supplemental Figure 1A and 3A). When we analyzed [3H]MTX transport by the L558A mutant in membranes loaded with cholesterol we found only a non-significant increase in this transport activity (Supplemental Figure 3A). In the estradiol-glucuronide vesicular transport experiments again none of the three mutants showed ABCG2-specific activity, and cholesterol did not improve their ESG transport function (Supplemental Figure 3B).

To further analyze the effect of cholesterol on the activity of the SBE mutants, we have measured the accumulation of different fluorescent ABCG2 substrates in intact Sf9 insect
cells. Accumulation of Hoechst 33342 or Pheophorbide A was determined by fluorometry or flow cytometry respectively, both in control cells and in cells loaded with 2 mM cholesterol-RAMEB. The actual expression levels of ABCG2 variants were examined in each experiment by Western blotting (data not shown). We found that the SBE mutants were able to transport both Hoechst 33342 and Pheophorbide A, but in contrast to the wild-type protein, cholesterol did not improve their dye extrusion capacity (Figure 2C and D).

III. Effect of cholesterol on the function of isolated and reconstituted ABCG2-SBE mutants

It has been shown earlier that increase in the cholesterol levels of the insect membranes did not significantly modulate the function of the ABCG2 R482G mutant, which would imply its apparent cholesterol-insensitivity. However, reconstitution of the purified ABCG2 R482G variant revealed that presence of cholesterol was also essential for the function of this mutant variant; however, lower cholesterol levels (amounts which are most probably present in native insect membranes) were sufficient to achieve its full activity as compared to wild-type ABCG2 (Telbisz et al., 2013). In order to analyze cholesterol sensing of the purified SBE mutants, we have generated N-terminally His6-tagged versions of the L558A and L555/558A variants. The His6-L558A and His6-L555A/L558A ABCG2 mutants were successfully expressed in Sf9 cells and we also found that tagging did not alter their functionality (data not shown).

The membrane isolation, as well as the purification and reconstitution of the L558A variant were successful. However, though the expression level of the His6-L555A/L558A variant in the Sf9 cells was comparable to that of the other variants, the purification yielded a much lower amount of this mutant (data not shown).

We analyzed the ATPase activity of the purified L558A variant, reconstituted in E. coli lipids in the absence and in the presence of cholesterol. Interestingly, this ABCG2 mutant had a negligible ATP hydrolysis in cholesterol free E. coli lipids similarly to the wild-type, while increasing cholesterol concentrations greatly accelerated both the basal (Fig. 3A) and the substrate-stimulated (data not shown) ATPase activities, again in harmony with the wild-type.

Surprisingly, the L555/558A mutant lost its functionality upon purification. There was no ATPase activity in proteoliposomes regardless the amount of cholesterol applied. When we investigated the dimerization state of the double mutant, we found that in contrast to the wild-type protein, the disulphide bridge linked homodimer (corresponding to the physiological state of ABCG2) could not be detected in the case of the double mutant after purification (Fig.
3B). Therefore the inactivity of the double mutant can be due to its impaired dimerization capability.

**IV. Effect of bile acids on the function of human wild-type ABCG2 expressed in Sf9 cell membranes**

Bile acids are continuously present in all tissue fluids, including blood plasma, where, after a meal, free bile acid levels can reach 100 μM. Bile acids participate in a continuous entero-hepatic circulation and their concentrations inside the intestinal epithelial cells or in the hepatocytes are considerably higher, especially in the bile-secreting canalicular membranes (Li and Chiang, 2012). The critical micelle concentrations of bile acids are in the range of 2-10 mM (Simonovic, 1997) and the hepatic bile acid secretion occurs by the formation of mixed micelles with phospholipids and cholesterol. In the next set of experiments, we aimed to determine whether, similarly to cholesterol, bile acids modulate the function of ABCG2.

