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Functional analysis of candidate ABC transporter proteins for sitosterol transport

C. Albrecht[a,*], J.I. Elliott[a], A. Sardini[a], T. Litman[b], B. Stieger[c], P.J. Meier[c], C.F. Higgins[a]

[a] *MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, Hammersmith Hospital Campus, Du Cane Rd., London W12 ONN, UK*
[b] *Department of Medical Physiology, University of Copenhagen, Denmark*
[c] *Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital, Zurich, Switzerland*

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Abstract

Two ATP-binding cassette (ABC) proteins, ABCG5 and ABCG8, have recently been associated with the accumulation of dietary cholesterol in the sterol storage disease sitosterolemia. These two 'half-transporters' are assumed to dimerize to form the complete sitosterol transporter which reduces the absorption of sitosterol and related molecules in the intestine by pumping them back into the lumen. Although mutations altering ABCG5 and ABCG8 are found in affected patients, no functional demonstration of sitosterol transport has been achieved. In this study, we investigated whether other ABC transporters implicated in lipid movement and expressed in tissues with a role in sterol synthesis and absorption, might also be involved in sitosterol transport. Transport by the multidrug resistance P-glycoprotein (P-gp; Abcb1), the multidrug resistance-associated protein (Mrp1; Abcc1), the breast cancer resistance protein (Bcrp; Abcg2) and the bile salt export pump (Bsep; Abcb11) was assessed using several assays. Unexpectedly, none of the candidate proteins mediated significant sitosterol transport. This has implications for the pathology of sitosterolemia. In addition, the data suggest that otherwise broad-specific ABC transporters have acquired specificity to exclude sitosterol and related sterols like cholesterol presumably because the abundance of cholesterol in the membrane would interfere with their action; in consequence, specific transporters have evolved to handle these sterols.

Keywords: ABC transporter; Sitosterol; Cholesterol; Confocal microscopy; Flow cytometry

1. Introduction

Sitosterolemia is a rare, autosomal recessive sterol storage disease characterised by tendon and tuberous xanthomas and by a strong predisposition to premature coronary atherosclerosis [1,2]. Increased levels of phytosterols (plant sterols) such as sitosterol and campesterol are found in blood, plasma, erythrocytes and especially in xanthomas and arteries of affected subjects. Increased intestinal absorption of phytosterols as well as decreased biliary and fecal excretion of cholesterol and phytosterols contribute to the abnormal lipid composition of blood and tissues from these patients. These phenotypes could be a consequence of either increased absorption or of decreased efflux of sterols.

Many adenosine triphosphate (ATP)-binding cassette (ABC) transporters mediate efflux of hydrophobic molecules from cells. Recently, mutations in two divergent genes encoding ABC transporters have been associated with sitosterolemia [3–5]. The 'half-transporters', ABCG5 and ABCG8, encoded by these genes are highly expressed in liver and intestine and presumably act together to form a complete transporter. It is assumed, but has not been demonstrated, that these transporters mediate sitosterol efflux and, when inactive, sitosterol accumulation is increased. Cholesterol feeding up-regulates their expression in mice, implicating them in the intestinal absorption and biliary excretion of sterols [3]. However, little is yet known about the transport of sterols across cell membranes. Since several ABC transporters have been implicated in lipid, phospholipid and cholesterol transport, it seemed likely that other members of the ABC transporter family may also

Abbreviations: ABC, ATP binding cassette; ATP, adenosine triphosphate; Bcrp, breast cancer resistance protein; BSA, bovine serum albumin; Bsep, bile salt export pump; calcein-AM, calcein-acetoxymethylester; CsA, cyclosporin A; FCS, fetal calf serum; IPS, isotonic perfusion solution; MDR, multidrug resistance; Mrp, multidrug resistance-associated protein; P-gp, P-glycoprotein; VBL, vinblastine

* Corresponding author. Tel.: +44-20-8383-8270; fax: +44-20-8383-8337.
E-mail address: c.albrecht@csc.mrc.ac.uk (C. Albrecht).

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play a role in pumping sitosterol in and other sterols out of cells.

The multidrug resistance P-glycoprotein (P-gp, also designated as ABCB1, encoded by the *MDR1* gene) transports a wide variety of hydrophobic compounds, including steroids, out of cells in a manner analogous to phospholipid flippases which maintain phospholipid asymmetry between leaflets of the lipid bilayer [6]. P-gp is known to transport analogues of membrane lipids with shortened acyl chains [7,8]. It has been suggested that P-gp may have a physiological role in the transport of natural membrane lipids including cholesterol [9–13], although this is still a matter of debate. The closely related *MDR3* (also designated as *ABCB4*) gene product is known to flip phosphatidyl choline across the canalicular membrane during bile formation [14–16]. The multidrug resistance-associated protein (Mrp1 also designated as ABCC1) also transports various lipid analogues (reviewed in Refs. [17,18]). Other ABC transporters involved in lipid transport include ABCR (also called rim protein or ABCA4), which transports a complex of retinaldehyde and phosphatidylethanolamine in the retina of the eye [19]; ABCA1 which is crucial for the elimination of excess tissue cholesterol (reviewed in Ref. [20]); ABCA6 which is potentially involved in lipid homeostasis [21]; peroxisomal ABC transporters which are involved in the import of long-chain fatty acids into peroxisomes [22,23] and ABCG1, the human homolog of the *Drosophila white* gene, which is implicated in the regulation of macrophage cholesterol and phospholipid transport [24,25].

The bile salt export pump (Bsep, also designated as Abcb11), is regarded as the key mediator of canalicular bile salt excretion. Bsep is known to affect the homeostasis of cholesterol and phospholipids. Thus, secretion of phospholipids and cholesterol usually correlates positively with the secretion of bile salts in vivo [26], and it is widely accepted that bile salt secretion drives the secretion of phospholipids and cholesterol into the bile. Disruption of the *Bsep* gene in mice also significantly increases biliary cholesterol and phospholipids [27]. Interestingly, the Bsep locus maps to 2q24, co-localises with the Lith1 locus of cholesterol gallstone susceptibility in mice [28], and is located in the proximity of the sitosterolemia locus which has been mapped to human chromosome 2p21 [29].

