proaches. An attempt has been made to predict the transport activity of P-gp, MRP2, PEPT1 or ASBT from the structure or physicochemical parameters of compounds.[129,130]

Transporter research can be used to profile the pharmacokinetic properties of candidates during drug development. Pravastatin and temocaprilat are good examples of success in differentiated marketing compared with other drugs due to a better understanding of the mechanism governing their transport.[35,86,87] Accordingly, methods allowing the rational prediction and extrapolation of *in vivo* drug disposition from *in vitro* data are also essential.[131] There are drugs that are recognized by several transporters localized on the same membrane, and multiple transporters are expected to be involved in membrane transport. Therefore, the contribution of each transporter to net membrane transport has to be taken into consideration when observations made in gene expression systems are extrapolated to *in vivo* situations. Sugiyama *et al.* have estimated the contribution of each transporter to the efflux of 17β-estradiol-D-glucuronide via the BBB using cDNA-transfected cells and specific inhibitors of each transporter.[132] A similar analysis has been applied to the renal uptake mechanism of pravastatin by Hasegawa *et al.*, and it is suggested that rat Oat3/*Slc22a8* is involved in the renal uptake of pravastatin[133]. Furthermore, it is suggested that, using specific inhibitors, rat Oat3 is mainly responsible for the uptake of benzylpenicillin and *p*-aminohippurate by the choroid plexus, and the efficient removal of its substrates from the cerebrospinal fluid.[134] Information about this contribution needs to be taken into account throughout the drug discovery and development process for predicting the extent to which the plasma concentrations of drugs are affected when there are large interindividual differences in transport activity due to genetic polymorphisms or a change in pharmacokinetics due to drug-drug interactions involving transporters. It is the responsibility of pharmaceutical companies to provide this information to clinicians to help them use drugs more safely.

Concluding Remarks

Research on drug transporters has made significant progress in recent years because of the increased use of molecular biology and genetic engineering techniques. Together with progress in the basic sciences, the information on transporters has grown steadily. The positive introduction of new technology or new analytical systems, in conjunction with estimates of the contribution made by each transporter to drug disposition *in vivo*, will lead to the more efficient development of new safer and more effective drugs.

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Drug Metab. Pharmacokin. 18 (1): 33-41 (2003).

Regular Article

Function of Uptake Transporters for Taurocholate and Estradiol 17β-D-Glucuronide in Cryopreserved Human Hepatocytes

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Summary: The uptake properties of taurocholate (TC) and estradiol 17β-D-glucuronide ($E_2$17βG) were examined in freshly isolated and cryopreserved human hepatocytes to discover if active transport is retained in cryopreserved human hepatocytes. Firstly, the uptake of TC and $E_2$17βG was measured before and after cryopreservation. The uptake of TC was found to be Na^+-dependent both in fresh and cryopreserved hepatocytes. The uptake activity in cryopreserved hepatocytes was found to range from 10 to 200% of that observed in freshly isolated cells. A kinetic analysis was performed to evaluate the transport activity of TC and $E_2$17βG and revealed that the Michaelis constant (K_m) for these compounds in cryopreserved human hepatocytes was 2–8 and 3–18 μM, respectively. This was within the range of K_m values previously found in human Na^+-taurocholate cotransporting polypeptides (NTCP) and organic anion transporting polypeptides (OATP) 2 and 8, respectively. The kinetic analyses also showed that the species difference between human and rat hepatocytes was more marked for the maximal uptake rate (V_{max}) (>22 and >22 times higher for TC and $E_2$17βG in rats than in humans, respectively) than that for K_m (2–12 and 0.7–4 times higher, respectively), compared with earlier data we obtained in primary cultured rat hepatocytes. Hence, we conclude that cryopreserved human hepatocytes, at least in part, retain their transporter functions and, therefore, can be a useful experimental system for examining the mechanism of the hepatic uptake of drugs.

Key words: human hepatocytes; cryopreservation; transporter; uptake

Introduction

Drug disposition in hepatocytes is initiated by the penetration of drugs through the sinusoidal membrane, followed by intracellular metabolism and/or biliary excretion.[1,2] Therefore, the hepatic uptake process is an important determinant of the hepatic clearance of drugs. Indeed, for several types of drugs and other xenobiotics, the hepatic uptake process has been demonstrated to be the rate-limiting step for systemic clearance.[3-5] For such compounds, the *in vitro* assessment of their uptake across the sinusoidal membranes is important for assessing hepatic clearance in intact liver.

It has been suggested that many drugs, bile acids and other xenobiotics are taken up into hepatocytes via drug transporters.[6-8] For drugs which are taken up into hepatocytes by such transporters, freshly isolated hepatocytes represent a useful experimental system for evaluating their uptakes.[2,9] However, the application of this system using human hepatocytes is hampered by their relatively poor availability. Recently, several laboratories have demonstrated that cryopreserved human hepatocytes can be used to evaluate human drug metabolism.[10-12] However, there have been few published reports on the drug transport properties of cryopreserved human hepatocytes.[13] Therefore, it is important to demonstrate the validity of cryopreserved hepatocytes as a tool for estimating the transport activity of xenobiotics.

More recent studies have identified the molecular

Received; October 30, 2002, Accepted; January 14, 2003
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Table 1. Background information on donors

Lot No. (Donor ID)	Age (years)	Sex	Race[a]	Tobacco Use	Alcohol Use	Substance Use	Viability[b] [%]
HH-063	33	male	C	yes	no	no	75
HH-068	44	female	C	no	yes	no	70
HH-069	63	female	C	yes	yes	no	89
HH-088	84	female	C	no	no	no	92
HH-093	68	female	C	no	no	no	93
HH-097	47	male	C	no	no	no	71
HH-099	74	female	C	no	no	no	69
HH-105	59	male	C	yes	yes	no	57
HH-107	69	female	C	no	no	no	58
HH-117	47	female	C	no	no	no	90

[a] C···Caucasian.
[b] Cell viability was confirmed by trypan blue exclusion test.

mechanism governing the hepatic uptake of these compounds. For example, Na^+-taurocholate cotransporting polypeptide (Ntcp) is mainly associated with Na^+-dependent uptake of bile acids in rats[14,15] while organic anion transporting polypeptide-1 (Oatp1) plays an important role in their Na^+-independent uptake.[16,17] Transporters involved in the uptake of anionic compounds can generally be categorized into two major groups: the Oatp family[14,18,19] and the organic anion transporter (Oat) family.[20-23] There is an overlapping substrate specificity between each of these families: the Oatp family mainly accepts amphipathic anions while the Oat family accepts small, hydrophilic organic anions.[24,25] Their substrates also include neutral compounds and organic cations.[24,26]

These families are, in general, conserved in humans although there are a few exceptions. OATP-A,[27] OATP-B,[28] OATP-C/OATP2/LST-1,[29-31] OATP-D,[28] OATP-E[28] and OATP8[32] are human transporters belonging to the OATP family whereas OAT1,[33,34] OAT2,[35] OAT3[34] and OAT4[36] belong to the OAT family. OATP-B, OATP-C/OATP2/LST-1, OATP8 and OAT2 are reported to be expressed in liver.[29-32,35]

Significant differences have been reported in drug transport characteristics between human and rat hepatocytes.[37] For example, the uptake of taurocholate in human hepatocytes is much lower than that in rat hepatocytes.[37] Taking this interspecies difference into consideration, human hepatocytes are needed as a tool to assess drug disposition in humans. The present study was carried out to evaluate the usefulness of cryopreserved human hepatocytes in drug transport studies by examining the uptake kinetics of the NTCP substrate, taurocholate (TC)[14,15] and OATP substrate, estradiol 17β-D-glucuronide ($E_2$17βG)[17,31,32] in freshly isolated and cryopreserved human hepatocytes.