**IV.a. Effect of bile acids on the ATPase activity and transport function of wild-type ABCG2**

In order to compare the effects of cholesterol and bile acids we performed studies with these sterols alone or in combination. In the control Sf9 cell membranes there is no stimulation of the ATPase activity with prazosin (a known transported substrate of ABCG2), whereas quercetin (also a transported substrate of the protein) has a measurable stimulatory effect even in these conditions. When the cholesterol content of the membranes is increased, a significant activation of the ATP hydrolysis by prazosin occurs, and an increase in quercetin-stimulated ATPase activity is also observed (see Figure 4A). When cholic acid (CA, 1 mM) is added to the control membranes, a significant reduction in the baseline ABCG2-ATPase (measured in the absence of added drug-substrates), and a slight stimulatory effect of prazosin is observed (the effect of quercetin is unaltered - Fig. 4B, left columns). However, the most pronounced effect of CA is observed in cholesterol-loaded membranes (Fig. 4B, right columns): the baseline ATPase activity is strongly reduced (almost to the level of Sf9 membranes expressing the inactive ABCG2-K86M mutant - see Figure 2B), while the drug-stimulated ATPase activity is unchanged. Thus, the relative drug-stimulated ATPase activity (ATP hydrolysis with drug substrates/basal ATP hydrolysis) is increased up to 6-8 fold.

In order to characterize these phenomena in more detail, we have examined the concentration-dependent effects of bile acids on the human wtABCG2-ATPase activity in cholesterol-loaded Sf9 cell membranes, closely reflecting the plasma membrane environment...
of mammalian cells. It has to be emphasized that bile acids were used under their critical micelle concentration values, thus a direct membrane effect was unlikely to occur.

As shown in Fig. 4C, all the investigated bile acids (glycocholate (GC), taurocholate (TC) cholic acid (CA) and ursodeoxycholic acid (UDC) and a synthetic derivative, CHAPS (which has a critical micelle concentration of 6-10 mM), effectively reduced the baseline ATPase activity of wild-type ABCG2, while substrate-stimulated ATPase was unaltered. Due to these effects, the relative drug-stimulated ATPase activity (measured in the presence of quercetin) was increased, and especially UDC produced a high ratio of drug-stimulated and basal ABCG2-ATPase activity (Fig. 4D). We have examined the drug-stimulated ATPase activity for several other known ABCG2 substrates, and found that bile acids did not increase the V_max of the ATPase, but due to the decreased baseline ATP hydrolytic activity, relative drug stimulation was increased. These effects for several substrates are documented in Table I. It should also be noted that the effect of the ABCG2 inhibitor Ko143 remained unaltered in the presence of bile acids.

In the following experiments we have examined the effects of various bile acids on the transport function of ABCG2 in vesicular uptake assays. In these studies we used radio-labeled methotrexate (MTX) and estradiol-glucuronide (ESG) as established ABCG2 substrates, and added various bile acids to the medium. We found that at low concentrations (25-50 µM) bile acids had no effect on ABCG2-dependent substrate transport, while higher bile acid concentrations (above 250 µM) inhibited both MTX (data not shown) and ESG transport activity (Supplemental Figure 4). Since in the vesicular transport measurements we used low substrate concentrations, a high ratio of bile acid/transported substrate may explain these latter results (see Discussion).

It has been documented earlier that bile acids can stimulate the transport activity of MRP2 (Bodo et al., 2003). This has been also observed here when measuring ATP-dependent ESG transport by MRP2 – the addition of 250 µM cholic acid doubled the ESG transport activity in the case of MRP2 and glycocholate also increased this transport by 25% (Supplemental Figure 4).

**IV.b. Examination of the transport of bile acids by ABCG2**

As described above, bile acids decrease the basal ATPase activity of ABCG2. However, this does not necessarily mean that they are not transported molecules (e.g. Hoechst 33342 a known transported ABCG2-substrate inhibits the ATPase function (Ozvegy et al., 2002)). In order to explore this question the transport of several bile acids in transporter-expressing inside-out Sf9 membrane vesicles was examined.
ATP-dependent uptake of different radiolabeled bile acids was measured in ABCG2-containing, cholesterol-loaded inside-out insect membrane vesicles (showing maximum transport activity e.g. for ESG). In parallel experiments we also examined similar bile acid transport by using membrane vesicles containing MRP2 (ABCC2) or BSEP (bile salt export pump, ABCB11), two known bile acid transporters. In all cases the transport of labeled GC, CA, ESG and MTX was examined, in concentrations already documented to provide well measurable uptake in the case of these transporters.

