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Lack of Interaction between Tauroursodeoxycholate and ATP-Binding Cassette Transporter Isoform G2 (ABCG2)

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Abstract: Ursodiol (UDCA) is useful for treating several cholestatic hepatic maladies, including intrahepatic cholestasis of pregnancy. Its taurine amidate (TUDC), which accumulates in the bile salt pool, could interact with ABCG2 (ATP-binding cassette transporter isoform G2), which is expressed in various tissues including the canalicular membrane of the hepatocyte and in the apical membrane of the placental syncytiotrophoblast. The purpose of this study was to determine the interaction between TUDC and ABCG2. ABCG2 was expressed in *Sf*9 cells, and ABCG2-mediated ATP-dependent transport was determined in sucrose-fractionated *Sf*9 membrane vesicles. The transport of estrone 3-sulfate (E_1S) into ABCG2-expressing membranes was ATP-dependent and was much greater in membrane vesicles expressing ABCG2 versus the negative control (empty virus lacking the ABCG2 coding region). To determine whether TUDC affects ABCG2-mediated ATP-dependent transport of E_1S, transport activity in the presence of TUDC (20−500 μM) was measured. No significant changes were observed in the ABCG2-mediated ATP-dependent E_1S transport. Furthermore, ABCG2-mediated TUDC transport was undetectable. Thus, TUDC does not affect ABCG2-mediated E_1S transport and is not an ABCG2 substrate.

Keywords: ABCG2; tauroursodeoxycholate; bile salts; estrone 3-sulfate; cholestasis; pregnancy

Introduction

ABCG2 (ATP-binding cassette transporter isoform G2) transports a variety of compounds including chemotherapeutics such as mitoxantrone, methotrexate, and several glucuronide, glutathione, and sulfate conjugates, including sulfated steroids and bile salts.[1−3] ABCG2 is expressed in the apical membrane of the placental syncytiotrophoblast, the canalicular membrane of the hepatocyte, and the intestine, brain, kidney, and bone marrow stem cell side population.[1,4] Suzuki et al. showed that P388 cells expressing or lacking ABCG2 showed insignificant differences with respect to transport of taurocholate or taurolithocholate sulfate, but the latter effectively inhibited ABCG2-mediated E_1S transport (IC_{50} 37 μM).[2] Additionally, Imai et al. found that taurolithocholate sulfate and taurolithocholate (30 μM each) inhibited E_1S transport by 70% and 75% respectively in membrane vesicles from K562/BCRP cells while taurocholate had little effect,[5] suggesting that certain unsulfated taurine-amidated bile salts interact with ABCG2.

Ursodiol (ursodeoxycholic acid, UDCA) is useful for treating several cholestatic hepatic maladies, including intrahepatic cholestasis of pregnancy.[6] Furthermore, ursodiol

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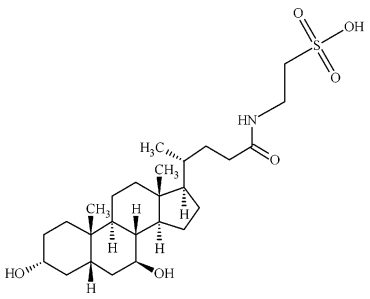

Figure 1. Structure of tauroursodeoxycholic acid.

has some direct antioxidant effects.[7] Upon continued ursodiol therapy, its taurine (tauroursodeoxycholate, TUDC) and glycine (glycoursodeoxycholate, GUDC) amidates accumulate in the bile salt pool, exerting therapeutic benefits.[6]

Interactions between ursodiol and the canalicular and syncytiotrophoblast ABC transporters have been previously established. Ursodiol is transported by the bile salt export pump (BSEP) which is expressed in the canalicular membrane,[8] but this transporter is largely absent from the syncytiotrophoblast.[9] While ursodiol inhibits MDR1,[10] it stimulates MRP2 (<100 μM)[11] and has no effect on ABCG2.[3] When it is used for the treatment of intrahepatic cholestasis of pregnancy, ursodiol increases the placental expression of MRP2.[12]

Ursodiol's taurine amidate, TUDC, is transported by BSEP with a K_m of 11.9 μM.[13] Furthermore, while TUDC also stimulates MRP2 (Gerk and Vore, unpublished data), it has no effect on MDR1 ($\leq$100 μM),[10] but its effects on ABCG2 have not been determined. Notably, direct therapeutic benefits of TUDC have been reported.[14] Although TUDC is not a sulfate conjugate, as a β-amino acid, it has a sulfonate group two carbons away from a primary amine (Figure 1). This yields a sulfonated structure that is almost completely ionized at physiologic pH (p$K_a \sim 1.4 \pm 0.5$; ACD/pK_a v8.02 from ACD/I-Lab service) and is thus fairly hydrophilic (clog$D$ @ pH 7.0 $= -1.4 \pm 1.0$; ACD/Log$D$ v8.02 from ACD/I-Lab service) and could be an ABCG2 substrate and/or inhibitor. Therefore, the purpose of this study was to determine the nature of the interaction between ABCG2 and TUDC.