Materials and Methods

Materials: [^{3}H]TC (111 GBq/mmol) and [^{3}H]$E_2$17βG (1628 GBq/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled TC and $E_2$17βG were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

Hepatocyte preparation: The human hepatocytes used in the study were isolated from human livers donated for transplantation purposes but not used for transplantation mainly due to the lack of appropriate recipients (**Table 1**). All livers were surgically removed from brain-dead donors whose hearts were functioning and who were free of known liver diseases. All livers were stored for less than 24 hours in University of Wisconsin solution. Hepatocytes were isolated by perfusion using a two-step collagenase digestion procedure.[38] After enzymatic dissociation, the hepatocytes were further separated from nonparenchymal cells by centrifugation through 30% Percoll. Freshly isolated hepatocytes, before cryopreservation, were used within 4 hours after isolation. The cells were stored on ice in Krebs-Henseleit buffer. For the studies using cryopreserved hepatocytes, the purified hepatocytes were cryopreserved in liquid nitrogen until analysis. The storage time during the cryopreservation process was as follows: HH-063, -068, -069, -088 and -117 were cryopreserved for approximately 12 months and HH-086, -093, -097, -099, -105 and -106 were cryopreserved for approximately 6 months before use. Immediately before the uptake studies, the hepatocytes (1 mL suspension) were thawed at 37°C then immediately suspended in 10 mL ice-cold Krebs-Henseleit buffer and centrifuged ($50 \times g$) for 2 minutes at 4°C, followed by removal of the supernatant. This procedure was repeated once more to remove cryopreservation buffer and then cells were resuspended in the same buffer to give a cell density of 2.0×10^6 or 4.0×10^6

viable cells/mL for the uptake studies.

Uptake studies: To measure the uptake of TC in the absence of Na^+, sodium chloride and sodium bicarbonate in Krebs-Henseleit buffer were replaced with choline chloride and choline bicarbonate, respectively, for all subsequent studies. Prior to starting the uptake studies with TC or $E_2 17\beta G$, 120 μL of the cell suspensions was prewarmed in an incubator at 37°C for 3 minutes.[39] In a pilot experiment, a 3-minute preincubation was confirmed to be sufficiently long to raise the temperature of cells to 37°C and longer preincubation, for up to 30 minutes, did not alter the uptake rate of $E_2 17\beta G$. The uptake studies were initiated by adding an equal volume of substrate solution to the cell suspension. At a designated time, the reaction was terminated by separating the cells from the substrate solution by centrifugal filtration.[39] An aliquot of 100 μL incubation mixture was collected and placed in a centrifuge tube (450 μL) containing 50 μL 2 N NaOH under a layer of 100 μL oil (density = 1.015, a mixture of silicone oil and mineral oil, Sigma-Aldrich, St. Louis, MO). The sample tube was then centrifuged for 10 sec using a tabletop microfuge (10,000 × g: Beckman Microfuge E™, Beckman Coulter, Inc. Fullerton, CA) during which the hepatocytes passed through the oil layer into the alkaline solution. After an overnight incubation in alkali to dissolve the hepatocytes, the centrifuge tube was cut and each compartment was transferred to a scintillation vial. The compartment containing the dissolved cells was neutralized with 50 μL 2 N HCl, mixed with scintillation cocktail and the radioactivity was counted using a liquid scintillation counter (LS6000SE, Beckman Coulter, Fullerton, CA).

Data analysis: The time-courses of the uptake of TC and $E_2 17\beta G$ were expressed as the uptake volume (μL/10^6 cells) for the radioactivity taken up into cells (dpm/10^6 cells) divided by their concentration of radioactivity in the incubation medium (dpm/μL). The initial uptake velocity of each drug was calculated using their uptake volume obtained at 0.5 and 2 min and expressed as the uptake clearance (CL_{uptake}: μL/min/10^6 cells). To measure the transporter-mediated CL_{uptake}, the uptake of TC and $E_2 17\beta G$ was measured under tracer conditions (in the presence of 1 μM TC or $E_2 17\beta G$) and excess conditions (in the presence of excess unlabeled TC (300 μM) or $E_2 17\beta G$ (100 μM)) and the CL_{uptake} was expressed as the CL_{uptake} under tracer conditions minus that under excess conditions.

The kinetic parameters for the uptake of TC and $E_2 17\beta G$ were calculated using the following equations:

$$v_0 = \frac{V_{max} \cdot S}{K_m + S} + P_{dif} \cdot S \quad (1)$$

$$v_0 = \frac{V_{max,1} \cdot S}{K_{m,1} + S} + \frac{V_{max,2} \cdot S}{K_{m,2} + S} + P_{dif} \cdot S \quad (2)$$

where v_0 is the initial uptake rate (pmol/min/10^6 cells), S is the substrate concentration (μM), $K_{m(,n)}$ is the Michaelis constant (μM), $V_{max(,n)}$ is the maximal uptake rate (pmol/min/10^6 cells) and P_{dif} is the nonsaturable uptake clearance (μL/min/10^6 cells). The above equation was fitted to the uptake data by a nonlinear least-squares method using a computer program, MULTI[40] to obtain the kinetic parameters. The input data were weighed as the reciprocal of the observed values and the Damping Gauss Newton method was used as the fitting algorithm.

Results

Uptake of TC: To examine the effect of cryopreservation on the drug uptake activity, the uptake of TC was measured before and after cryopreservation for hepatocytes isolated from five different donors (**Table 2**). Typical time profiles for cryopreserved hepatocytes from two donors are shown in **Fig. 1**. Time-dependent uptake was observed in the freshly isolated hepatocytes, with reduced uptake in the presence of excess unlabeled TC (**Fig. 1**). The effects of cryopreservation were different for the two lots of cryopreserved cells (**Fig. 1**). In HH-093, TC uptake in the presence of Na^+ was similar before and after cryopreservation while, in HH-099, it was markedly reduced after cryopreservation (**Fig. 1**).

In the presence of Na^+, the highest CL_{uptake} of TC (21 μL/min/10^6 cells) was observed in freshly isolated hepatocytes of HH-106, this uptake being reduced to 42% of that after cryopreservation (**Table 2**). A middle range of CL_{uptake} (7–13 μL/min/10^6 cells) was found in fresh hepatocytes of HH-097, HH-099, and HH-105 (**Table 2**), the reduction in uptake after cryopreservation being more marked in HH-099 whereas it was minimal in HH-097 (**Table 2**). The lowest CL_{uptake} (1.7 μL/min/10^6 cells) was found in HH-093 and the reduction was minimal in this lot.

In the absence of Na^+, the TC uptake in HH-093 and HH-099 was much lower than that in its presence (5–40% of control; **Fig. 1**). Such Na^+-dependence in CL_{uptake} was found in the majority of hepatocyte samples (**Table 2**). The uptake of TC in the absence of Na^+ was also reduced by excess unlabeled TC in HH-093 whereas this effect was minimal in HH-099 (**Fig. 1**).

Analysis of the mean CL_{uptake} of 5 samples revealed that more than half of the total uptake of TC is the Na^+-dependent portion both in freshly isolated and cryopreserved hepatocytes (54 and 71% was Na^+-dependent for freshly isolated and cryopreserved hepatocytes, respectively: **Table 2**). Na^+-dependent and -independent uptake was reduced to 59 and 29% after cryopreservation, respectively (**Table 2**).

Uptake of $E_2 17\beta G$: The uptake of $E_2 17\beta G$ in

Table 2. Comparison of uptake clearance for taurocholic acid (TC) in freshly isolated and cryopreserved human hepatocytes.[a]

		freshly isolated [μL/min/10^6 cells]		cryopreserved [μL/min/10^6 cells]		cryopreserved/fresh ratio
HH-093	$Na^+(+)$[b]	1.68±1.23		2.55±0.60	]*	1.52±1.17
	$Na^+(-)$[c]	0.467±0.942		1.01±0.46	]	2.16±4.47
	Na^+-dependent[d]	1.21±1.55		1.54±0.76		1.27±1.75
HH-097	$Na^+(+)$	7.07±4.50		6.55±1.61	]*	0.926±0.632
	$Na^+(-)$	2.10±0.65		2.17±1.11	]	1.033±0.618
	Na^+-dependent	4.97±4.55		4,38±1.96		0.881±0.898
HH-099	$Na^+(+)$	13.4±4.2	]**	1.90±0.18	]**	0.142±0.046**[f]
	$Na^+(-)$	0.733±1.201	]	0.420±0.238	]	0.573±0.993
	Na^+-dependent	12.7±4.4		1.48±0.92		0.117±0.083*
HH-105	$Na^+(+)$	7.03±2.06		3.08±0.93	]*	0.438±0.184*
	$Na^+(-)$	6.03±1.48		1.21±1.27	]	0.201±0.216*
	Na^+-dependent	0.993±2.539		1.87±1.57		1.883±5.068
HH-106	$Na^+(+)$	20.7±2.0	]**	8.67±3.12	]*	0.419±0.156**
	$Na^+(-)$	13.5±1.1	]	1.84±3.79	]	0.136±0.281**
	Na^+-dependent	7.24±2.28		6.83±4.91		0.943±0.740
mean[g]	$Na^+(+)$	9.98±7.29		4.55±2.92		
	$Na^+(-)$	4.57±5.47		1.33±0.69		
	Na^+-dependent	5.42±4.84		3.22±2.35		

[a] Uptake clearances were calculated as described in the Methods section. All studies were carried out in triplicate and data are represented by mean±S.D.
[b] Transporter-mediated uptake clearances estimated in the presence of Na^+.
[c] Transporter-mediated uptake clearances estimated in the absence of Na^+.
[d] $Na^+(+)-Na^+(-)$.
[e] *…$p<0.05$, **…$p<0.01$: significantly different between $Na^+(+)$ and $Na^+(-)$ by Dunnet's test.
[f] *…$p<0.05$, **…$p<0.01$: significantly different between in freshly isolated and cryopreserved hepatocytes by Dunnet's test.
[g] mean±S.D. of 5 lots.