To date, no functional measurements of sitosterol transport by different ABC transporters have been reported. As many ABC transporters play crucial roles in lipid transport, we studied ABC transporter proteins known to be expressed in cell membranes of tissues with a potential role in sitosterol transport. P-gp is highly expressed in the canalicular membrane of the liver and the gut epithelium [30]; the breast cancer resistance protein (Bcrp; also known as mitoxantrone resistance-associated protein MXR, designated as ABCG2 according to the HUGO nomenclature) mRNA is found in the small intestine, colon and liver as well as other tissues [31]. Mrp1 is ubiquitously expressed [17] and is located on the basolateral membrane of polarised cells [32]; Bsep is expressed exclusively in the liver and has been localised by immunohistochemistry to the canalicular membrane of hepatocytes [27,33,34]. The functional data obtained in this study show that P-gp, Mrp1, Abcg2 and Bsep are unlikely to be involved in sitosterol transport. These findings complement previous studies of sitosterolemic patients and give new insights into the substrate specificity and physiological role of individual ABC transporters.

2. Materials and methods

2.1. Mice

Mdr1a/b$^{-/-}$ mice were initially purchased from Taconic Farms (Germantown, USA). Mdr1a and1b mutations had been backcrossed for at least seven generations onto the FVB background at Taconic Farms. FVB, FVB.mdr1a/b$^{-/-}$ and C57BL/10 mice were bred in the Biological Services Unit at the MRC Clinical Sciences Centre. Mice were used at 5 to 12 weeks of age.

2.2. Cell culture

NIH3T3 murine fibroblasts which had been permanently transfected with the human *MDR1* gene, have been described previously [35,36]. Parental NIH3T3 cells and MDR1-overexpressing NIH3T3 fibroblasts (NIH3T3-MDR1) were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco Life Technologies) supplemented with 10% fetal calf serum (FCS). NIH3T3-MDR1 cells were maintained in media supplemented with 1 μg/ml colchicine (Sigma) to ensure P-gp expression. Colchicine was withdrawn from the medium 24 h prior to carrying out transport studies.

2.3. Calcein-AM assay for P-gp activity in NIH3T3 cells

2.3.1. Confocal microscopy

Calcein fluorescence in individual cells was measured by epifluorescence confocal microscopy as described previously [37], using a BioRad μRadiance microscope mounted on a Zeiss Axiovert 100 and a 40 × plan neofluor 1.3 NA oil immersion objective. The experiments were performed with an excitation line of 488 nm; the emitted light was collected through a HQ500LP filter. Briefly, NIH3T3 cells were plated at low density 48 h before the experiment on poly-L-lysine coated cover slips and maintained in DMEM containing 10% FCS with (NIH3T3-MDR1) or without (NIH3T3) 1 μg/ml colchicine. Colchicine was removed 24 h prior to the assays. Experiments were performed with a isotonic perfusion solution (IPS) containing 140 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, 5 mM KCl and 1 g/l D-glucose, pH 7.3. Fluorescence changes were detected as a function of time by collecting sequential images, usually at 20 s intervals, after adding calcein-acetoxymethylester (AM; 2.5 μM, Molecular Probes). Accumulation of calcein, measured by fluorescence

increase, is inversely related to the rate of transport of P-gp and was calculated as described previously [37]. β-sitosterol was tested in final concentrations of 2–100 μM.

2.3.2. Flow cytometry

Cells were washed with phosphate buffered saline and incubated at room temperature for 10 min with IPS containing 0.1 μM calcein-AM and one of the following components: 20 μM cyclosporin A (CsA; Sigma), an inhibitor of P-gp; 2–22 μM vinblastine (Sigma), a substrate for P-gp; 90 μM methotrexate (Sigma), which is not transported by P-gp; 2–100 μM β-sitosterol or 100 μM cholesterol. Ethanol (0.1%) served as a diluent control. Cells were washed once with IPS, detached with Trypsin-EDTA and centrifuged for 5 min at 400 × *g*. The cell pellet was resuspended in 500 μl IPS and fluorochrome efflux was assessed by flow cytometry. Live cells were gated by eye on the basis of forward scatter and side scatter characteristics and analysed using CellQuest software (Beckton Dickinson).

2.4. ATPase assay for P-gp

P-gp-associated ATPase activity was determined according to Litman et al. [38], by quantitating the release of inorganic phosphate from ATP, using a sensitive, colorimetric assay originally described by Chifflet et al. [39] and later modified by Doige et al. [40]. Human P-gp containing Sf9 microsomes (K228, BD GenTest) were thawed on ice before resuspension in ice-cold ATPase assay medium (3 mM ATP, 50 mM KCl, 2.5 mM MgSO4; 3 mM dithiothreitol, 25 mM Tris–HCl, pH 7.0) containing 0.5 mM EGTA (to inhibit Ca-ATPases), 2 mM ouabain (to inhibit the (Na+K)-ATPase), and 3 mM azide (to inhibit mitochondrial ATPase). Each series of experiments was carried out in a 96-well microtiter plate, with reaction volumes of 50 μl/well corresponding to 5 μg protein/well. Incubation with verapamil, a known activator of the P-gp ATPase, and with β-sitosterol at various concentrations was started by transferring the plate from ice to 37 °C for 1 h, and terminated by the addition of