As documented in Figure 5, a significant ATP-dependent glycocholate (GC) uptake was measured in the case of MRP2 and BSEP, while ABCG2 showed no transport activity. Cholic acid was efficiently transported by BSEP and not by MRP2 or ABCG2. These direct transport measurements also indicate that bile acids in the applied concentrations (100 μM) have no pronounced effect on the stability of the inside-out Sf9 membrane vesicles used in these experiments. In the case of ESG, MRP2 had the highest transport activity, while MTX was best transported by ABCG2. These results indicate that ABCG2 is most probably not a professional bile acid transporter.

V. Effects of bile acids on the function of ABCG2 R482 and SBE mutants

We have documented above that mutations in the amino acid position 482, and the replacement of leucines to alanines in the potential steroid-binding element (SBE) of ABCG2 strongly influence the cholesterol sensing capability of the protein. In order to explore the effect of bile acids, we performed these experiments in cholesterol-loaded Sf9 cell membranes by examining selected representatives of the R482 variants. These were the R482G and R482S variants, being fully active already at low membrane cholesterol levels, and the R482K and R482I mutants, which showed similar cholesterol-sensing capability to the wtABCG2 (see above). We have also examined the effects of bile acids on the LxxL mutants (see Figure 6).

When measuring the effects of bile acids on the ABCG2-ATPase activity in isolated Sf9 membranes we found that, in contrast to the wt protein (Figures 4C, 4D and 6A), in the case of the R482G or S variants low concentrations of cholic acid did not significantly alter ABCG2-ATPase activity, while using higher bile acid concentrations (above 0.5 mM) both the baseline and the substrate-stimulated ATPase activities decreased (see Figure 6B for the R482G mutant, R482S is not shown). Thus the relative substrate stimulation was not increased by bile acids in these variants (see also Fig. 6D). In the case of the R482K and I mutants a variable alteration in the substrate stimulation was observed by different bile acids (Fig. 6D).
Figure 6C shows that cholic acid does not influence ATP hydrolysis of the L555A/L558A mutant, that is, both the basal and the substrate stimulated activities remained unaltered. Glycocholate and taurocholate also did not influence the activity of the L555A/L558A mutant (data not shown). Similarly, the activity of the L555A was not altered by the presence of bile acids. In the case of the L558A mutant a similar effect of bile acids was observed as in the case of the R482G variant: cholic acid and taurocholate inhibited both the basal and the substrate stimulated ATPase activity (Supplemental Figure 5), but the relative substrate activation was practically unchanged (Figure 6D). Glycocholate had no effect either on the baseline or the substrate stimulated activity of the L558A mutant (Supplemental Figure 5).
DISCUSSION

Human ABCG2 has an important role in detoxification. Therefore better understanding of the regulation of its function may allow the modulation of ADME-Tox (absorption, distribution, metabolism, excretion and toxicity) properties thus may improve anticancer therapies. Membrane cholesterol has been shown to modulate the activity of ABCG2 (Pal et al., 2007; Storch et al., 2007; Telbisz et al., 2007); however, the protein sites for cholesterol recognition have not yet been identified.

In order to achieve a better understanding of the interaction between sterols and ABCG2, we have generated several mutant variants of the transporter, expressed them in insect cells and characterized their sterol sensitivity. Previously, Arg 482 was found to be critical in cholesterol-sensing of ABCG2 (Telbisz et al., 2007). Here we investigated R482 mutants with various amino acid side chain characters. Based on our present results, cholesterol-sensing capability of these variants could be grouped into two clusters. Members of the first cluster, also including the wild-type protein (R), contain large (hydrophobic or positively charged) amino acids, represented by R482I, M, K and Y. The function of these ABCG2 protein variants is highly cholesterol-dependent as they are significantly activated by increased membrane cholesterol levels (Figure 1A and B). Interestingly, the activating effect of cholesterol is the most pronounced in the case of the R482K and Y mutants, as these variants are practically unable to transport Hoechst 33342, unless high levels of cholesterol is present in the cell membranes (Figure 1B). In contrast, R482 mutants represented by relatively small (polar, uncharged, or negatively charged) amino acids formed another cluster regarding cholesterol sensing. The basal ATP hydrolysis in these variants was slightly accelerated by increased membrane cholesterol levels (Supplemental Table I), while their substrate stimulation and transport function was practically unaltered by cholesterol loading (Figure 1).