Experimental Section

Materials. Tritium labeled estrone 3-sulfate ammonium salt, ^{3}H-E$_1$S (50 Ci/mmol), was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). ^{3}H-TUDC (9 Ci/mmol) was provided by Dr. Alan Hofmann (University of California, San Diego). Unlabeled tauroursodeoxycholate sodium (TUDC) was obtained from Calbiochem (La Jolla, CA). Unlabeled E$_1$S was obtained from Steraloids, Inc. (Newport, RI). Fumitremorgin C (FTC) was obtained from Alexis Biochemicals (San Diego, CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Chemicals (Fair Lawn, NJ) unless otherwise indicated below.

Preparation of Membrane Vesicles. The recombinant ABCG2 baculovirus was generated by cloning bases 205−2172 of NM_004827 into the pFastBac1 vector (Invitrogen, Carlsbad, CA) between the *Bam*HI and *Hin*dIII restriction sites (Enzymax, Lexington, KY), recombined into the baculovirus genome using the BaculoDirect Baculovirus Expression System (Invitrogen), and transfected into *Sf*9 insect cells using CellFectin reagent (Invitrogen). The recombinant baculovirus was harvested, amplified, and titered by a viral plaque assay. As a background control, a recombinant baculovirus lacking the ABCG2 insert, empty virus (EV), was also prepared as above.

*Sf*9 cells (5×10^8 cells) in suspension were infected with the titered viral stock at a multiplicity of infection of 4, and cell membranes for transport experiments were harvested 48 h later by layering on 38% sucrose and collecting the layer at the buffer−sucrose interface.[15] Membranes were vesiculated, snap frozen by dropping into liquid nitrogen, stored at −80 °C, and labeled as ABCG2 or EV infected cell membranes. Van Aubel et al. reported an inside-out orientation for 65% of the membrane vesicles, resulting from a similar preparation.[16] Protein concentrations were determined by a modification of the Lowry protein assay using bovine serum albumin as a standard.[17]

Expression of ABCG2 was determined by Western blotting using 0.5 μg of sucrose-fractionated membrane protein or 25 μg of protein homogenate from normal human placental tissue (which physiologically expresses ABCG2) as a positive control. Proteins were denatured in the presence of sodium lauryl sulfate at 37 °C for 30 min before loading onto a Tris/glycine polyacrylamide Novex precast gel (Invitrogen), separated by standard electrophoresis in the presence of NuPage sample reducing agent (Invitrogen), and transferred

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onto nitrocellulose membranes (Schleicher and Schuell, Keene, NJ). Membranes were blocked overnight at 4 °C using Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE). Binding of the primary antibody (mouse anti-human ABCG2 (BXP-21); Alexis Biochemicals, San Diego, CA) and the secondary antibody (goat anti-mouse IR Dye 800; Li-Cor) was performed in the Odyssey blocking buffer at room temperature for 1 h, in the dark. The resulting fluorescent complex was detected using the Odyssey Infrared Imaging System and the Odyssey Application Software, version 1.2 (Li-Cor).

Transport Studies in *Sf*9 Membrane Vesicles. Transport experiments were performed as previously described,[15,18,19] in up to three different batches of membranes. Briefly, the experiments were carried out in Tris-sucrose buffer, containing 5 mM ATP or AMP, 10 mM $MgCl_2$, 10 mM phosphocreatine, and 100 μg/mL creatine phosphokinase in the presence or absence of unlabeled TUDC (0−500 μM), E_1S (200 μM), or FTC (10 μM), a selective inhibitor of ABCG2,[20] in dimethyl sulfoxide (0.5%). ATP-dependent transport of ^{3}H-E_1S (60 nM) or ^{3}H-TUDC (115 nM) into membrane vesicles (15 μg/25 μL) was measured in incubations at 37 °C for 5 min or at times as indicated. Transport was stopped by adding 3.5 mL of ice-cold stop buffer and filtering through Durapore 0.4 μm filters (Millipore Corporation, Bedford, MA). Filters were collected and mixed with scintillation cocktail (Ecoscint, National Diagnostics, Atlanta, GA), and ^{3}H was detected by liquid scintillation counting. Three to six replicate determinations were performed under each different condition.