HH-093 and HH-099 is shown in **Fig. 2**. For both, the uptake was saturated by excess unlabeled $E_2$17βG in freshly isolated and cryopreserved hepatocytes. The CL_{uptake} of $E_2$17βG was also calculated and is shown in **Table 3**. In freshly isolated hepatocytes, the rank order in the CL_{uptake} of $E_2$17βG (HH-097, HH-099, HH-105 ≫ HH-093, HH-106) was not the same as that in the CL_{uptake} of TC (HH-106>HH-099>HH-097, HH-105>HH-093). A reduction in CL_{uptake} of $E_2$17βG following cryopreservation was observed in some lots and, as in the case of TC uptake, the magnitude of this reduction did not depend on the absolute value for the CL_{uptake} in freshly isolated hepatocytes (**Table 3**).

The mean CL_{uptake} of $E_2$17βG of 5 samples was reduced to 68% after cryopreservation (**Table 3**).

Kinetic analysis of the uptake: Eadie-Hofstee plots for the uptake of TC and $E_2$17βG in cryopreserved hepatocytes from three (for TC) and five (for $E_2$17βG) donors are shown in **Fig. 3**. Both a saturable and nonsaturable components were found for each compound (**Fig. 3**). The data obtained in the present study was fitted to the equation (1) with a smaller AIC value than equation (2) and, therefore, kinetic parameters calculated only by the equation (1) are shown (**Table 4**). The obtained K_m values were 2–8 μM for TC and 3–18 μM for $E_2$17βG (**Table 4**). The mean values for the kinetic parameters obtained from multiple lots of hepatocytes revealed a saturable component for TC and $E_2$17βG uptake, which was estimated by V_{max}/K_m, as 74 and 66% of the total uptake (V_{max}/K_m+P_{dif}), respectively (**Table 4**).

Discussion

The results of our studies suggest that active transport is retained in cryopreserved human hepatocytes. Of the five human hepatocyte preparations, the CL_{uptake} in cryopreserved hepatocytes, expressed as a percentage of the activity of the hepatocytes before cryopreservation, ranged from 12% to 188% for sodium-dependent TC uptake, and from 38% to 195% for $E_2$17βG uptake (**Tables 2** and **3**). In some preparations, the transport activity of these compounds in cryopreserved hepatocytes exceeded that in freshly-isolated ones although the mechanism for this phenomenon remains to be clarified (**Table 2** and **3**). However, the increase of the transporter activity after cryopreservation was not statistically significant (**Tables 2** and **3**). The change in CL_{uptake}, before and after cryopreservation, exhibited an inter-lot variability although the exact mechanism for this remains to be clarified. Both saturable and nonsaturable components were observed in the uptake of these compounds (**Fig. 3**). The K_m for the saturable portion of the

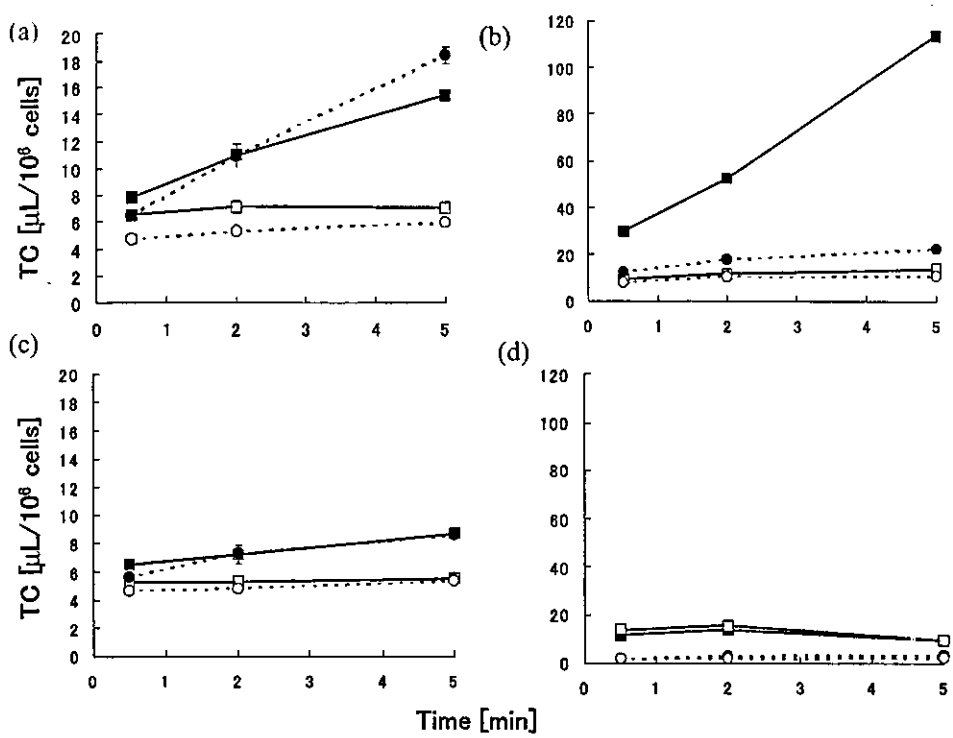

Fig. 1. Time profile for the uptake of TC in the presence (a, b) or absence (c, d) of Na^+ in freshly isolated or cryopreserved human hepatocytes.
Uptake of TC in freshly isolated hepatocytes (■, □) or cryopreserved hepatocytes (●, ○) was measured by incubating cells with 1 μM (●, ■) or 300 μM (○, □) TC at 37°C in Na^+(+) or Na^+(−) Krebs-Henseleit buffer. The uptake in lot No. HH-093 (a, c) and HH-099 (b, d) is shown here and other results are summarized in **Table 2**. Each point and bar represents mean ± S.E. of 3 separate determinations.

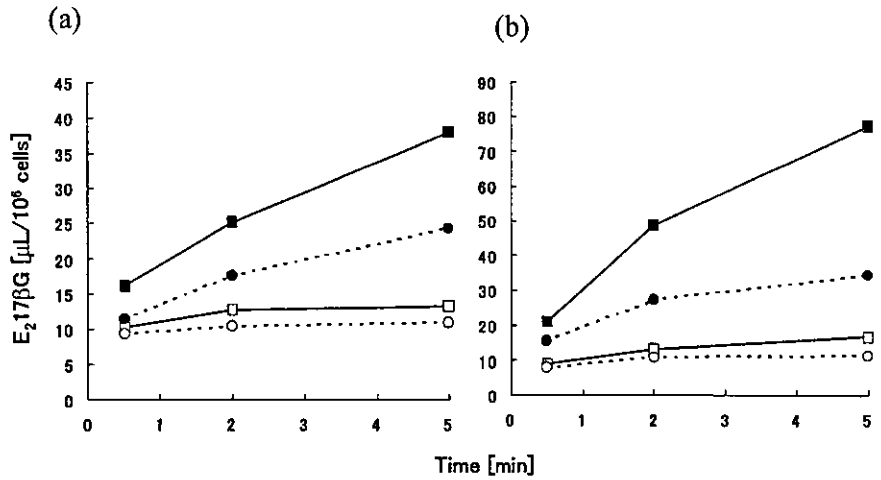

Fig. 2. Time profile for the uptake of $E_2$17βG in freshly isolated or cryopreserved human hepatocytes.
Uptake of $E_2$17βG in freshly isolated hepatocytes (■, □) or cryopreserved hepatocytes (●, ○) was measured by incubating cells with 1 μM (●, ■) or 100 μM (○, □) $E_2$17βG at 37°C in Na^+ (+) Krebs-Henseleit buffer. The uptake in lot No. HH-093 (a) and HH-099 (b) is shown here and other results are summarized in **Table 3**. Each point and bar represents mean ± S.E. of 3 separate determinations.