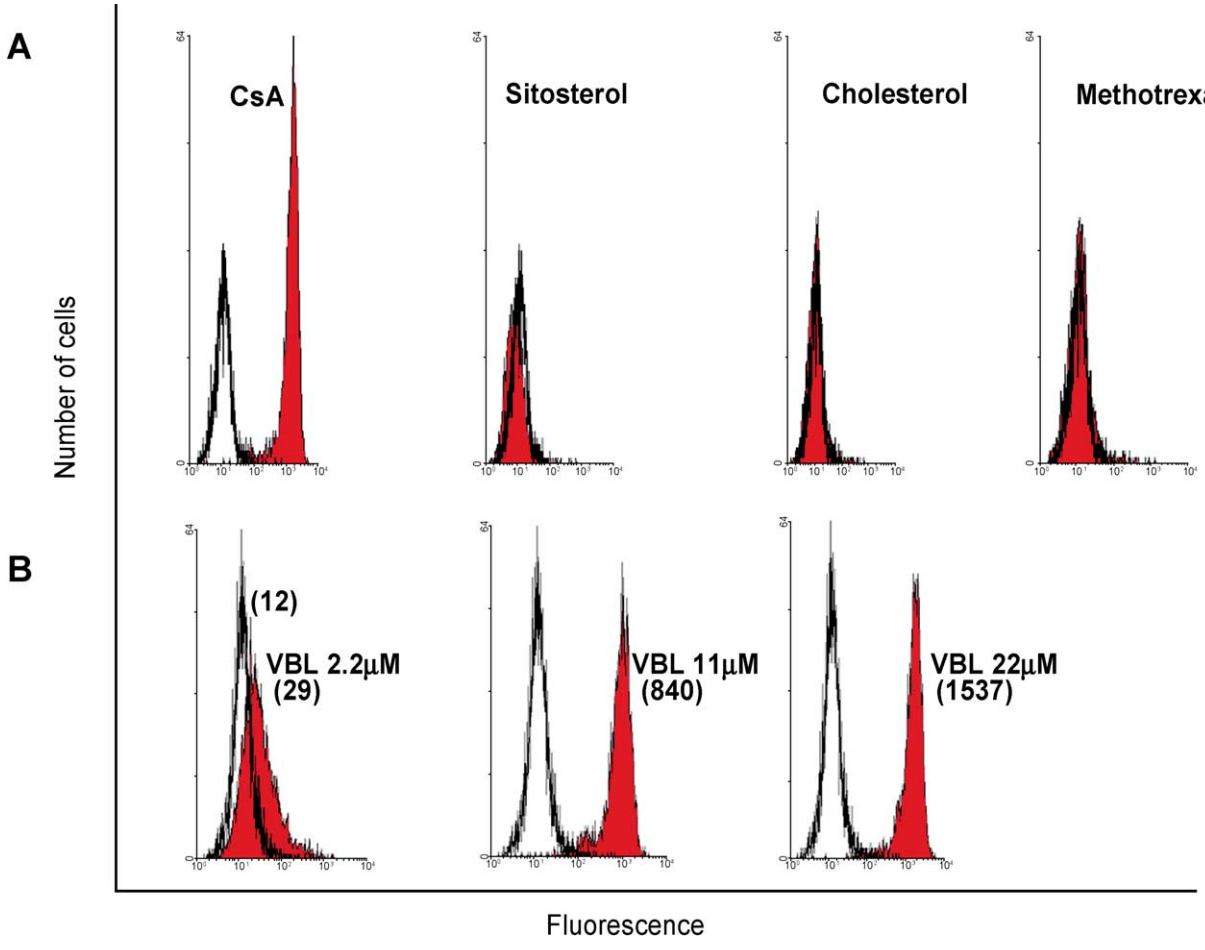

Fig. 1. Effect of sitosterol on P-gp activity in NIH3T3-MDR1 cells. Murine NIH3T3 cells permanently transfected with human MDR1 cDNA (NIH3T3-MDR1) were incubated with 0.1 μM calcein-AM for 10 min and fluorochrome accumulation was measured in the absence of an inhibitor or substrate (A and B, black lines), or in the presence of 20 μM cyclosporin A (CsA; panel A1, red), 100 μM β-sitosterol (panel A2, red), 100 μM cholesterol (panel A3, red), 90 μM methotrexate (panel A4, red), or different concentrations of vinblastine (VBL; panels B1–3, red), respectively. Cells were analysed by flow cytometry. As expected, CsA and VBL increased fluorescence (shifted curves to the right), demonstrating inhibition of P-gp activity, while sitosterol, cholesterol and methotrexate had no effect. Numbers listed in panel B indicate mean fluorescence values of the gated cell population.

200 μl ice-cold stopping medium (ammonium molybdate, 0.2% (w/v); sulfuric acid, 1.3% (v/v); SDS, 0.9% (w/v); trichloroacetic acid, 2.3% (w/v); freshly prepared ascorbic acid, 1% (w/v)) to each well. After 30 min incubation at room temperature, the released phosphate was quantitated colorimetrically using a microplate reader (Bio-Tek Instruments) at 750 nm. Background and control experiments with ATPase assay buffer alone, samples incubated on ice, and samples incubated with 500 μM vanadate (inhibiting the P-gp ATPase) were obtained in parallel and subtracted from the measurements.

2.5. Analysis of P-gp, Mrp1 and Abcg2 activity by flow cytometry in murine lymphocytes

Murine lymphocytes (10^7/ml) were incubated in the presence of fluorochrome for 30 min at 37 °C in DMEM (Sigma) supplemented with 1% bovine serum albumin (BSA; Sigma), in the presence or absence of 2–100 μM β-sitosterol (Sigma) or the following inhibitors: 20 μM CsA, an inhibitor of P-gp; 70 μM MK571 (gift from Dr. B. Sarkadi), a specific inhibitor of Mrp1; or 10 μM fumitremorgin C (gift from Dr. S. Bates), a specific inhibitor of Abcg2. 0.1% ethanol served as a diluent control. In order to discriminate different lymphocyte populations, $CD4^{QUANTUM\ RED}$ or $CD8^{PE}$ antibodies (Beckton Dickinson) were added for the final 10 min of the incubation. Cells were washed once with DMEM/BSA, resuspended in 200 μl DMEM/BSA and fluorochrome efflux was assessed by flow cytometry. Live cells were gated by eye on the basis of forward scatter and side scatter characteristics and analysed using CellQuest software (Beckton Dickinson). The following fluorochromes were used: BODIPY-taxol (Molecular Probes) for P-gp; 0.025 μM Fluo-4 AM (Molecular Probes) or 0.0125 μM