Transport corrected for that in the presence of AMP was termed ATP-dependent transport, and that corrected for background (EV transport) was termed ABCG2-mediated transport. Data were plotted and analyzed using Prism version 4 (GraphPad Software Inc., San Diego, CA). Effects of FTC and TUDC on ABCG2-mediated ATP-dependent ^{3}H-E_1S transport were analyzed by one-way analysis of variance ($\alpha = 0.05$) followed by Dunnett's multiple comparison test (Prism version 4).

Results and Discussion

Expression of ABCG2 in Membrane Vesicles. Human ABCG2 protein (70 kDa) was detected in the normal human placental tissue homogenate (25 μg) and three different batches of ABCG2-expressing *Sf*9 membrane vesicles (0.5 μg) by Western blotting (Figure 2). ABCG2 was not detected in any of the three batches of EV membrane vesicles.

Figure 2. Western blot of ABCG2 expressed in three batches of ABCG2-expressing or EV *Sf*9 membrane vesicles (0.5 μg of protein each) and normal human placental tissue homogenate (25 μg of protein). No ABCG2 was detected in the three batches of EV membrane vesicles.

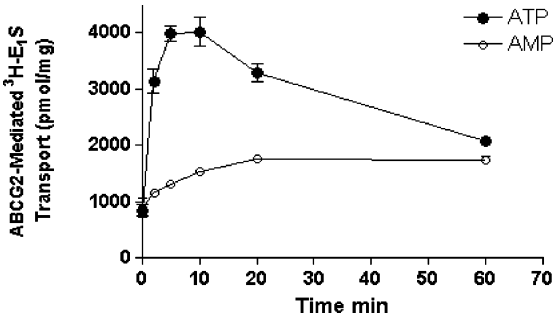

Figure 3. Transport of ^{3}H-E_1S (60 nM) in ABCG2-expressing sucrose-fractionated *Sf*9 membrane vesicles as a function of time in the presence (closed circles) or absence (open circles) of ATP. Each data point represents mean ± SD from triplicate determinations.

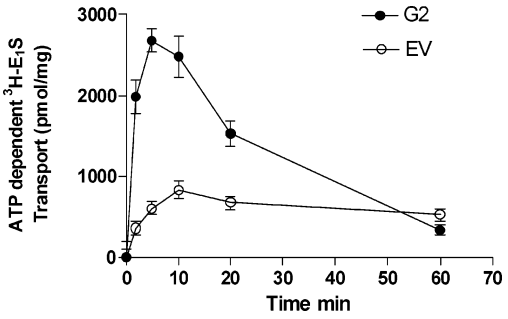

Figure 4. Transport of ^{3}H-E_1S (60 nM) in ABCG2-expressing (closed circles) or EV (open circles) membrane vesicles as a function of time. Each data point represents mean ± SD from triplicate determinations.

^{3}H-E_1S Transport in Membrane Vesicles. The transport of ^{3}H-E_1S into ABCG2-expressing *Sf*9 plasma membranes was determined in the presence or absence of an ATP-regenerating system. The transport of ^{3}H-E_1S into ABCG2-expressing membranes was ATP-dependent (Figure 3), although some non-ATP-dependent binding was observed at zero time. Transport of ^{3}H-E_1S increased up to 5 min and was much greater in membrane vesicles expressing ABCG2 versus EV (Figure 4). Transport of ^{3}H-E_1S was found to be linear with respect to protein concentration and occurred into an osmotically sensitive space (data not shown).

Effects of TUDC on ^{3}H-E_1S Transport. To determine whether TUDC affects ABCG2-mediated ATP-dependent

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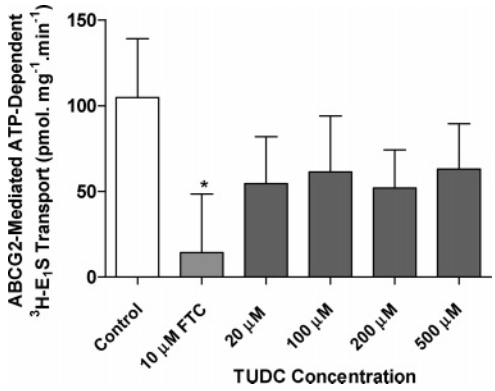

Figure 5. ABCG2-mediated ATP-dependent ^{3}H-E$_1$S transport in the presence of FTC (10 μM) and TUDC (20−500 μM) vs control, at 5 min. Each data point represents mean ± SD from 3−6 replicate determinations. Comparisons were made using Dunnett's test following one-way analysis of variance. The asterisk (*) represents $p < 0.05$ versus control.