TC uptake (2–8 μM) was close to that reported in human NTCP transfected cells (6 μM).[41] In cryopreserved human hepatocytes, Na^+-dependent uptake of TC, at least partly mediated by NTCP, ranged from 60% to 79% of the total uptake (**Table 2**). $E_2$17βG is reported to be a substrate for the OATP family, of which OATP-C/OATP2 and OATP8 have been identified as being expressed in the liver. The K_m for uptake in cryopreserved hepatocytes (3–18 μM) was also similar to that reported for OATP-C/OATP2 (8 μM)[31] and

OATP8 (5 μM).[32]

Among the five hepatocyte preparations, the rank order of the CL_{uptake} of TC and $E_2$17βG was different, supporting the fact that the major transporter(s) for these two substrates is different. This rank order for $E_2$17βG was changed markedly before and after cryopreservation. This might be explained by the possibility that multiple transporters are involved in the uptake of $E_2$17βG and the effect of cryopreservation on their expression level and/or function may vary.

Table 3. Comparison of uptake clearance for estradiol 17β-D-glucuronide ($E_2$17βG) in freshly isolated and cryopreserved human hepatocytes.[a]

	freshly isolated [μL/min/10^6 cells]	cryopreserved [μL/min/10^6 cells]	cryopreserved/ fresh ratio
HH-093	4.29±1.15	3.43±0.69	0.80±0.27
HH-097	21.5±14.1	14.5±2.0	0.67±0.45
HH-099	15.7±2.4	5.97±1.25	0.38±0.10*[b]
HH-105	14.9±1.2	6.84±0.93	0.46±0.07**
HH-106	5.94±2.64	11.6±3.35	1.95±1.04
mean[c]	12.5± 7.2	8.47±4.49	

[a] Uptake clearances were calculated as described in the Methods section. All uptake clearances mean transporter-mediated uptake clearances. All studies were carried out in triplicate and data are represented by mean±S.D.
[b] *···$p<0.05$, **···$p<0.01$: significantly different between in freshly isolated and cryopreserved hepatocytes by Dunnet's test.
[c] mean±S.D. of 5 lots.

Large inter-lot variations in the transport activity observed (**Table 2** and **3**) may be due to intrinsic inter-individual differences, in part, by the genetic polymorphism of transporters such as OATP-C/OATP2[42,43] or due to an artifact produced during the isolation and/or cryopreservation of hepatocytes. However, it also should be noted that such variations were observed in freshly isolated hepatocytes. In addition, drug disposition profiles in humans are generally thought to be more variable than those in animals.[44] Therefore, we cannot conclude that the variations observed in this study were solely due to an artifact. However, we cannot find a clear factor governing the inter-lot difference in the CL_{uptake} of TC and $E_2$17βG as far as sex, tobacco usage and alcohol usage (**Table 1**) are concerned, due to the limited amount of liver samples available.

The kinetic analyses of TC and $E_2$17βG uptake in cryopreserved human hepatocytes have shown that inter-lot differences were observed both in V_{max}/K_m and P_{dif} (**Table 4**). The range in the V_{max}/K_m values may be due to interindividual differences in the expression level and/or function of transporters although it may be caused by other factors such as the cell integrity being affected during the cell isolation and/or cryopreservation process. It may be also due to inter-lot differences in the driving force for transporters (i.e. Na^+ gradient between the inside and outside of cells for NTCP) although this was not confirmed in the present study. Considering that multiple transporters accept $E_2$17βG as a substrate,[31,32] a large variation in the K_m of $E_2$17βG

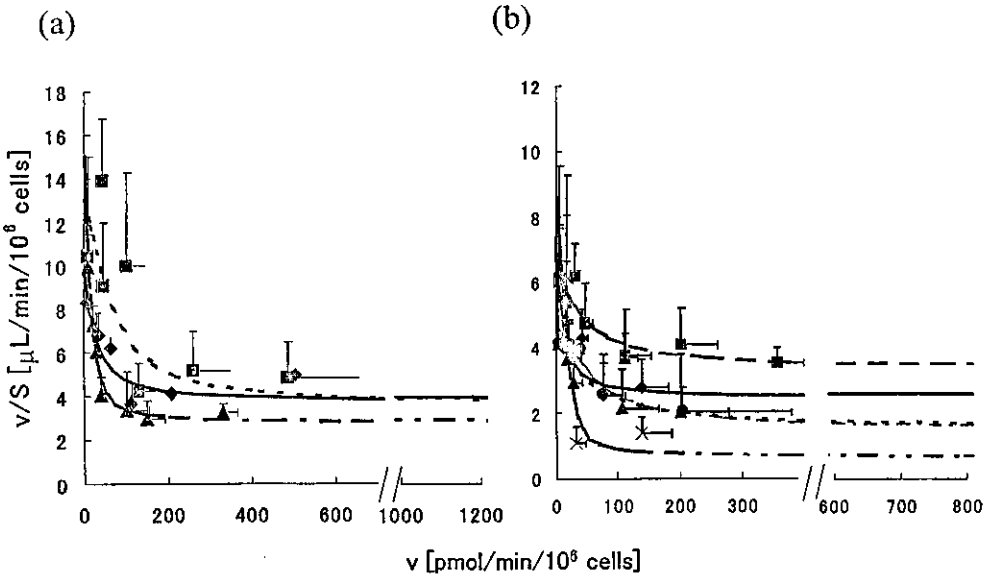

Fig. 3. Eadie-Hofstee plots for the uptake of TC (a) and $E_2$17βG (b) in cryopreserved human hepatocytes, HH-063 (◆), HH-068 (■), HH-069 (▲), HH-088 (●) and HH-117 (×).

Uptake of TC and $E_2$17βG by cryopreserved hepatocytes was measured at concentrations of 1, 3, 5, 10, 30, 50, and 100 μM in the presence of Na^+. Each point and bar represents mean±S.E. of 3 separate determinations. In each graph, the lines represent the fitted curves obtained using equation (1).

Table 4. Kinetic parameters for the uptake of taurocholic acid (TC) and estradiol 17β-D-glucuronide ($E_2$17βG) in cryopreserved human hepatocytes.[a]

Substrate	Lot No.	K_m [μM]	V_{max} [pmol/min/10^6 cells]	V_{max}/K_m [μL/min/10^6 cells]	P_{dif} [μL/min/10^6 cells]
TC	HH-063	5.25±3.48	35.8±19.5	6.82±5.85	3.45±0.47
	HH-068	7.71±5.91	73.1±49.9	9.48±9.73	3.54±0.91
	HH-069	1.55±0.41	18.9±2.6	12.2±3.6	2.81±0.12
average		4.84±1.79	42.6±16.0	9.50±2.69	3.27±0.23
	rat[b,d]	17.7±2.8	1630±150	92.1±16.09	
$E_2$17βG	HH-063	3.09±2.64	18.8±11.5	6.08±6.41	3.74±0.46
	HH-068	11.5±10.1	38.3±33.1	3.33±4.11	3.18±0.51
	HH-069	6.31±6.44	22.1±19.3	3.50±4.71	3.47±0.45
	HH-088	18.1±10.2	60.2±35.1	3.33±1.87	1.49±0.39
	HH-117	3.21±2.27	26.0±12.5	8.10±5.73	0.688±0.33
average		8.44±2.86	33.1±7.5	4.87±0.96	2.51±0.60
	rat[c,d]	12.9±1.3	1300±100	101±13	

[a] Kinetic parameters were obtained from the uptake studies shown in **Fig. 4**. All data are shown as mean±S.E. values.
[b] Ref 15) V_{max} and V_{max}/K_m are expressed as pmol/min/mg protein and μL/min/mg protein, respectively.
[c] Ref 17) V_{max} and V_{max}/K_m are expressed as pmol/min/mg protein and μL/min/mg protein, respectively.
[d] 1-mg-protein rat hepatocytes correspond to approximately 10^6 cells in our studies.

emphasizes the necessity for further studies to clarify the variability in the contribution of each transporter to the uptake of this substrate in each hepatocyte preparation.