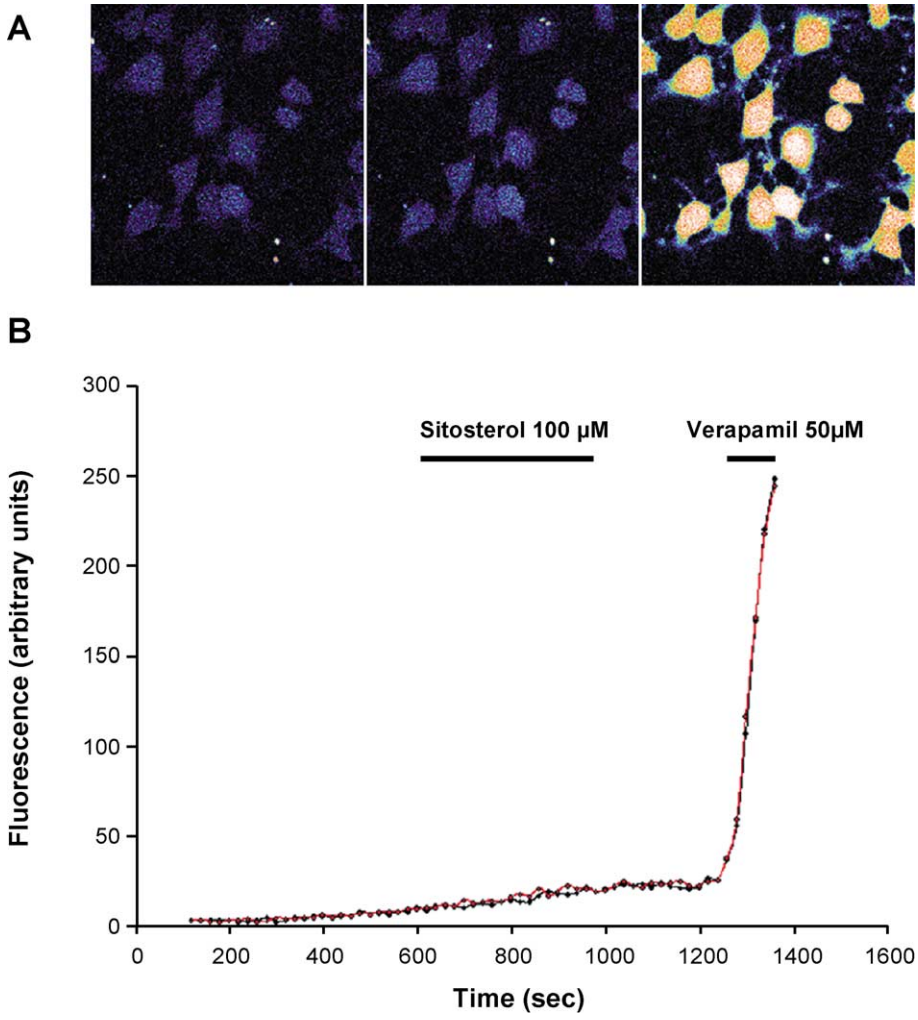

Fig. 2. Effect of sitosterol on rate of transport by P-gp. Calcein accumulation by individual NIH3T3-MDR1 cells was measured by epifluorescence confocal microscopy. (Panel A) Fluorescence intensity, shown color-coded on a scale from blue (low) to white (high), is proportional to the concentration of accumulated calcein and inversely related to P-gp activity. Images of individual cells loaded with 2.5 μM calcein-AM are shown in the absence of β-sitosterol (left panel), in the presence of 100 μM β-sitosterol (middle panel), and in the presence of 50 μM verapamil (right panel) 2 min after application of β-sitosterol and verapamil, respectively. (Panel B) Quantitative analysis for several individual cells was performed measuring the average fluorescence intensity from an area within each cell as a function of time (for clarity, the analysis of only two cells is shown). Sitosterol or verapamil were added where indicated by horizontal bars.

calcein-AM for Mrp1; 0.5 μM mitoxantrone (Sigma) for Abcg2.

2.6. Transport by Bsep in Sf9 insect cells

ATP-dependent bile acid uptake into Sf9 cell vesicles expressing rat Bsep was performed as described previously [34,41]. Briefly, Sf9 cells were infected with Bsep expressing baculovirus generated with the Bac-to-Bac system (Life Technologies). The total membrane fraction was isolated and vesicles resuspended in taurocholate uptake buffer consisting of 50 mM sucrose, 100 mM KNO_3 and 10 mM HEPES/Tris, pH 7.4. Expression of Bsep in membrane vesicles was demonstrated by Western blotting. Uptake of 2 μM [^{3}H]-taurocholate (NEN Life Science Products) was performed in the buffer system by rapid filtration technique [42] in the absence or presence of 1–10 μM β-sitosterol; 1% BSA was added to increase the solubility of β-sitosterol. A potential inhibitory effect of β-sitosterol on ATP-dependent, Bsep-mediated taurocholate transport in Sf9 vesicles was investigated at initial (45 s), intermediate (5 min) and long (2 h) periods of incubation with β-sitosterol; experiments were performed in triplicate. ATP-dependent uptake was calculated as the difference in uptake in the presence and absence of ATP.

3. Results

To assess the transport of sitosterol by the different ABC transporters, a variety of assays and the use of different cell types expressing the various transporters were required.

3.1. Sitosterol is not a substrate for P-gp

To study whether sitosterol interacts with P-gp, we first assayed its ability to inhibit, competitively, P-gp-mediated transport in NIH3T3 cells overexpressing P-gp (NIH3T3-MDR1) using the calcein assay. Calcein-AM is a non-fluorescent P-gp substrate that crosses the cell membrane passively and, following cleavage by cytoplasmic esterases, is converted to fluorescent calcein. As calcein is not a substrate for P-gp, cellular fluorescence is inversely related to the activity of P-gp [37,43].