In earlier studies (Velamakanni et al., 2008), a potential steroid-binding element (SBE, aa 555-558) in ABCG2 was suggested to be responsible for sterol sensing in this transporter. In their study (Velamakanni et al., 2008) found that the ABCG2-L555A/L558A mutant does not have an altered cholesterol sensing, but progesterone and estradiol binding as well as transport were abolished. In our present work we have expressed and analyzed in detail the SBE (or LxxL motif) mutants, L555A, L558A and L555/558A of human ABCG2. We found that the mutants are active, showing a vanadate- and Ko143 (an ABCG2-specific inhibitor) -sensitive ATPase activity, and are able to transport several established wtABCG2 substrates (Figure 2).
Additionally, though the SBE mutants were active, they showed altered substrate-specificity, as compared to the wild-type ABCG2 protein (Sections II.a and b.).

When examining the effect of cholesterol on their function, we found that although a slight increase in the baseline ATP hydrolysis of the L555A and L558A mutants occurred in cholesterol-enriched membranes (fold activation was 1.2 +/- 0.1 and 1.5 +/- 0.1 respectively), their relative substrate stimulation (ratio of ATP hydrolysis in the presence and absence of substrates) did not change (Figure 2B and Supplemental Fig.2). Accordingly, in direct transport experiments we found that all three SBE mutants were already fully active without excess cholesterol (Figure 2C,D). Moreover the L555A/L558A mutant was absolutely insensitive to cholesterol-loading in the ATPase activity measurements (Figure 2B). This apparent cholesterol independence of the L555A/L558A mutant contradicted the results described in the study of Velamakanni et al., which may be due to the fact that they investigated a triple mutant of ABCG2, having R482G besides the L555A/L558A mutation, while we performed our experiments using the wild-type ABCG2 (482R) as a ‘background’. As demonstrated earlier, and also in this paper, the R482G variant is already fully active in cholesterol “deficient” Sf9 membranes. In order to solve this contradiction, we have also generated the triple mutant R482G/L555A/L558A of ABCG2, and expressed this protein in insect cells. Still, this variant was also not activated by cholesterol.

Since even very low levels of membrane sterols may affect ABCG2 function, we have purified and reconstituted the L558A and L555A/L558A mutants in cholesterol free liposomes. Surprisingly, we found that the L558A mutant also needs cholesterol for its full activity. Unfortunately cholesterol sensing of the double mutant could not be defined as it became inactive during purification. In summary, we suggest that the aforementioned two regions, both localized to the transmembrane domain, modulate both cholesterol sensing and substrate recognition of ABCG2.

The effects of sterols have been studied in detail in the case of the ABCB1/MDR1 protein (Garrigues et al., 2002). Increased cholesterol accelerated the basal ATPase activity of this multidrug transporter, while sterols had no major effect on substrate stimulated ATP hydrolysis. It has also been shown that membrane cholesterol differentially modulated the effect of MDR1 substrates, depending on the size of these molecules. Kimura et al. (Kimura et al., 2007a) found that the presence of cholesterol increased the recognition of small molecular weight MDR1 substrates (molecular mass below 800 Da), but had no impact on the transport of larger molecules (molecular mass higher than 900 Da), and cholesterol itself was not transported by MDR1. Based on these findings a review suggested a cholesterol fill-in
model (Kimura et al., 2007b), that is in the case of larger substrates the binding pocket of MDR1 is completely filled in, and there is no room for cholesterol. However, in the case of smaller substrates an empty space remains in the binding pocket, which can be filled up with cholesterol.

In the case of wild-type ABCG2 we have tested a large number of substrates and found that cholesterol increased the activation in all cases. When analyzing the size of these compounds we found that all these molecules belong to the “smaller” category, therefore the effect of cholesterol found in the case of ABCG2 may be in harmony with the cholesterol fill-in model. Larger compounds (e.g. cyclosporin A) mostly behave as inhibitors of ABCG2, thus this question is difficult to answer. Based on our current results we suggest that in the case of ABCG2, larger side chains at position aa482 and 555 or 558 may keep the gate of the substrate binding pocket clogged, and cholesterol is required to widen the channel for the entry of the substrates.