Our results are consistent with the known species-differences in active transport. Sandker *et al.* have reported that the uptake of TC and ouabain in freshly-isolated human hepatocytes was much lower than that in rat hepatocytes.[37] In our previous analysis, the K_m for TC in primary cultured rat hepatocytes was 18 μM,[15] which is higher than that in humans (4.8 μM) (**Table 4**), whereas the V_{max} in rats was 1630 pmol/min/mg protein,[15] which was much higher than that observed in this study in human hepatocytes (42.6 pmol/min/10^6 cells) (**Table 4**). Thus, the difference in TC uptake between rats and humans is mainly due to the difference in V_{max} values. Similarly, the K_m and V_{max} of $E_2$17βG uptake in rat hepatocytes obtained in our previous analysis were 12.9 μM and 1300 pmol/min/mg protein, respectively,[17] with the difference in V_{max} from human hepatocytes (33.1 pmol/min/10^6 cells) being more marked than that in K_m (8.44 μM) (**Table 4**). This differs from the case involving the transport activity across the bile canalicular membranes, which is mainly affected by interspecies differences in K_m rather than in V_{max}.[44]

Using isolated hepatocytes, overall drug clearance, including transporter-mediated uptake and metabolism, can be estimated.[2] Moreover, isolated hepatocytes are equipped with numbers of enzymes and coenzymes involved in drug metabolism under conditions close to those in the intact liver.[45] Therefore, isolated hepatocytes are a good tool for a more precise estimation of *in vivo* drug metabolism. Recently, to avoid drug-drug interactions (DDI), many *in vitro* assays using microsomes and/or expression systems of metabolizing enzymes have been performed. For this purpose, isolated hepatocytes are a better tool because this experimental system is close to the intact liver and also enables the prediction of transporter-mediated DDI.

In conclusion, cryopreserved human hepatocytes retain, at least in part, their transporter function for TC and $E_2$17βG. Cryopreserved human hepatocytes appear to be a useful tool for examining the mechanism of hepatic uptake of drugs.

Acknowledgment: This work was partly supported by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 278, No. 25, Issue of June 20, pp. 22644–22649, 2003
Printed in U.S.A.

ABCG2 Transports Sulfated Conjugates of Steroids and Xenobiotics*

Received for publication, December 5, 2002, and in revised form, April 7, 2003
Published, JBC Papers in Press, April 7, 2003, DOI 10.1074/jbc.M212399200

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The mechanism for the cellular extrusion of sulfated conjugates is still unknown. In the present study, we investigated whether human wild type ABCG2 transports estrone 3-sulfate (E_1S) using membrane vesicles from cDNA-transfected mouse lymphoma cell line (P388 cells). The uptake of [^{3}H]E_1S into ABCG2-expressing membrane vesicles was stimulated by ATP, and the K_m value for [^{3}H]E_1S was determined to be 16.6 μM. The ABCG2-mediated transport of [^{3}H]E_1S was potently inhibited by SN-38 and many sulfate conjugates but not by glucuronide and glutathione conjugates or other anionic compounds. Other sulfate conjugates such as [^{3}H]dehydroepiandrosterone sulfate (DHEAS) and [^{35}S]4-methylumbelliferone sulfate (K_m = 12.9 μM) and [^{35}S]6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole (E3040) sulfate (K_m = 26.9 μM) were also transported by ABCG2. Although [^{3}H]methotrexate, [^{3}H]17β-estradiol-17β-D-glucuronide, [^{3}H]2,4-dinitrophenyl-*S*-glutathione, and [^{14}C]4-methylumbelliferone glucuronide were transported by ABCG2, this took place to a much lesser extent compared with [^{3}H]E_1S. It was suggested that ABCG2 preferentially transports sulfate conjugates and that E_1S and DHEAS are the potential physiological substrates for this transporter.

Human ABCG2, also referred to as placenta-specific ABC transporter[1]/breast cancer resistance protein/mitoxantrone resistance-associated protein, belongs to the ATP-binding cassette (ABC) transporter family (1–3). The structure of ABCG2 differs from that of MDR1 P-glycoprotein and multidrug resistance-associated protein (MRP/ABCC) family proteins, which are two major groups of cancer multidrug resistance ABC transporters. Although MDR1/ABCB1 contains two tandem repeats of transmembrane and ABC domains, and many of the MRP family proteins (such as MRP1–3/ABCC1–3) contain the additional third transmembrane domain prior to the two tandem repeats of transmembrane and ABC domains, ABCG2 consists of only one ABC and one transmembrane domain and, therefore, is referred to as a half-sized ABC transporter (1–3). ABCG2 was initially identified as an mRNA expressed in placenta (2) and as a non-MDR1 and non-MRP type resistance factor from cell lines which were selected in the presence of anthracyclines and mitoxantrone (1, 3). Although most of the half-sized ABC transporters are located on the intracellular organelle membrane, immunohistochemical studies revealed that ABCG2 is expressed on the plasma membrane (4). The fact that the intracellular concentration of substrate anticancer drugs is reduced in ABCG2-expressing cells (5) is consistent with its localization on the plasma membrane.

Although the function of ABCG2 has been studied extensively in terms of multidrug resistance, the physiological and/or pharmacological functions of this transporter have not been clarified yet. In normal human tissues, ABCG2 has been found to be expressed on the apical membrane of trophoblast cells in placenta, the apical membrane of enterocytes, the bile canalicular membrane of hepatocytes, and the apical membrane of lactiferous ducts in the mammary gland (6). These results suggest that ABCG2 may play an important role in protecting these tissues against the exposure to xenobiotics by extruding them across the apical membrane. In addition, ABCG2 has been demonstrated to be expressed in a wide variety of stem cells and to be a molecular determinant of the side-population phenotype (7). Recently, it was demonstrated that the disruption of the *ABCG2* gene results in the loss of the number of side-population cells in the bone marrow and skeletal muscle (8). Moreover, *ABCG2* (−/−) hematopoietic cells were more sensitive to mitoxantrone in the drug-treated transplanted mice, suggesting its protective role against cytotoxic substrates (8). It was recently demonstrated that *ABCG2* (−/−) mice were more sensitive to pheophorbide, a breakdown product of chlorophyll, resulting in the phototoxic lesions on the light-exposed skin (9). The function of ABCG2 in determining the drug disposition has been investigated by examining the disposition of topotecan, a substrate for ABCG2 and MDR1 P-glycoprotein, in *mdr1a/1b* (−/−) mice (10). Jonker *et al.* (10) reported that the oral absorption of topotecan was increased by the administration of GF120918, an ABCG2 inhibitor. GF120918 has been demonstrated to alter the disposition of topotecan by preventing intestinal (re)absorption and/or biliary excretion (10).

Concerning the cellular extrusion of xenobiotics and/or their metabolites, the role of MDR1 and MRP family proteins has

* This work was supported by Grants-in-aid for Scientific Research on Priority Areas ABC proteins 10044243 and on Priority Areas epithelial vectorial transport 12144201 from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

A part of the present study has been presented in the 53rd annual meeting of the American Association for the Study of Liver Diseases held in Boston, November 1st through 5th, 2002, and has been published in an abstract form (35).

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[1] The abbreviations used are: ABC transporter, ATP-binding cassette transmembrane transporter; MRP, multidrug resistance-associated protein; GSH, reduced glutathione; DTT, dithiothreitol; E_1S, estrone 3-sulfate; DHEAS, dehydroepiandrosterone sulfate; PNPS, *p*-nitrophenyl sulfate; TLC-S, taurolithocholate sulfate; 4-MUS, 4-methylumbelliferone sulfate; 4-MUG, 4-methylumbelliferone glucuronide; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole; E3040S, E3040 sulfate; E3040G, E3040 glucuronide; $E_2$17βG, 17β-estradiol-17β-D-glucuronide; LTC_4, leukotriene C_4; DNP-SG, 2.4-dinitrophenyl-*S*-glutathione; PBS, phosphate-buffered saline; ATPγS, adenosine 5′-*O*-(thiotriphosphate); GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

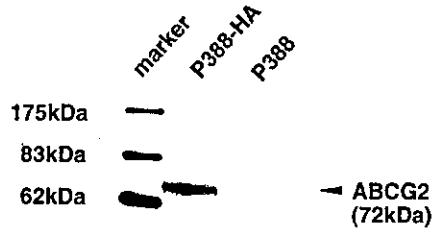

FIG. 1. **Western blot analysis of ABCG2 in membrane vesicles.** Membrane vesicles isolated from *ABCG2*-transfected (P388-HA) and control P388 cells (25 μg of protein) were separated on an 8.5% polyacrylamide gel containing 0.1% SDS. The proteins transferred to the polyvinylidene difluoride membrane by electroblotting were detected by monoclonal antibodies against human ABCG2.