Fig. 1A shows calcein accumulation measured by flow cytometry in NIH3T3-MDR1 cells which express active P-gp in the absence of inhibitor (black lines), or following treatment with either 20 μM CsA (panel 1, red), 100 μM β-sitosterol (panel 2, red), 100 μM cholesterol (panel 3, red), or 90 μM methotrexate (panel 4, red). Panel 1 shows that CsA, a known inhibitor of P-gp, increased fluorescence significantly (shifting the peak to the right), demonstrating that, as expected, it does inhibit P-gp activity and therefore increases fluorescent calcein accumulation by the cells. CsA had no effect on the uptake of calcein-AM by the parental NIH3T3 cell line (data not shown), confirming that its effects are through inhibition of P-gp. The possibility that CsA exhibited a toxic effect at the concentrations and time period used was excluded by monitoring propidium iodide uptake (data not shown). In contrast to CsA, β-sitosterol, even at concentrations as high as 100 μM, had no effect on P-gp activity (panel 2, red, complete overlay of both curves) implying that sitosterol does not interact with P-gp. Likewise, cholesterol, structurally very similar to sitosterol, and methotrexate, which is assumed not to be transported by P-gp, did not affect calcein accumulation (panels 3 and 4, complete overlay of both curves).

In order to examine the effect of a P-gp substrate in our experimental setup, we tested vinblastine (VBL) in a concentration range between 2.2 and 22 μM (Fig. 1B). As expected, VBL interacted with P-gp and inhibited competitively calcein-AM efflux in a concentration dependent

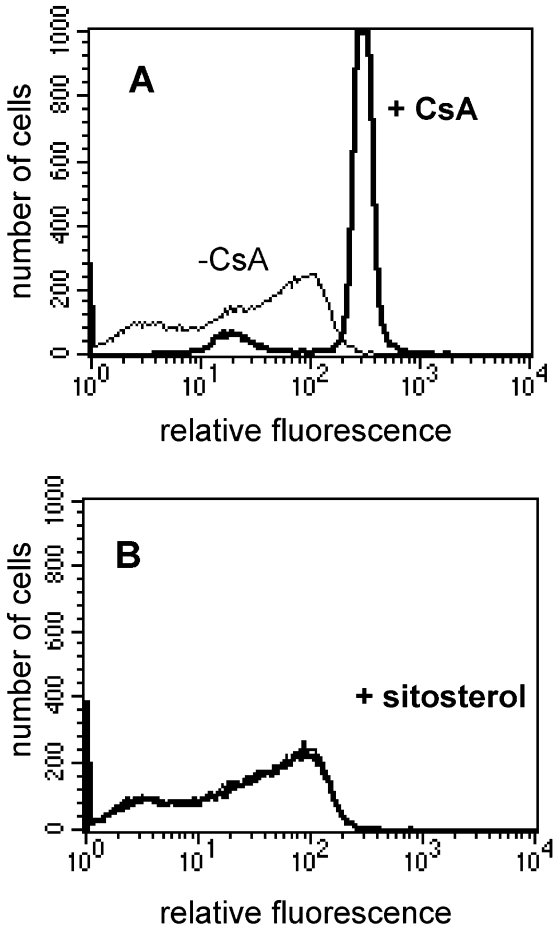

Fig. 3. Effect of sitosterol on P-gp activity in murine lymphocytes. Mesenteric murine lymph node cells were incubated with 0.02 μM BODIPY-taxol for 30 min, CD4 cells separated and fluorochrome accumulation measured in either the absence of inhibitor (thin lines), in the presence of 20 μM cyclosporin A (CsA; panel A, thick line) or in the presence of 100 μM β-sitosterol (panel B, thick line). Cells were analysed by flow cytometry. CsA increased fluorescence, as expected, demonstrating inhibition of P-gp activity, while sitosterol had no effect.

manner (panels 1–3) resulting in significantly increased fluorescence as illustrated by a shift of the peak to the right and increased mean fluorescence values for the gated population (numbers in brackets). This finding implies that our transport assay is capable of identifying substrates for and inhibitors of P-gp and suggests that neither sitosterol nor cholesterol interact with P-gp.

Since the above assay only measured steady-state accumulation, we also monitored the effect of sitosterol on the rate of calcein-AM uptake by confocal microscopy. Fig. 2A

