

Human Hepatobiliary Transport of Organic Anions Analyzed by Quadruple-Transfected Cells

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ABSTRACT

Hepatobiliary elimination of many organic anions is initiated by OATP1B1 (OATP2, LST-1, OATP-C), OATP1B3 (OATP8), and OATP2B1 (OATP-B), which are the predominant uptake transporters of human hepatocytes. Thereafter, the unidirectional efflux pump ABCC2 (multidrug resistance protein 2) mediates the transport of organic anions, including glutathione conjugates and glucuronosides, into bile. In this study, we generated a Madin-Darby canine kidney (MDCKII) cell line stably expressing recombinant OATP1B1, OATP1B3, and OATP2B1 in the basolateral membrane and ABCC2 in the apical membrane. Double-transfected MDCKII cells stably expressing ABCC2 together with OATP1B1, OATP1B3, or OATP2B1 served as control cells. The quadruple-transfected cells exhibited high rates of vectorial transport of organic anions, including bromosulfophthalein, cholecystokinin peptide (CCK-8), and estrone 3-sulfate. The quadruple-transfected cells enabled the identification

of substrates for uptake or vectorial transport that may be missed in studies with a double-transfected cell line, as exemplified by CCK-8, which is a substrate for OATP1B3 but not for OATP1B1 or OATP2B1. The broad substrate spectrum covered by the three hepatocellular OATP transporters enables representative analyses of the uptake of many organic anions into human hepatocytes. The broad spectrum of organic anions transported vectorially by the quadruple-transfected cells also provides valuable information on the substrate selectivity of ABCC2, without the need for studies in inside-out membrane vesicles containing the ABCC2 protein. The quadruple-transfected MDCKII-ABCC2/OATP1B1/1B3/2B1 cells may thus be useful for the identification of substrates and inhibitors, including drug candidates, undergoing uptake and secretion by human hepatocytes, under conditions that may be better defined than in primary cultures of human hepatocytes.

The liver is the major organ for the detoxification and elimination of substances from the blood circulation. The hepatocellular uptake of a large number of organic anions from the blood is mediated in humans by OATP1B1 (formerly termed LST-1, OATP2, or OATP-C; Abe et al., 1999; Hsiang et al., 1999; König et al., 2000a), OATP1B3 (formerly termed OATP8; König et al., 2000b), and OATP2B1 (formerly known as OATP-B; Tamai et al., 2000; Kullak-Ublick et al., 2001). Once taken up into the hepatocyte, many amphiphilic anions and/or their conjugates can be excreted into human bile via the canalicular export pump ABCC2 (MRP2) (Büchler et al., 1996; Evers et al., 1998; Cui et al., 1999; König et al., 1999; Suzuki and Sugiyama, 2000).

The uptake of substances into hepatocytes and the subsequent export into bile can be viewed as a vectorial transport process. This vectorial transport in human liver can be mimicked in an experimental model system using MDCKII cells,

which grow in a polarized fashion with distinct apical and basolateral membrane domains when cultured on an appropriate filter membrane support. MDCKII cells stably transfected with cDNAs encoding a human hepatocyte uptake transporter, expressed in the basolateral membrane domain, and a human hepatocyte export pump, expressed in the apical membrane domain, have been established in recent years (Cui et al., 2001; Sasaki et al., 2002). This is exemplified by the double-transfected MDCKII cell lines ABCC2/OATP1B3 (Cui et al., 2001) and ABCC2/OATP1B1 (Sasaki et al., 2002). Using these double-transfected cell lines, the vectorial transport of established substrates, such as 17 β -glucuronosyl estradiol, bromosulfophthalein (BSP), and leukotriene C₄, was shown (Cui et al., 2001; Sasaki et al., 2002). Cholecystokinin-8 sulfate (CCK-8) was identified recently as a substrate for ABCC2, and vectorial transport of CCK-8 was demonstrated in double-transfected ABCC2/OATP1B3 MDCKII cells (Letschert et al., 2005). These studies demonstrated that such double-transfected polarized cell lines may serve as useful tools for the characterization of the substrate selectivity of transport proteins and for the identification of inhibitors