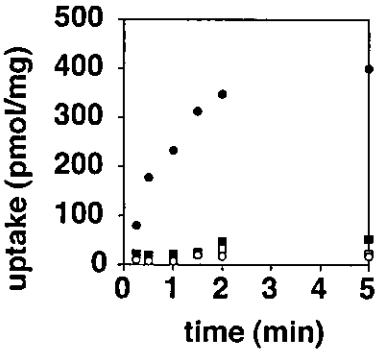

FIG. 2. **Time profiles for [^{3}H]E_1S uptake by membrane vesicles.** Membrane vesicles (5 μg of protein) prepared from *ABCG2*-transfected P388 cells (*circles*) or vector-transfected P388 cells (*squares*) were incubated at 37 °C in medium containing 500 nM unlabeled and 52 nM ^{3}H-labeled E_1S in the presence (*closed symbols*) and absence (*open symbols*) of 5 mM ATP. Each *point* and *vertical bar* represents the mean ± S.E. of triplicate determinations (*closed symbols*) or the mean values of two determinations (*open symbols*). Where *vertical bars* are not shown, the S.E. is within the limits of the *closed symbols*.

been documented (11–15). It has been established that MDR1 preferentially extrudes hydrophobic cationic and/or neutral compounds, whereas MRP family proteins preferentially transport organic anions, including conjugated metabolites (11–15). Indeed, it is well known that MRP family proteins act synergistically with conjugative enzymes to detoxify xenobiotics (11–13, 15, 16). For example, the glucuronide and glutathione conjugates formed in hepatocytes by UDP-glucuronosyl transferases and glutathione *S*-transferases, respectively, are excreted into the bile via MRP2, an apically located efflux transporter (11–13, 15, 16). Although the substrate specificity of UDP-glucuronosyl transferases and sulfotransferases resemble each other, limited information is available for the cellular extrusion of sulfated conjugates. Our results indicated that xenobiotic sulfates are not significantly transported by MRP2 but, rather, stimulated the function of MRP2 (17, 18). MRP1, a basolaterally located efflux transporter, has been shown to transport estrone 3-sulfate (E_1S), and this transport is extensively stimulated in the presence of reduced glutathione (GSH) (19). Very recently, it was demonstrated that dehydroepiandrosterone sulfate (DHEAS) is extruded from MRP2-expressing cells (20). In the present study, we examined whether ABCG2 transports sulfated conjugates using membrane vesicles from wild type human ABCG2-expressing mouse lymphoma (P388) cells.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]E_1S (43.1 Ci/mmol), [^{3}H]17β-estradiol-17β-D-glucuronide ($E_2$17βG, 55.0 Ci/mmol), [^{3}H]leukotriene C_4 (LTC_4, 136.9 Ci/mmol), [^{3}H]DHEAS (60 Ci/mmol), and [^{3}H]taurocholate (2.00 Ci/mmol) were purchased from PerkinElmer Life Science, Inc. (Boston, MA).

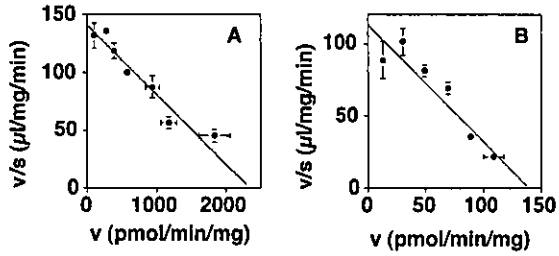

FIG. 3. **Concentration dependence of ABCG2-mediated [^{3}H]E_1S uptake.** The uptake of [^{3}H]E_1S (34 nM) by ABCG2-expressing membrane vesicles was determined at 37 °C for 1 min in medium containing unlabeled E_1S (*A*) and the uptake of 25 nM [^{3}H]E_1S with 500 nM unlabeled E_1S by ABCG2-expressing membrane vesicles was determined at 37 °C for 1 min in medium containing different concentrations of ATP (*B*). Results are shown as an Eadie-Hofstee plot. The *solid line* represents the fitted line obtained by non-linear regression analysis. Each *point* and *bar* represents the mean ± S.E. of triplicate determinations. Where *vertical bars* are not shown, the S.E. is within the limits of the symbols.

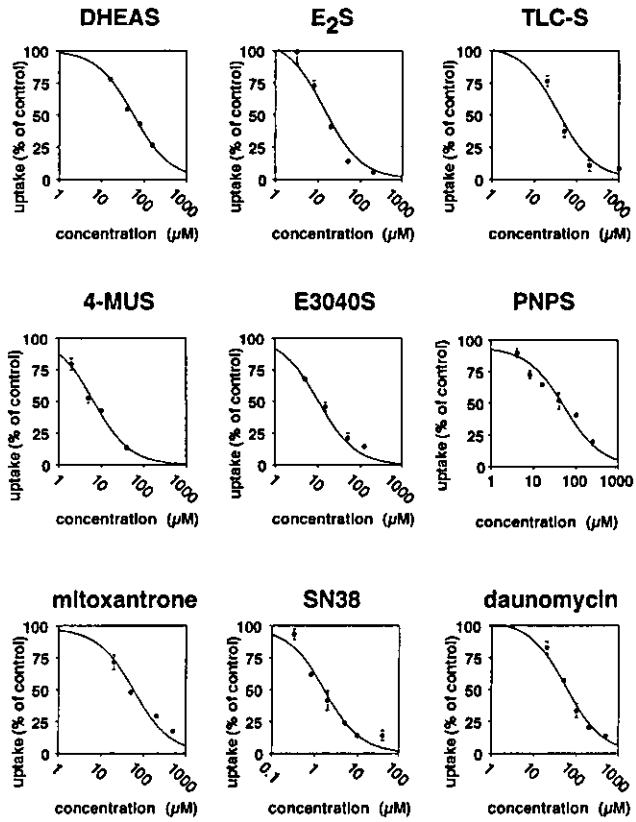

FIG. 4. **Effect of compounds on ABCG2-mediated [^{3}H]E_1S uptake by membrane vesicles.** [^{3}H]E_1S uptake by membrane vesicles prepared from *ABCG2*-transfected cells and vector-transfected P388 cells was determined at 37 °C for 2 min in medium containing 500 nM unlabeled and 33 nM [^{3}H]labeled E_1S, with or without the compounds at the indicated concentrations. The uptake values were calculated by subtracting the values for control membrane vesicles from those for ABCG2-expressing membrane vesicles in the presence of 5 mM ATP. Data represent the mean ± S.E. of triplicate determinations. Where *bars* are not shown, the S.E. is minimal. *, significantly lower than the control ($p < 0.05$) by analysis of variance (ANOVA) followed by Dunnett's test. **, significantly lower than the control ($p < 0.01$) by ANOVA followed by Dunnett's test.

[^{3}H]Methotrexate (29 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]Taurolithocholate sulfate (TLC-S) was prepared using [^{3}H]taurine (30.3 Ci/mmol, PerkinElmer Life Science, Inc.) according to a method described previously (21). [^{35}S]4-Methylumbelliferone sulfate (4-MUS) and [^{14}C]4-methylumbelliferone glucuronide (4-MUG) were biosynthesized by incubating 4-methylumbelliferone in rat liver cytosol and microsomes, respec-

TABLE I
Inhibition of ABCG2-mediated [^{3}H]E_1S transport by sulfated conjugates and anticancer drugs

Approximate IC_{50} values were estimated from the data shown in Fig. 4. [^{3}H]E_1S uptake by membrane vesicles prepared from *ABCG2*-transfected cells and vector-transfected P388 cells was determined at 37 °C for 2 min in medium containing 500 nM unlabeled and 33 nM ^{3}H-labeled E_1S, with or without the compounds at the indicated concentrations. The uptake values were calculated by subtracting the values for control membrane vesicles from those for ABCG2-expressing membrane vesicles in the presence of 5 mM ATP.