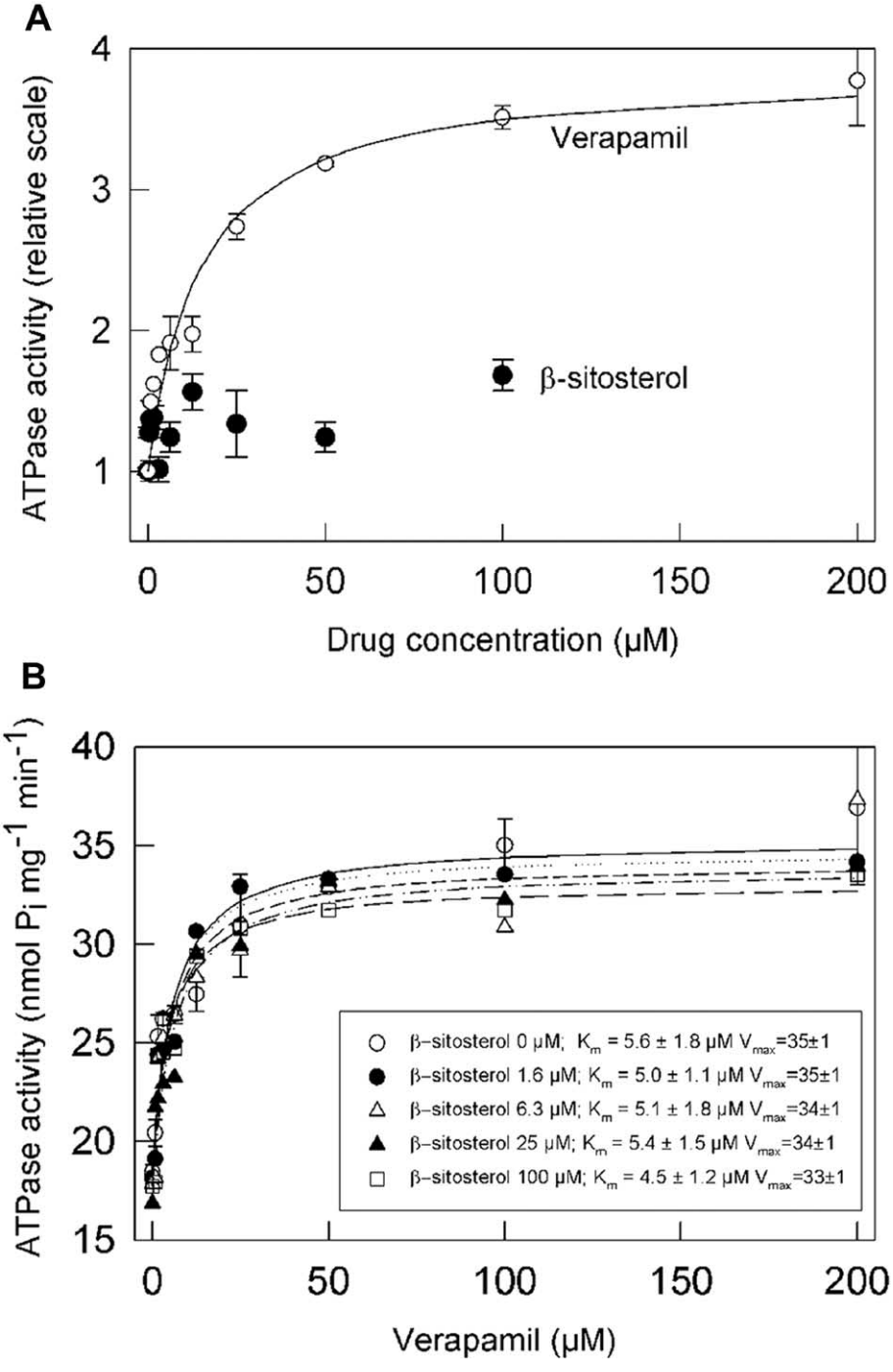

Fig. 4. Effect of sitosterol on ATPase activity. (Panel A) Relative ATPase activity in membranes isolated from P-gp-transfected Sf9 cells was measured for verapamil (○) and β-sitosterol (●) at various concentrations. The verapamil curve has been fitted to the Michaelis–Menten equation: $V = V_0 + (V_{max} - V_0)\, S/(S + K_m)$. (Panel B) Kinetics of modulation of the verapamil-stimulated P-gp ATPase activity by sitosterol was determined at 0 μM (○), 1.6 μM (●), 6.3 μM (△), 25 μM (▲) and 100 μM (□) β-sitosterol. The curves have been fitted to the Michaelis–Menten equation, and the kinetic parameters obtained, K_m and V_{max} are shown in the insert. Sitosterol affected neither the P-gp-associated ATPase activity (panel A) nor modulated the verapamil-activated ATPase activity of P-gp (panel B).

shows images of individual cells in the presence of 2.5 μM calcein-AM before application of 100 μM β-sitosterol (left panel), 2 min after β-sitosterol application (middle panel), and 2 min after application of 50 μM verapamil (right panel). Verapamil, a known inhibitor of P-gp-mediated transport activity, increased fluorescence (calcein-AM uptake) as expected, while in contrast, sitosterol had no effect on P-gp activity. Quantitative analysis for several individual cells was performed by measuring the average fluorescence intensity from an area within the cell as a function of time (Fig. 2B). No significant effect of β-sitosterol on the rate of calcein-AM efflux was detected; in contrast, and as expected, verapamil increased fluorescence markedly (Fig. 2A and B). Thus, even at high concentrations, sitosterol did not affect the rate of transport activity of P-gp.

In order to test the effects of sitosterol on P-gp activity in a non-transfected cell system (murine lymphocytes), we used a second fluorophore, BODIPY-taxol (Molecular Probes), which is a specific P-gp substrate (Elliott, J., S. Raguz, and C.F. Higgins, 2002, manuscript submitted; [44]). Fig. 3 shows that, as expected, 20 μM CsA inhibited BODIPY-taxol uptake (panel A), while β-sitosterol, even at concentrations as high as 100 μM, had no effect on P-gp activity as illustrated by the complete overlay of both curves (panel B).

Finally, we aimed to evaluate the direct effect of β-sitosterol at the level of the catalytic site of P-gp assuming that its ATPase activity is directly coupled to the transport function of the protein (reviewed in Ref. [45]). In order to study the direct effect of β-sitosterol, ATPase activity in membranes isolated from P-gp-transfected Sf9 cells was tested. As Fig. 4A demonstrates, β-sitosterol did not affect the P-gp-associated ATPase activity in the concentration range studied. Neither was β-sitosterol able to modulate the verapamil activated ATPase activity of P-glycoprotein (Fig. 4B), as neither the K_m (the verapamil concentration at which half-maximal ATPase activation is reached), nor the V_{max} (maximum level of verapamil-induced ATPase activity) for verapamil were affected. These results indicate that sitosterol does not interact with the catalytic site of P-glycoprotein.

3.2. Sitosterol does not affect Mrp1 activity

To test whether Mrp1 is able to transport sitosterol, we used mesenteric lymph node cells from Mdr1a/b$^{-/-}$ mice to exclude interference of P-gp in the assay. We have previously shown that these cells from Mdr1a/b$^{-/-}$ mice express both Mrp1 and Abcg2, but not P-gp and that the uptake of Fluo-4-AM and calcein-AM in Mdr1a/b$^{-/-}$ mice is mediated solely by Mrp1 (i.e. not by Abcg2) and is specifically inhibited by MK571 (Elliott, J., S. Raguz, and C.F. Higgins, 2002, manuscript submitted). Results are shown for CD4-positive cells. Similar results were obtained for CD8-positive cells.

Fig. 5A shows data for lymphocytes from Mdr1a/b$^{-/-}$ mice incubated with calcein-AM in the presence (thick line) or absence (thin line) of MK571. As expected, MK571 inhibited Mrp1 activity and resulted in a marked increase in the uptake of calcein-AM (thick line). In contrast, β-sitosterol (Fig. 5B) did not affect Mrp1-dependent efflux of calcein-AM even at concentrations up to 100 μM (complete overlay of both curves). Similar results were obtained using the fluorophore Fluo-4AM, another fluorescent substrate of Mrp1 (data not shown). Thus sitosterol does not appear to interact with Mrp1-mediated transport.