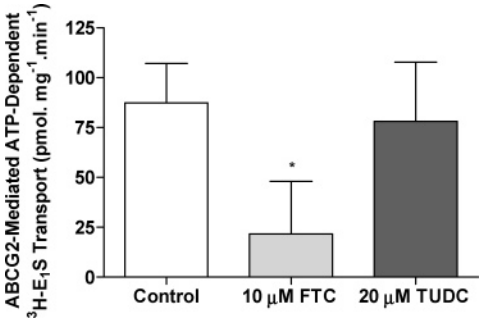

Figure 6. ABCG2-mediated ATP-dependent ^{3}H-E$_1$S transport in the presence of FTC (10 μM) and TUDC (20 μM) vs control, at 5 min. Each data point represents mean ± SEM for means resulting from 3−6 replicate determinations each in three independent batches of membranes. Comparisons were made using Dunnett's test following one-way analysis of variance. The asterisk (*) represents $p < 0.05$ versus control.

transport of ^{3}H-E$_1$S, we measured the transport activity in the presence of FTC (a selective inhibitor of ABCG2) and varying concentrations of TUDC (Figure 5). ABCG2-mediated ATP-dependent ^{3}H-E$_1$S transport was significantly decreased in the presence of FTC (10 μM), as expected for ABCG2-mediated transport.[20] There was no significant change in the ABCG2-mediated ATP-dependent ^{3}H-E$_1$S transport in the presence of TUDC (20−500 μM) as compared to the control (Figure 5). Furthermore, these results were confirmed in two additional batches of membrane vesicles (Figure 6), showing that ABCG2-mediated ATP-dependent ^{3}H-E$_1$S transport was consistent across batches.

^{3}H-TUDC Transport in Membrane Vesicles. To determine whether TUDC is an ABCG2 substrate, we evaluated the ABCG2-mediated ATP-dependent transport of ^{3}H-TUDC, in the presence or absence of unlabeled TUDC (100 μM) or E$_1$S (200 μM). We did not detect any significant ABCG2-mediated ATP-dependent ^{3}H-TUDC transport, in the presence or absence of the unlabeled agents (Table 1).

Table 1. ABCG2-Mediated ATP-Dependent ^{3}H-TUDC Transport in Membrane Vesicles[a]

unlabeled agents	concentration (μM)	^{3}H-TUDC transport (fmol·mg^{-1}·min^{-1})
control	—	0 ± 124
TUDC	100	92 ± 91
E$_1$S	200	167 ± 108

[a] Data expressed as mean ± SD of ^{3}H-TUDC transport (115 nM) from triplicate determinations, at 5 min, corrected for activity in the absence of ATP as well as the absence of ABCG2 protein, as described in the Experimental Section. Transport in ABCG2-expressing membrane vesicles was not significant.

In conclusion, TUDC does not appear to affect ABCG2-mediated ATP-dependent transport of E$_1$S and does not appear to be transported by ABCG2. Even though TUDC is a sulfonated compound, our results show that it is not a substrate or inhibitor of ABCG2. Thus, we expect that ursodiol or TUDC therapy for intrahepatic cholestasis of pregnancy would not directly inhibit ABCG2-mediated placental export of sulfated steroids like E$_1$S, and should not alter any ABCG2-mediated xenobiotic efflux. Further studies are required to determine the effects of ursodiol or TUDC on placental ABCG2 expression and to assess the therapeutic relevance of these findings.

Abbreviations Used

ABC, ATP-binding cassette; ABCG2, ABC transporter isoform G2; BSEP, bile salt export pump; MDR, multidrug resistance protein (P-glycoprotein); MRP, multidrug resistance associated protein; TUDC, tauroursodeoxycholate; GUDC, glycoursodeoxycholate; UDCA, ursodeoxycholic acid; E$_1$S, estrone 3-sulfate; EV, empty virus (lacking the ABCG2 coding region); FTC, fumitremorgin C.

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