	Approximate IC_{50} value
	μM
DHEAS	55
E_2S	14
4-MUS	6
E3040S	10
PNPS	53
TLC-S	37
SN-38	1.6
Mltoxantrone	61
Daunomycin	59

TABLE II
Effect of organic anions including glucuronide and glutathione conjugates on ABCG2-mediated [^{3}H]E_1S transport

For compounds that did not potently inhibit the ABCG2 function, their inhibitory effects are shown at the maximum concentration used in the present study. [^{3}H]E_1S uptake by membrane vesicles prepared from *ABCG2*-transfected cells and vector-transfected P388 cells was determined at 37 °C for 2 min in medium containing 500 nM unlabeled and 33 nM ^{3}H-labeled E_1S, with or without the compounds at the indicated concentrations. The uptake values were calculated by subtracting the values for control membrane vesicles from those for ABCG2-expressing membrane vesicles in the presence of 5 mM ATP.

	Percentage of control	Maximum concentration tested
	%	μM
Pravastatin	76 ± 1[a]	1000
Taurocholate	107 ± 4	150
Methotrexate	98 ± 1	600
Doxorubicin	72 ± 4[a]	200
$E_217\beta G$	93 ± 3	75
4-MUG	84 ± 9[a]	500
E3040G	76 ± 4[a]	250
Estrone	56 ± 3[a]	200
LTC_4	113 ± 2	2
DNP-SG	78 ± 2[b]	80

[a] Significantly lower than the control ($p < 0.01$).
[b] Significantly lower than the control ($p < 0.05$).

tively. In the case of [^{35}S]4-MUS, the reaction buffer contained 5 mM HEPES, 1 mM $MgCl_2$, 4 mM 4-methylumbelliferone, 1 mg/ml rat liver cytosol, and 50 μCi of [^{35}S]phosphoadenosine 5′-phosphosulfate (1.82 Ci/mmol, PerkinElmer Life Science, Inc.) in a final volume of 1.0 ml. For 4-MUG, the reaction buffer contained 5 mM HEPES, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM D-saccharic acid-1,4-lactone, 0.01%(v/v) Triton X-100, 4 mM 4-methylumbelliferone, and 5 μCi of [^{14}C]UDP-glucuronic acid (252 mCi/mmol, ICN Biomedicals, Inc., Irvine, CA) in a final volume of 1.0 ml. After incubation for 60 min at 37 °C, both reactions were terminated by adding ice-cold 9 ml of methanol. After centrifugation, the supernatant was subsequently reduced by centrifugal concentration and applied to a pre-coated thin-layer chromatography plate (Silica-Gel 60F254, with fluorescent indicator; 0.25 mm in thickness, Merck, Whitehouse Station, NJ). The plates were developed in 1-butanol/ethanol/water (80:10:20, v/v). After development, the spots were located by UV light. The region associated with radiolabeled 4-MUS or 4-MUG was scraped and extracted with methanol. The radiochemical purity levels for [^{35}S]4-MUS and [^{14}C]4-MUG prepared by this method were found to be 97.6% and 95.0%, respectively.

Unlabeled and ^{3}H-labeled 2,4-dinitrophenyl-S-glutathione (DNP-SG) was prepared using unlabeled and ^{3}H-labeled GSH (22.5 Ci/mmol, PerkinElmer Life Science, Inc.) according to the method described previously (22, 23). ^{35}S-Labeled 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole (E3040) sulfate (E3040S) and ^{14}C-labeled glucuronide (E3040G) were prepared from [^{35}S]phosphoadenosine 5′-phosphosulfate (3.00 Ci/mmol, PerkinElmer Life Science, Inc.) and [^{14}C]UDP-glucuronic acid (325.9 mCi/mmol, ICN Biomedicals, Inc.), respectively, based on a method described previously (17). Unlabeled E_1S, estradiol 3-sulfate (E_2S), estrone, $E_217\beta G$, DHEAS, taurocholate TLC-S, 4-MUG, 4-MUS, LTC_4, methotrexate, *p*-nitrophenyl sulfate (PNPS), daunomycin, and mitoxantrone were purchased from Sigma. Unlabeled E3040G and E3040S were donated by Eisai. Co., Ltd. SN-38 was supplied by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan).

Transport Studies with Membrane Vesicles—Membrane vesicles were prepared from 2 × 10^8 vector- and wild type human ABCG2-transfected mouse lymphoma (P388) cells (24) according to the method described previously (25, 26). The membrane vesicles were frozen in liquid nitrogen and then transferred to a deep freezer (−100 °C) until required.

For the Western blot analysis, 20 μg of protein of membrane vesicles was dissolved in 10 μl of 0.25 M Tris-HCl buffer containing 2% SDS, 30% glycerol, 6% 2-mercaptoethanol, and 0.01% bromphenol blue, pH 6.8, and was separated on a 7.5% SDS-polyacrylamide gel electrophoresis with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (New England BioLabs, Beverly, MA). Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, MA) using a blotter (Bio-Rad Laboratories, Richmond, CA) at 15 V for 1 h. The membrane was blocked with PBS containing 5% skim milk for 1 h at room temperature. After blocking, the membrane was incubated for 1 h at room temperature in PBS containing 5% skim milk and 100-fold diluted BXP-21 (Kamiya Biomedical Company, Seattle, WA). Then, the membrane was washed with PBS containing 0.1% Tween 20 and allowed to bind to 5000-fold diluted Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) for 1 h. Subsequently, the membrane was rinsed four times with PBS containing 0.1% Tween 20 for 5 min. The enzyme activity was assessed by using 5000-fold diluted Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, Inc.) with an Odyssey infrared imaging system (LI-COR, Inc., Lincoln, NE).

The transport studies were performed using a rapid filtration technique (25, 26). Briefly, 15 μl of transport medium (10 mM Tris-HCl, 250 mM sucrose, 10 mM $MgCl_2$, pH 7.4) containing radiolabeled compounds, with or without unlabeled substrate, was preincubated at 37 °C for 3 min and then rapidly mixed with 5 μl of membrane vesicle suspension (5–10 μg of protein). The reaction mixture contained 5 mM ATP or other nucleotides, along with the ATP-regenerating system (10 mM creatine phosphate and 100 μg/μl creatine phosphokinase). The transport reaction was terminated by the addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4). The stopped reaction mixture was filtered through a 0.45-μm membrane filter (GVWP, Millipore Corp., Bedford, MA) and then washed twice with 5 ml of stop solution. Radioactivity retained on the filter was determined in a liquid scintillation counter (LSC-3500, Aloka Co., Tokyo, Japan). The ATP-dependent uptake of ligands was calculated by subtracting the ligand uptake in the absence of ATP from that in its presence.

RESULTS

Expression of ABCG2—The expression level of the wild type human ABCG2 in a mouse lymphoma cell line was determined by Western blot analysis using anti-human ABCG2 (BXP-21) monoclonal antibody. As shown in Fig. 1, ABCG2 was only detectable in the transfected cells, and its molecular mass was ~72 kDa, which is consistent with the previously reported molecular mass (24). The Western blot analysis with an antibody against MRP1 (MRPr1) revealed that the strength of the band at 175 kDa is the same between control and ABCG2-transfected cells (data not shown). Although we could detect the band in the Western blot analysis of crude membrane fraction from mouse liver and intestine, but not that of the membrane vesicles from the parental P388 cells, with BXP-21 monoclonal antibody (data not shown), we cannot deny the possibility that the presence of an unknown amount of related mouse transporter may significantly influence the results of the transport studies. It should be noted that the use of mouse lymphoma cells has disadvantages due to this possibility.