3.3. Sitosterol does not affect Abcg2 activity

To study Abcg2 activity, we used the fluorochrome mitoxantrone in mesenteric lymph node cells from Mdr1a/

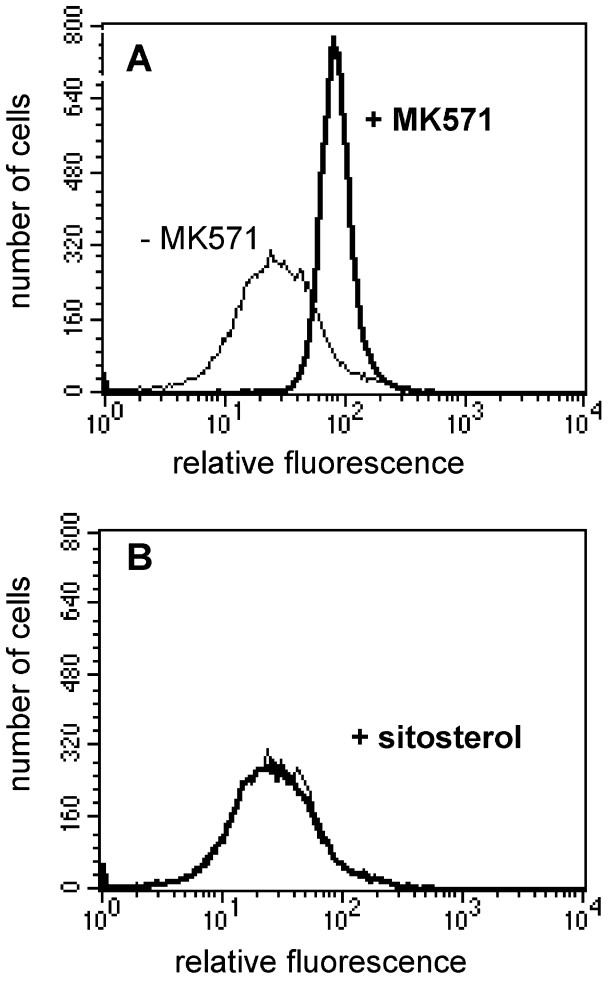

Fig. 5. Effect of sitosterol on Mrp1 activity. Mesenteric lymph node cells from Mdr1a/b$^{-/-}$ mice were incubated with 0.0125 μM calcein-AM for 30 min and fluorochrome accumulation measured in CD4 cells either in the absence of inhibitor (thin lines), or in the presence of 70 μM MK571 (Fig. 4A, thick line) or in the presence of 100 μM β-sitosterol (Fig. 4B, thick line). Cells were analysed by flow cytometry. MK571 increased fluorescence, as expected, demonstrating inhibition of Mrp1 activity, while sitosterol had no effect.

b$^{-/-}$ mice. Mitoxantrone is a specifying Abcg2 substrate [46,47], which is not transported by Mrp1; the transport of mitoxantrone is specifically inhibited by fumitremorgin C ([48], Elliott, J., S. Raguz and C.F. Higgins, 2002, manuscript submitted). Fig. 6A shows that, Mdr1a/b-deficient CD4 cells exhibited fumitremorgin C-sensitive efflux of mitoxantrone (thick line). In contrast, sitosterol did not affect Abcg2 transport activity (Fig. 6B).

3.4. Sitosterol does not inhibit Bsep activity

Membrane vesicles isolated from Bsep expressing Sf9 cells demonstrated marked ATP-dependent uptake of taurocholate, whereas control vesicles from wild-type cells did not (data not shown). ATP-dependent uptake of 2 μM taurocholate by these vesicles was tested in the absence or presence of 1–10 μM β-sitosterol (Table 1). No inhibitory effects of sitosterol on Bsep-mediated taurocholate transport were found, indicating that sitosterol is not a substrate for the canalicular bile salt transporter.

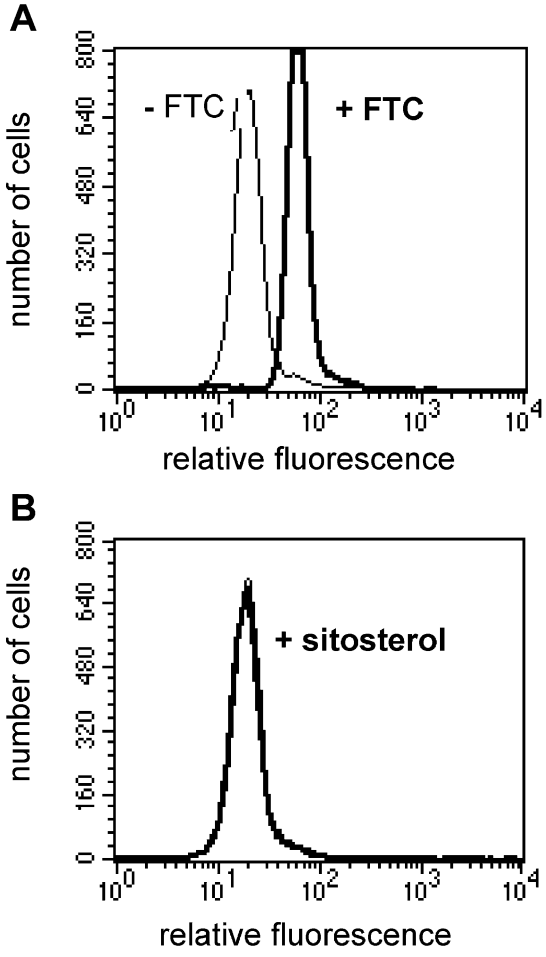

Fig. 6. Effect of sitosterol on Abcg2 activity. Mesenteric lymph node cells from Mdr1a/b$^{-/-}$ mice were incubated with 0.5 μM mitoxantrone for 30 min and fluorochrome accumulation was measured in CD4 cells either in the absence of inhibitor (thin lines), in the presence of 10 μM fumitremorgin C (FTC; Fig. 5A, thick line), or in the presence of 100 μM β-sitosterol (Fig. 5B, thick line). Cells were analysed by flow cytometry. FTC increased fluorescence, as expected, demonstrating inhibition of Abcg2 activity while sitosterol had no effect.