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ABBREVIATIONS: OATP, organic anion transporting polypeptide; MDCKII, Madin-Darby canine kidney cells, strain II; BSP, bromosulfophthalein; CCK-8, cholecystokinin-8 sulfate; MRP2, multidrug resistance protein 2 (ABCC2).

tors acting on uptake or efflux of substrates (Cui et al., 2001; Sasaki et al., 2002; Letschert et al., 2005). Studies with MDCKII double-transfectants expressing rat *Oatp1b2* together with rat *Abcc2* provided evidence that the in vivo biliary clearance of substances may be predicted by such in vitro systems (Sasaki et al., 2004).

Human hepatocytes simultaneously express three major uptake transporters of the OATP family in basolateral membrane: OATP1B1, OATP1B3, and OATP2B1 (König et al., 2000a,b; Kullak-Ublick et al., 2001; HaGenBuch and Meier, 2004). As an in vitro model for the analysis of uptake and vectorial transport of various organic anions by human hepatocytes, we developed, in this study, an MDCKII cell line expressing the three predominant hepatocellular OATPs together with the apical export pump ABCC2, namely an ABCC2/OATP1B1/1B3/2B1 quadruple-transfected cell line. This cellular model allows for testing of the hepatocellular uptake of compounds and provides an insight into the concerted action of these three uptake transporters with the export pump ABCC2. The efficient intracellular accumulation of a variety of substrates of the three different OATPs in these cells provides information on the substrate selectivity of ABCC2 by measurement of compounds effluxed into the apical chamber of cultured quadruple-transfected MDCKII cells. Inhibitors of ABCC2, entering the cells via one of the three OATPs, may be identified by the intracellular accumulation of a labeled prototypic substrate, as shown earlier with ^3H -labeled BSP in the less advanced system of double-transfected MDCKII cells expressing OATP1B3 and ABCC2 (Cui et al., 2001). In our present study, we demonstrate the functionality of the ABCC2/OATP1B1/1B3/2B1 cell line by using the transport substrates BSP, estrone 3-sulfate, CCK-8, and fluvastatin.

Materials and Methods

Chemicals. [^3H]BSP (0.6 TBq/mmol) and [^3H]fluvastatin (1.55 Ci/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany) by custom synthesis. [^3H]Estrone 3-sulfate (2.12 TBq/mmol) and [^3H]CCK-8 (3.52 TBq/mmol) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Unlabeled fluvastatin was purchased from Sequoia Research Products (Oxford, UK). Unlabeled BSP and estrone 3-sulfate were obtained from Sigma (Taufkirchen, Germany). G418 (Geneticin) disulfate, hygromycin, Zeocin, and blasticidin were from Invitrogen (Groningen, Netherlands).

Antibodies. For detection of ABCC2, the antiserum EAG5 (Büchler et al., 1996; Keppler and Kartenbeck, 1996) and the mouse monoclonal antibody M₂III-6 (Alexis Biochemicals, San Diego, CA) were used. OATP1B1 was detected using the antiserum ESL (König et al., 2000a). OATP1B3 was detected by the antiserum SKT (König et al., 2000b). For detection of OATP2B1, the antiserum SPA was raised in rabbits using a peptide corresponding to the carboxyl-terminal amino acids 688 to 709 of the human OATP2B1 sequence (SPAVEQQLLVSGPGKKPEDSRV; NCBI accession number NP_009187) coupled via an additional N-terminal tyrosine to keyhole limpet hemocyanin (Peptide Specialty Laboratories, Heidelberg, Germany). Horseradish peroxidase-conjugated goat anti-rabbit antibodies were from Bio-Rad (München, Germany). Cy3-conjugated goat anti-mouse IgG was obtained from Jackson Laboratories (West Grove, PA), and Alexa Fluor 488-conjugated goat anti-rabbit IgG was from Molecular Probes (Eugene, OR).