In the next series of experiment, we investigated the modulatory effect of bile acids on the function of wild-type ABCG2. We found that bile acids and their derivatives, much below their critical micelle concentrations, strongly reduce the basal ABCG2-ATPase activity of the wild-type protein, without significantly affecting its substrate-stimulated ATPase (Figure 4C). Moreover, we observed that when membranes were loaded with cholesterol, cholic acid decreased the baseline ATP hydrolysis down to the background level, that is, to an ATP hydrolysis in membranes expressing the inactive mutant ABCG2-K86M (see Figure 2B). As a consequence, in the presence of bile acids the relative substrate stimulation was greatly increased (Figures 4D).

A key advantage of the insect cell overexpression system is that isolated membrane preparations contain much higher level of ABCG2 than membranes isolated form mammalian overexpression systems (Ozvegy et al., 2001), thus providing an efficient tool for examining the modulation of ABCG2 ATPase or transport activity (Hegedus et al., 2009). Based on our present observations we suggest that in order to obtain maximum activation with ABCG2 substrates, cholesterol-loaded insect membranes should be used in the presence of bile acids. This provides an improved ATPase assay for testing potential substrate interactions of ABCG2.

Interaction between the ABCG2 transporter and bile acids has already been investigated in several studies, though with contradictory results (see Introduction). In our experiments we could not detect a direct transport of the investigated bile acids in membrane vesicles (Fig. 5), while we found an inhibitory effect of higher bile acid concentrations on the ABCG2-
dependent vesicular uptake (Suppl. Figure 4). A direct effect of bile acids on the membrane vesicle integrity was clearly excluded by the findings that under the same conditions bile acids significantly increased MRP2-dependent vesicular estradiol-glucuronide transport (see (Bodo et al., 2003) and Supplemental Fig. 4), and both MRP2- and BSEP-containing vesicles performed efficient bile acid uptake (Figure 5).

Our data thus do not support the findings of some earlier reports, but can be explained by the different expression systems and experimental conditions used. Even Blazquez et al. (Blazquez et al., 2012) suggested that since the expression of this protein in the liver is significantly lower than that of the “professional” bile acid transporter BSEP, the role of ABCG2 in this function is questionable (while placental bile acid transport by ABCG2 may be relevant). Accordingly, Abcg2 (-/-) mice do not develop the symptoms of cholestasis (Mennone et al., 2010), thus ABCG2 may have only a moderate effect on bile acid transport. Based on our results we suggest that although bile acids modulate the basal ATP hydrolysis by ABCG2, they are not exported from the cells at a significant rate by this transporter. A low affinity, low transport activity type interaction of ABCG2 with bile acids may explain this phenomenon.

Interestingly, we found that R482 and the L555-L558 positions are also crucial in bile acid recognition of ABCG2 (Figure 6 B-D and Suppl. Fig. 5). Mutations at these sites removed the major effects of bile acids on the basal ATPase activity, thus suggesting a site-specific modulatory effects of bile acids.

As a conclusion, sterols may significantly affect the function of ABCG2, especially in the liver. In hepatocytes ABCG2 is abundantly expressed, and is a key transporter for xenobiotics into the bile. Cholesterol significantly activates the transport function of ABCG2; therefore, increased cholesterol level may promote enhanced detoxification. Currently we cannot exclude if cholesterol – although with a low efficiency – is also a transported substrate of this protein. Our present data reveal that bile acids, although probably not efficiently transported by ABCG2, also significantly modulate its function. In the liver, where a large amount of bile acids is formed from cholesterol, bile acids may cause a reduction of a futile ATP consumption by ABCG2, represented as a baseline ATPase activity. In addition to the functional regulation, although not examined in the present report, it should be mentioned that sterols may also modulate ABCG2 transcription and expression levels through interactions with nuclear receptors, e.g. PPARγ, PXR and CAR (Jigorel et al., 2006).

Based on the present results we suggest a model describing a complex post-translational regulation of the ABCG2 function in the liver (Figure 7). While cholesterol and bile acids are
mostly transported by “professional” active ABC transporters, that is ABCG5/G8, BSEP and MRP2 into the bile, the drug transport function of ABCG2 is significantly modulated by sterols. Based on these findings we also suggest that the amino acid positions characterized in this study may serve as potential targets for a pharmacological modulation of ABCG2 function.
ACKNOWLEDGEMENTS:

The technical help by Zsuzsanna Andrási, Éva Krizsán, Katalin Mihály and Györgyi Vermes is gratefully appreciated. We thank SOLVO Biotechnology Ltd. for providing BSEP-containing Sf9 membrane vesicles.