Uptake of [^{3}H]E_1S into Membrane Vesicles—The time profiles for the uptake of [^{3}H]E_1S by membrane vesicles from P388 cells are shown in Fig. 2. The uptake of [^{3}H]E_1S into membrane

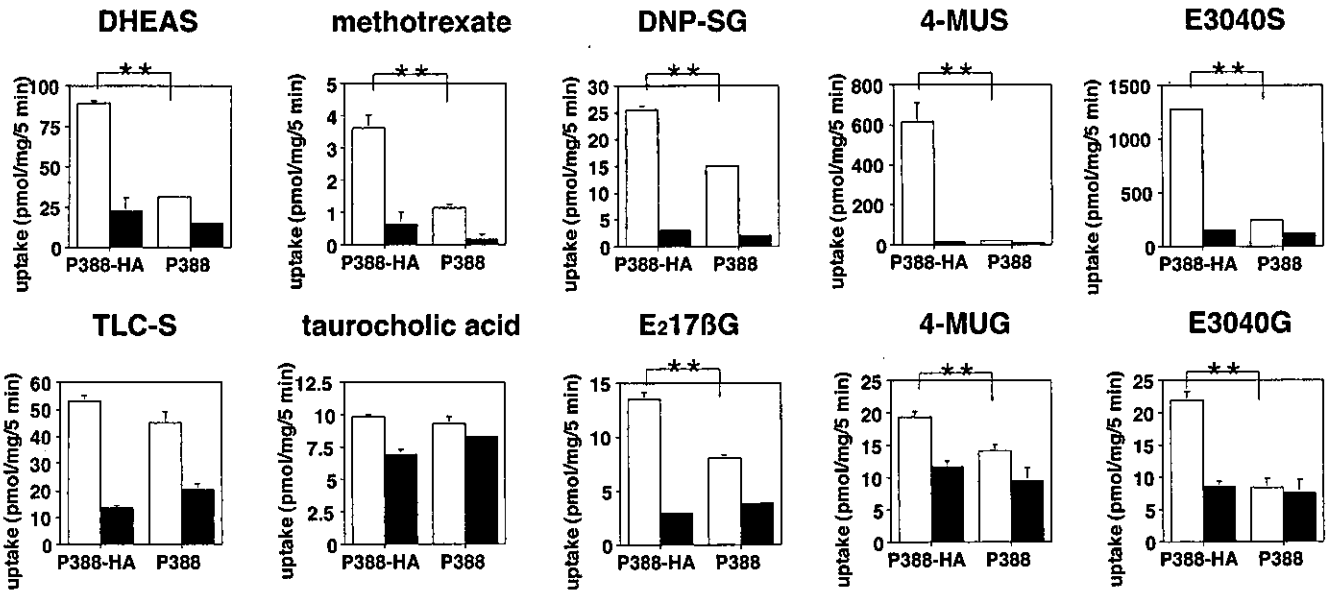

FIG. 5. **Transport of organic anions by ABCG2.** The uptake of [^{3}H]DHEAS (0.5 μM), [^{3}H]methotrexate (MTX, 0.5 μM), [^{3}H]$E_2$17βG (0.5 μM), [^{3}H]TLC-S (0.5 μM), [^{3}H]taurocholate (TC; 0.5 μM), [^{3}H]DNP-SG (0.5 μM), [^{35}S]4-MUS (5.0 μM), [^{14}C]4-MUG (4.1 μM), [^{35}S]E3040S (2.5 μM), and [^{14}C]E3040G (2.5 μM) into membrane vesicles prepared from *ABCG2*-transfected (P388-HA) and vector-transfected P388 cells was determined at 37 °C for 5 min in medium containing 5 mM ATP (*open bar*) or AMP (*closed bar*). Each *bar* represents the mean ± S.E. of triplicate determinations. Where bars are not shown, the S.E. is minimal. *, significantly higher in ABCG2-expressing membrane vesicles ($p < 0.05$). **, significantly higher in ABCG2-expressing membrane vesicles ($p < 0.01$).

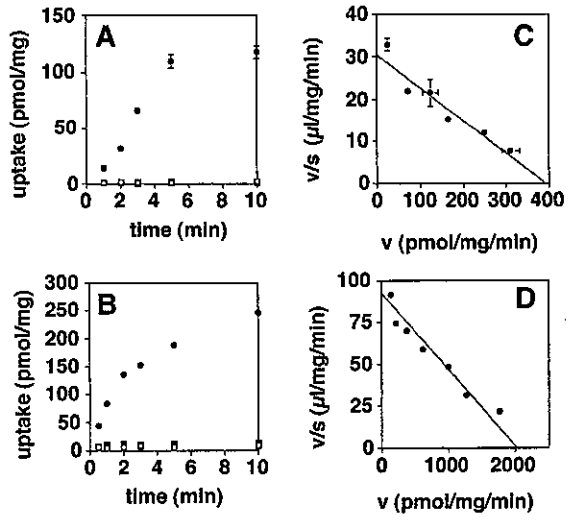

FIG. 6. **Uptake of [^{35}S]4-MUS and [^{35}S]E3040S by ABCG2.** *A* and *B* show the time profiles for the ABCG2-mediated uptake of [^{35}S]4-MUS (*A*) and [^{35}S]E3040S (*B*). Membrane vesicles (10 μg of protein) prepared from *ABCG2*-transfected P388 cells (*circles*) or vector-transfected P388 cells (*squares*) were incubated at 37 °C in medium containing 0.7 μM ^{35}S-labeled 4-MUS or 0.7 μM ^{35}S-labeled E3040S in the presence (*closed symbols*) and absence (*open symbols*) of 5 mM ATP. *C* and *D* show the saturation of the ABCG2-mediated transport of [^{35}S]4-MUS (*C*) and [^{35}S]E3040S (*D*). The uptake of [^{35}S]4-MUS (0.7 μM) or [^{35}S]E3040S (0.3 μM) by ABCG2-expressing membrane vesicles was determined at 37 °C for 3 min as to [^{35}S]4-MUS and for 1 min as to [^{35}S]E3040S in medium containing unlabeled 4-MUS or E3040S, respectively. Results are shown as an Eadie-Hofstee plot. The *solid line* represents the fitted line obtained by nonlinear regression analysis. Each *point* and *bar* represents the mean ± S.E. of triplicate determinations. Where *vertical bars* are not shown, the S.E. is within the limits of the symbols.

vesicles from ABCG2-transfected P388 cells, but not into that from vector-transfected cells, was markedly stimulated by ATP (Fig. 2). Indeed, the uptake of [^{3}H]E_1S into ABCG2-expressing membrane vesicles at 2 min was more than 20-fold higher than that into control membrane vesicles (Fig. 2). We also confirmed that the vesicle-associated [^{3}H]E_1S represents uptake into the intravesicular space, rather than binding to the vesicle surface, by confirming the osmotic sensitivity (data not shown).

Characterization of ABCG2-mediated Transport of [^{3}H]E_1S—The ATP-dependent uptake of [^{3}H]E_1S into ABCG2-expressing membrane vesicles was saturable (Fig. 3*A*). Nonlinear regression analysis of the ATP-dependent uptake of [^{3}H]E_1S revealed that the uptake can be described by a single saturable component with a K_m of 16.6 ± 3.4 μM and a V_{max} of 2.34 ± 0.24 nmol/min/mg of protein, respectively. Kinetic analysis revealed that the K_m of ATP was 1.23 ± 0.20 mM (Fig. 3*B*), which is higher than the previously reported value for the ATP concentration producing the half maximum membrane ATPase activity (0.3 mM) in the presence of 100 μM prazosin or producing the half maximal velocity of SN-38 transport (~0.8 mM) (27, 28).

GTP and UTP also stimulated the uptake of [^{3}H]E_1S to the same extent as ATP, whereas the stimulatory effect of CTP was ~50% that of ATP. In contrast, nonhydrolyzable analogues such as ATPγS or GTPγS, along with AMP, GMP, or UMP, did not support the ABCG2-mediated transport of [^{3}H]E_1S. However, from the present results, we cannot discuss details on the ATPase activity of ABCG2, which has been demonstrated by Ozvegy *et al.* (27, 28).

To further characterize the ABCG2-mediated transport of [^{3}H]E_1S, we examined the effect of inhibitors. Sulfated conjugates (DHEAS, E_2S, TLC-S, PNPS, 4-MUS, and E3040S) significantly inhibited the ABCG2-mediated transport of [^{3}H]E_1S (Fig. 4 and Table I). The inhibitory effect of anti-tumor drugs (such as SN-38, mitoxantrone, and daunomycin), along with that of estrone, was also observed (Fig. 4 and Tables I and II). In contrast, glutathione conjugates (LTC_4 and DNP-SG), glucuronide conjugates ($E_2$17βG, 4-MUG, and E3040G), or nonconjugated organic anions (pravastatin, methotrexate, and taurocholate) did not potently reduce the uptake of [^{3}H]E_1S (Table II). We also found that the ABCG2-mediated transport of [^{3}H]E_1S was not affected by 10 mM dithiothreitol (DTT), 10 mM DTT and 4 mM GSH, and 10 mM DTT and 4 mM nonreducing *S*-methyl-GSH (data not shown).

Determination of the Substrate Specificity of ABCG2—In ad-