cDNAs Encoding Human ABCC2 and OATP Proteins. The cDNA encoding ABCC2 (GenBank/EMBL accession number

X96395), originally cloned by Büchler et al. (1996) and Cui et al. (1999), was subcloned into the expression vector pcDNA3.1(+) (Invitrogen). The cDNA encoding OATP1B1 (GenBank/EMBL accession number NM_006446), cloned by König et al. (2000a), was subcloned into the expression vector pcDNA3.1/Hygro(−) (Invitrogen). The cDNA encoding OATP1B3 (GenBank/EMBL accession number NM_019844), cloned by König et al. (2000b), was subcloned into the expression vector pcDNA3.1/Zeo(+) (Invitrogen). The cDNA encoding OATP2B1 was cloned from human adult brain by polymerase chain reaction using the following OATP2B1-specific primers (forward, 5'-TCCAGCAGTCATGGGACCCCA-3'; reverse, 5'-CCCAA-GACAGCTCACACTCG-3') based on the original sequence published by Tamai et al. (2000) (GenBank/EMBL accession number NM_007256) and cloned into the vector pCR2.1-TOPO (Invitrogen). The cDNA sequence was 100% identical to the reference sequence (GenBank/EMBL accession number NM_007256) and was subcloned into the expression vectors pcDNA3.1/Hygro(−) and pcDNA6/V5-HisB (Invitrogen).

Generation of Stably Transfected Cell Lines. MDCKII cells were cultured at 37°C and 5% CO₂ in minimum essential medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. As control cells, we used MDCKII cells transfected with the empty vector (Cui et al., 1999). For generation of the ABCC2/OATP2B1 double-transfected MDCKII cells, the ABCC2 single-transfected MDCKII cells (Cui et al., 1999) were transfected with the expression vector pcDNA3.1/Hygro(−) containing the *SLCO2B1* cDNA by the metafectene method (Biontex, München, Germany). Selection was carried out with hygromycin (0.5 mg/ml). Cells of the clone showing the highest expression of ABCC2 and a homogeneous expression of OATP2B1 were designated ABCC2/OATP2B1 double-transfected MDCKII cells and chosen for further studies.

For generation of the quadruple-transfected MDCKII cells, first the ABCC2/OATP1B1 double-transfected MDCKII cells (Fehrenbach et al., 2003) were transfected with the *SLCO1B3* cDNA; selection was carried out with Zeocin (1 mg/ml). Then, the clone stably expressing ABCC2, OATP1B1, and OATP1B3 was transfected with the expression vector pcDNA6/V5-HisB containing the cDNA encoding OATP2B1 and selection was carried out with blasticidin (10 µg/ml). Cells of the clone showing the highest expression of ABCC2 and, in addition, strong and widespread expression of OATP1B1, OATP1B3, and OATP2B1 were designated ABCC2-OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells and used for further studies.

Cell Culture Conditions. For long term cultivation, all MDCKII transfectants were cultured in minimum essential medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin as described above. The vector-transfected MDCKII cells and the ABCC2 single-transfected MDCKII cells were grown in medium supplemented with G418 (1 mg/ml). The ABCC2/OATP1B1, ABCC2/OATP1B3, and ABCC2/OATP2B1 double-transfected MDCKII cells were grown in medium supplemented with G418 (1 mg/ml) and hygromycin (0.5 mg/ml). The ABCC2-OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells were grown in medium that contained Zeocin (0.1 mg/ml), and blasticidin (5 µg/ml) in addition to G418 (1 mg/ml) and hygromycin (0.5 mg/ml). The selection agents were only used during long-term culture but not after seeding of the cells on ThinCert filter membrane supports (see below).