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 Participated in research design: Telbisz, Hegedüs, Özvegy-Laczka and Sarkadi
 Conducted experiments: Telbisz, Hegedüs and Özvegy-Laczka
 Performed data analysis: Telbisz, Hegedüs and Özvegy-Laczka
 Wrote or contributed to the writing of the manuscript: Hegedüs, Özvegy-Laczka, Váradi and Sarkadi
REFERENCES


FOTNOTES:

This work was supported by the Hungarian Research Fund grant [NK 83533], and grants from KMOP-1.1.2-07/1-2008-0003, NKTH-STEMKILL, and NIH-ARD ACHILLES.

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FIGURE LEGENDS

FIGURE 1: ATPase and transport activity of the ABCG2-R482 mutants - effect of cholesterol. Panel A: Vanadate-sensitive ATP hydrolysis of wtABCG2 and its R482 mutant variants was determined in insect cell membranes (open bars) and in the membranes loaded with cholesterol (black columns). The ratio of substrate stimulated ATPase activity (measured in the presence of 100 μM prazosin) and the baseline ATP hydrolysis (no added substrate) is shown. Columns represent data obtained from two independent experiments with four parallels, bars ± SD values. *: Student’s t-test, significant difference, p < 0.05

Panel B: Hoechst 33342 (1 μM) uptake was determined in insect cells transfected with baculoviruses encoding different ABCG2-R482 mutants or the wild-type protein. Cellular fluorescence was determined in fluorescence spectrophotometer. Transport activity was calculated as ((F100-F0)/F100)*100, where F100: fluorescence in the presence of 1 μM Ko143, F0: fluorescence in the absence of the inhibitor. *: Student’s t-test, significant difference, p < 0.05

FIGURE 2: Expression and ATPase activity of SBE mutants in Sf9 cells. Panel A: Western blot detection of the SBE mutants in insect membranes using the BXP-21 antibody. Samples were loaded in equal amounts to the gel. Panel B: ATP hydrolysis of the SBE mutants in control and cholesterol-loaded membranes. ATPase activity was determined either in the absence of added substrates (open bars), in the presence of 1 μM nilotinib (black columns) or 10 μM Ko143 (striped bars). Data were compiled from two independent experiments with four parallels and average ± SD values are indicated.

Panels C and D: Effect of cholesterol on the transport activity of wtABCG2 and the SBE mutant variants in insect cells. Fluorescent dye uptake was determined in insect cells transfected with baculoviruses encoding different ABCG2-SBE mutants or wild-type protein. Cellular fluorescence was determined in FACsCalibur cytometer (2 μM Pheophorbide A) or in fluorescence spectrophotometer (1 μM Hoechst 33342). Transport activity was calculated as described in the Experimental procedures or at Figure 1. For Pheophorbide A uptake, data were normalized to the activity measured in the absence of added cholesterol. Average ± SD values of three independent experiments are shown.
FIGURE 3 Panel A: ATPase activity of purified wtABCG2, ABCG2-L558A and L555/558A in proteoliposomes. N-terminally His6-tagged ABCG2 was isolated, purified and reconstituted in 1 mg/ml E.coli lipid extract plus various amounts of cholesterol, as indicated on the x axis. ATPase activity was measured in the absence of added substrates. Data points show the average of three independent experiments ± SD values.

Panel B: Lack of dimerization of the purified the L555/558A mutant. Purified wtABCG2 or L555/558A was treated with 4 mM oxidized DTT for 5 minutes at 37°C, and then dissolved in sample buffer (62.5 μM Tris HCl pH 6.8, 2 % SDS, 10 μM EDTA-Na pH 6.8, 10 % glycerol, 2M urea, 0.14 mg/ml bromophenol blue). Electrophoresis and Western blotting have been performed as described in the Methods section. ABCG2 was visualized by the BXP-21 antibody.