Table 1
Effect of sitosterol on ATP-dependent uptake of taurocholate into rat Bsep expressing Sf9 cell vesicles

Incubation time	Control	β-sitosterol (1 μM)	β-sitosterol (10 μM)
	Taurocholate uptake (pmol/mg protein)		
Experiment 1			
45 s	17.1 ± 1.63	19.2 ± 1.36	17.5 ± 1.60
5 min	76.4 ± 1.78	75.1 ± 3.47	74.3 ± 2.00
2 h	45.8 ± 2.58	47.5 ± 1.20	43.9 ± 2.36
Experiment 2			
45 s	13.9 ± 1.49	12.4 ± 1.15	15.7 ± 2.14
5 min	60.9 ± 3.67	57.6 ± 3.83	56.2 ± 7.05
2 h	25.7 ± 3.11	26.5 ± 2.76	25.3 ± 3.65

Membrane vesicles were isolated from Sf9 insect cells expressing rat Bsep. Initial (45 s), intermediate (5 min) and late (2 h) ATP-dependent Bsep-mediated taurocholate transport was measured without β-sitosterol application (control) and after addition of 1 μM or 10 μM β-sitosterol. Experimental data from triplicate determinations for two separate membrane preparations are shown.

4. Discussion

An increasing number of ABC transporters have been implicated in the movement of lipids, phospholipids or other hydrophobic compounds across cell membranes and are attractive candidates for sitosterol transport in addition to ABCG5 and ABCG8 which play a role in sitosterolemia [3–5,49,50]. It as assumed, but to date has never been functionally demonstrated, that sitosterol is definitely or exclusively transported by ABCG5 and ABCG8. In order to assess whether other ABC transporter candidate proteins mediate sitosterol transport across cell membranes, we studied their ability to transport sitosterol both in live cells as well as in direct transport assays using Sf9 cell membrane vesicle systems. The transporters tested were selected because of their known role in lipid or phospholipid transport and their expression in tissues involved in sterol synthesis, absorption or excretion (predominantly liver and intestine). None of the ABC transporters tested, P-gp, Mrp1, Abcg2, and Bsep, appear to transport sitosterol.

Since cells express multiple transporters with partially overlapping substrate specificities, it is surprising that other ABC transporters do not compensate for the lack of sitosterol transport in the absence of ABCG5 and/or ABCG8 in sitosterolemic patients. Sitosterol has a chemical structure very similar to that of cholesterol (Fig. 7), and, given the broad specificity of many ABC lipid transporters, it seemed likely that sitosterol would be transported via the same transport proteins as cholesterol. This hypothesis is supported by the finding that sitosterolemic individuals also hyperabsorb dietary cholesterol and other sterols, including

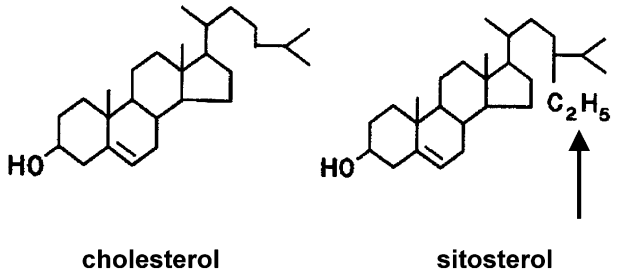

Fig. 7. Comparison of the structures of cholesterol and sitosterol. Arrow indicates the ethyl group at carbon atom 24 of sitosterol which represents the only difference between the two compounds.

shellfish and plant sterols [3,5]. The unexpected finding here, that none of the ABC transporters tested appear to transport sitosterol, provides a potential explanation.

There is some indirect evidence that P-gp transports cholesterol. For example, esterification of plasma membrane cholesterol is inhibited after application of a variety of compounds known to inhibit P-gp activity [9–11], and increased expression of P-gp has been associated with enhanced esterification of plasma membrane cholesterol [12]. However, in these published studies, most of the compounds tested were not necessarily specific inhibitors of P-gp, and may have been inhibiting other transporters. Furthermore, a recurrent problem in studying the role of P-gp in cholesterol transport is the importance of cholesterol as structural component of the cell membrane for the maintenance of P-gp activity [51–53]. Thus, it has not been possible to measure cholesterol transport by P-gp directly.

The demonstration here that sitosterol in not a substrate for P-gp has potential implications for cholesterol uptake. Sitosterol is chemically very similar to cholesterol, and thus our findings strongly suggest that cholesterol is unlikely to be a substrate for P-gp or any of the other ABC transporters tested. It is, of course, possible that the additional ethyl group at carbon atom 24 (Fig. 7, arrow), the only structural difference between sitosterol and cholesterol, may generate specificity of the compound. However, P-gp has few clear structural determinants to its specificity, so this seems unlikely. This was also confirmed by our experiments where cholesterol, as sitosterol, failed to show an inhibitory effect upon P-gp transport activity. It therefore appears that most ABC transporters, which otherwise have broad specificity for hydrophobic molecules, may have acquired specificity to exclude sterols such as cholesterol, presumably because the abundant cholesterol in the cell membrane would otherwise compete for transport. Where there is a need for sterol transport, specific ABC transporters have evolved to accommodate this biological requirement.

In summary, the four ABC transporters investigated in this study are unlikely to play a significant role in sitosterol transport. This has implications for the specificity and the biological roles of these ABC transporters, suggesting that they are unlikely to be involved in cholesterol transport. Additionally, with respect to the pathology of sitosterolemia the data suggest that specialization has occurred such that specific ABC transporters have adapted to handle sitosterol and related sterols distinct from the relatively broad specificity of other ABC transporters.

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