Immunoblot Analysis. Crude membrane fractions from transfected cells were prepared as described previously (Cui et al., 1999). The proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose membranes (GE Healthcare). The antiserum EAG5 for detection of ABCC2 was used at a dilution of 1:5000, the antisera ESL and SKT for detection of OATP1B1 and OATP1B3, respectively, were used at a dilution of 1:8000. The antiserum SPA directed against OATP2B1 was used at a dilution of 1:40,000. Horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum was used at a dilution of 1:3000.

Immunofluorescence Microscopy. Human liver samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Serial cryosections of $6\text{-}\mu\text{m}$ thickness were cut and allowed to dry at room temperature for 30 min. After fixation in phosphate-buffered formaldehyde (10%, w/v) for 10 min, incubation with the primary and secondary antibodies was performed.

MDCKII cells were grown on ThinCerts (diameter, 6 mm; pore size, $0.4\text{ }\mu\text{m}$; pore density, $1 \times 10^8/\text{cm}^2$; Greiner Bio-One, Frickenhausen, Germany) as described previously (Letschert et al., 2005) for 3 days at confluence and induced with 10 mM sodium butyrate for 24 h (Cui et al., 1999). Fixation and permeabilization were performed as described previously (Cui et al., 2001). The ABCC2-specific antibody M₂III-6 was used at a dilution of 1:25, and the antisera ESL and SKT were used at a dilution of 1:50. For the detection of OATP2B1, the antiserum SPA was used at a dilution of 1:200. Both the Cy3-conjugated goat anti-mouse and the Alexa Fluor 488-conjugated goat anti-rabbit IgG were used at a dilution of 1:300. Confocal laser scanning microscopy was performed using a LSM 510 Meta apparatus from Carl Zeiss (Jena, Germany).

Transport Studies. MDCKII cells were grown on ThinCerts (diameter, 24 mm; pore size, $0.4\text{ }\mu\text{m}$; pore density, $1 \times 10^8/\text{cm}^2$) and induced with butyrate as mentioned above. The cells were washed in prewarmed (37°C) transport buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.5 mM CaCl_2 , 5 mM glucose, and 12.5 mM HEPES, pH 7.3). The ^3H -labeled substrate was dissolved in transport buffer and added either to the apical or to the basolateral compartment at the concentration indicated. After incubation at 37°C , the radioactivity in the opposite compartment was measured by sampling aliquots from the apical or basolateral compartment. Cells were washed two times with ice-cold transport buffer containing 0.5% bovine serum albumin and three times in ice-cold transport buffer. Intracellular accumulation of radioactivity was calculated by lysing the cells with 0.2% SDS and measuring the radioactivity in the lysate.

The transcellular leakage was determined by addition of $1\text{ }\mu\text{M}$ [^3H]inulin to the basolateral compartment and measurement of the radioactivity appearing in the apical compartment after an incubation period of 30 min. The transcellular leakage was 1 to 2% of the radioactivity added for all MDCKII cell clones examined in this study.

Transport of estrone 3-sulfate into inside-out membrane vesicles was performed using nick spin columns as described previously (Keppler et al., 1998). Membrane vesicles were prepared from MDCKII vector-transfected control cells and MDCKII-ABCC2 single transfected cells described by Cui et al. (1999) and Letschert et al. (2005). ATP-dependent transport was determined by subtracting values obtained in the presence of $5'\text{-AMP}$ from those in the presence of ATP.

Results

Expression and Localization of Recombinant ABCC2 and OATP Proteins in MDCKII Cells. A scheme of the localization of the four transporters that were expressed in MDCKII cells is shown in Fig. 1. In the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells, the apical export pump ABCC2 and all three basolateral hepatic uptake transporters, OATP1B1, OATP1B3, and OATP2B1, were expressed (Fig. 1C). The levels of ABCC2 and the OATP proteins in the transfected MDCKII cells were analyzed by immunoblotting (Fig. 2). The OATP1B1 protein was detected by the antiserum ESL as two bands with apparent molecular masses of 56 and 84 kDa in the ABCC2/OATP1B1 double-transfected MDCKII cells and in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells. The OATP1B3 protein was present predominantly as fully glycosylated