FIGURE 4: Effect of bile acids on ABCG2 ATPase function. Panel A: Vanadate-sensitive ATPase activity in Sf9 membrane vesicles containing wtABCG2 was determined either in the absence (Panel A) or in the presence of 1 mM cholic acid (Panel B) both in control and 2 mM cholesterol-RAMEB-treated membranes. Open columns represent baseline ATPase activity (no added compound), black or striped columns show ATP hydrolysis in the presence of 100 μM prazosin or 5 μM quercetin, respectively. Average of two independent experiments each with two parallels ± SE values.

Panels C and D: Relative ATPase activity of wtABCG2 in cholesterol-loaded Sf9 membranes - effect of increasing amounts of bile acids. Panel C: ATP hydrolysis in cholesterol-loaded ABCG2-containing Sf9 membranes is shown as a relative value compared to the activity measured in the absence of added substrates and bile acids. Panel D: ATP hydrolysis in the presence of 5 μM quercetin compared to the baseline ATPase activity (no drug-substrate, no bile acids added). Measurements were performed in quadruplicates, mean ± SE values are shown.

FIGURE 5: ATP-dependent uptake of different bile acids and ABCG2-substrates into cholesterol-loaded Sf9 vesicles. Inside-out membrane vesicles containing wtABCG2, MRP2 or BSEP were incubated with [3H]-labelled glycocholate (GC, 100 μM), cholic acid (CA, 100 μM), methotrexate (MTX, 100 μM) and estradiol-glucuronide (ESG, 50 μM). Mean values of ATP-dependent uptake obtained from at least two independent experiments with four parallels ± SD values are shown.
FIGURE 6: Effects of bile acids on the ATPase activity of wtABCG2 and its mutant variants. Panels A-C: Increasing amounts of cholic acid were added to insect cell membranes previously loaded with cholesterol. Basal (black squares) or substrate-stimulated ATP hydrolysis was determined (open squares, measured in the presence of 1 μM quercetin (panels A and B), or 1 μM nilotinib (panel C)). The figure shows the average of at least two independent measurements, four parallels ± SE values. Panel D: Effect of bile acids on the substrate stimulation of the ATPase activity of wtABCG2 and its mutant variants. Membranes were incubated with or without 1 mM bile acid, and the ratio of ATP hydrolysis in the presence of 1 μM quercetin (wt, R482G, S, K and I) or 1 μM nilotinib (SBE mutants) and the baseline ATPase activity were calculated. Columns represent average of at least four data points, bars show SD values.

FIGURE 7: Schematic representation of a complex regulation of ABCG2 function in hepatocytes.
Table I: Effect of cholic acid on the stimulation or inhibition of the ABCG2-ATPase in cholesterol-loaded, isolated Sf9 membranes by various ABCG2 interacting compounds.

<table>
<thead>
<tr>
<th>ABCG2 substrates</th>
<th>Concentration (μM)</th>
<th>Relative ATPase activity compared to the baseline</th>
<th>with 1 mM cholic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>5</td>
<td>control: 2</td>
<td>5.4</td>
</tr>
<tr>
<td>ZD1839</td>
<td>1</td>
<td>control: 1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>0.1</td>
<td>control: 1.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Topotecan</td>
<td>100</td>
<td>control: 1</td>
<td>1.7</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>50</td>
<td>control: 0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Sulfasalazin</td>
<td>5</td>
<td>control: 2</td>
<td>2.8</td>
</tr>
<tr>
<td>Prazosin</td>
<td>50</td>
<td>control: 1.8</td>
<td>6</td>
</tr>
<tr>
<td>Ko143</td>
<td>1</td>
<td>control: 0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Figure 3

A

ATPase turnover (mol P/min/mol ABCG2) vs. cholesterol concentration (mM)

- L558A
- L555A/L558A
- wtABCG2

B

Western blot showing wtABCG2, L555A/L558A, aggregate, and dimer with molecular weights (kDa) indicated:
- Ms (kDa)
  - 250
  - 140
  - 100
  - 70
  - 50
  - 35

Sample volumes (µl): 2, 0.8, 4, 8
Figure 7

ABCG2:
- cholesterol increases activity
- bile acids decrease slippage

Bile canaliculus

ABCG5/8  MRP2  ABCB11  ABCG2

cholesterol

bile acids

don't transport

xenobiotics, drugs, drug conjugates, uric acid, hormones, etc.

direct transport  modulatory effect

hepatocyte

tight junction