protein with an apparent molecular mass of 120 kDa in the ABCC2/OATP1B3 double-transfected MDCKII cells and in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells (Fig. 2B). A minor portion of recombinant OATP1B3 was detected with an apparent molecular mass of approximately 84 kDa, which corresponds to its underglycosylated form (König et al., 2000b; Letschert et al., 2004). The OATP2B1 protein was detected with apparent molecular masses of approximately 84 and 56 kDa in the ABCC2/OATP2B1 double-transfected MDCKII cells and in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells (Fig. 2C). OATP2B1 seemed to be more strongly expressed in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells than in the ABCC2/OATP2B1 double-transfected MDCKII cells. ABCC2 was strongly expressed in all transfectants except MDCKII vector-transfected control

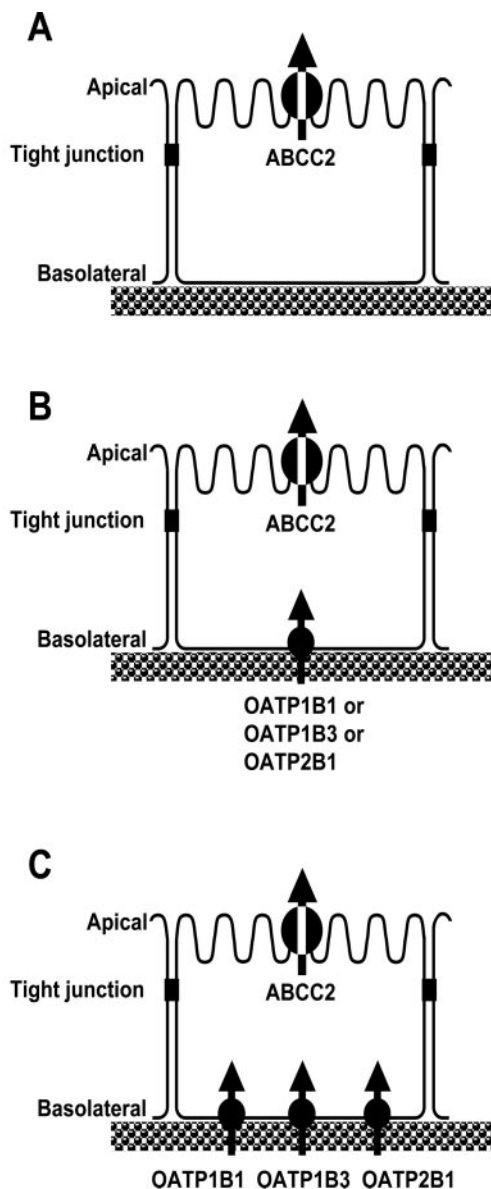


Fig. 1. Scheme of MDCKII cells stably expressing recombinant ABCC2 and OATP proteins. ABCC2 single-transfectant (A), double-transfectants expressing ABCC2 together with OATP1B1 or OATP1B3 or OATP2B1 (B), and quadruple-transfectant expressing ABCC2 together with OATP1B1, OATP1B3, and OATP2B1 (C).

cells (Fig. 2D). In the vector-transfected control cells, none of the four transport proteins was detectable.

The cellular localization of the recombinant transporter proteins in the MDCKII transfectants and in human liver was studied by confocal laser scanning immunofluorescence microscopy (Fig. 3). Using the monoclonal antibody M₂III-6 directed against ABCC2 (red fluorescence), we performed a costaining with the OATP proteins (green fluorescence) in the MDCKII transfectants and in human liver cryosections. In human liver, ABCC2 was localized to the bile canaliculi representing the apical domain of the hepatocyte membrane (Fig. 3, A–C), whereas OATP1B1 (Fig. 3A), OATP1B3 (Fig. 3B), and OATP2B1 (Fig. 3C) were localized to the basolateral (sinusoidal) membrane domain of the hepatocytes.

ABCC2 was localized exclusively to the apical membrane domain of the ABCC2/OATP1B1, ABCC2/OATP1B3, and ABCC2/OATP2B1 double-transfected MDCKII cells, as well as of the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells, whereas the OATP proteins were localized to the basolateral membrane (Fig. 3, D–O). The strict difference in domain-specific localization was particularly evident in the vertical sections of the cells by the lack of colocalization of ABCC2 with OATP1B1 (Fig. 3, G and M), OATP1B3 (Fig. 3, H and N), or OATP2B1 (Fig. 3, I and O). The percentage of quadruple-transfected MDCKII cells staining positive for transporter proteins detected by confocal laser scanning im-

munofluorescence microscopy amounted to 45% for ABCC2, 82% for OATP1B1, 96% for OATP1B3, and up to 100% for OATP2B1, based on counting of at least 700 cells per clone.

Transcellular Transport of Organic Anions by ABCC2/OATP1B1/1B3/2B1 Quadruple-transfected MDCKII Cells. The transport properties were compared among the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells, double-transfected ABCC2/OATP1B1, ABCC2/OATP1B3, or ABCC2/OATP2B1 MDCKII cells, single-transfected ABCC2 MDCKII cells, and vector-transfected control cells. [³H]BSP was used as model compound because it is an established substrate for ABCC2 (Cui et al., 2001), OATP1B1, OATP1B3 (König et al., 2000b), and OATP2B1 (Kullak-Ublick et al., 2001). The transcellular transport of [³H]BSP as a vectorial process in the MDCKII transfectants is shown in Fig. 4. The ABCC2 single-transfected MDCKII cells showed no significant transport compared with the vector-transfected control cells. In the ABCC2/OATP1B1, ABCC2/OATP1B3, ABCC2/OATP2B1 double-transfected MDCKII cells, and in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells, a basolateral to apical transport of [³H]BSP was observed, whereas the respective apical to basolateral transport was negligible. We compared the transcellular transport rates and intracellular accumulation of [³H]BSP at two substrate concentrations (Fig. 5). At 100 nM [³H]BSP, an intracellular [³H]BSP accumulation was observed and only minimal transcellular transport was detected in the double-transfected MDCKII cells (Fig. 5, A and B). The transcellular transport as well as the intracellular accumulation was highest in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells. Using [³H]BSP at a concentration of 5 μ M, that is in the range of the *K_m* of OATP2B1 for [³H]BSP (Kullak-Ublick et al., 2001) and ABCC2 (Cui et al., 2001), all three double-transfected MDCKII cell lines showed transcellular [³H]BSP transport, whereas the highest transcellular [³H]BSP transport was seen with the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells (Fig. 5, C and D). [³H]CCK-8 was tested as a substrate because it is considered a selective substrate of OATP1B3 (Ismair et al., 2001) and a substrate of ABCC2 (Letschert et al., 2005). [³H]CCK-8 was efficiently transported transcellularly by the ABCC2/OATP1B3 double-transfected MDCKII cells and by the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells (Fig. 6). Only a minor portion of the [³H]CCK-8 taken up by the cells was retained intracellularly because the efficient apical export via ABCC2.

Vectorial transport of [³H]estrone 3-sulfate was detected in all three double-transfected MDCKII cells and in ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells (Fig. 6, C and D), suggesting that [³H]estrone 3-sulfate is not only a substrate for OATP1B1, OATP1B3, and OATP2B1, but also for ABCC2. The transport of [³H]estrone 3-sulfate by ABCC2 was confirmed by transport studies using membrane vesicles prepared from MDCKII-ABCC2 single transfectants (Fig. 7). The ABCC2-mediated [³H]estrone 3-sulfate transport in the presence of ATP was 3.1-fold enhanced compared with controls.

[³H]Fluvastatin was accumulated intracellularly in the double-transfected cells and in ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells (Fig. 6F). However, no significant vectorial transport of [³H]fluvastatin was observed in the transfectants compared with the control cells. (Fig. 6E).

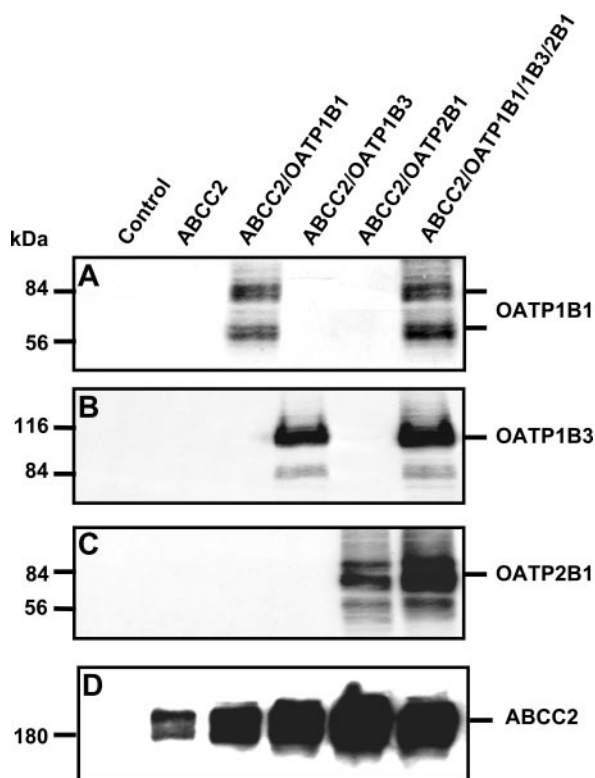


Fig. 2. Immunoblot of ABCC2, OATP1B1, OATP1B3, and OATP2B1 proteins in stably transfected MDCKII cells. Crude membrane fractions from MDCKII cells transfected with the control vector, with ABCC2, with ABCC2 and OATP1B1, with ABCC2 and OATP1B3, with ABCC2 and OATP2B1, and with ABCC2 together with OATP1B1, OATP1B3, and OATP2B1 cDNA (quadruple-transfectant) were separated by SDS-PAGE. OATP1B1 was detected by the antiserum ESL (König et al., 2000a) (A), OATP1B3 was detected by the antiserum SKT (König et al., 2000b) (B), OATP2B1 by the antiserum SPA (C), and ABCC2 by the antiserum EAG5 (Schaub et al., 1999) (D) (see *Materials and Methods*).

The kinetic constants for the intracellular accumulation of [^3H]BSP and [^3H]fluvastatin are summarized in Table 1. [^3H]Fluvastatin was transported with high affinity by all three OATPs. OATP2B1, with a K_m value of $0.7 \pm 0.3 \mu\text{M}$ showed the highest affinity for [^3H]fluvastatin relative to OATP1B1 (K_m $2.4 \pm 0.9 \mu\text{M}$) and OATP1B3 (K_m $7.0 \pm 2.5 \mu\text{M}$). Coexpression of all three OATP transporters in the quadruple-transfected MDCKII cells resulted in an enhanced transport capacity indicated by the V_{max} values, but the substrate affinity was in the range of the separate OATPs (Table 1). The K_m value of $4.8 \pm 0.2 \mu\text{M}$ for [^3H]BSP determined in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells was also in the same range as the K_m determined in the respective double-transfected MDCKII cells (Table 1).

Discussion

In the present study, we established a quadruple-transfected MDCKII cell line stably expressing the uptake transporters OATP1B1, OATP1B3, and OATP2B1, together with the efflux pump ABCC2. As shown by immunoblot analysis, all four transport proteins were expressed at high levels

in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected MDCKII cells (Fig. 2). Confocal laser scanning immunofluorescence microscopy indicated the basolateral localization of OATP1B1 and OATP1B3 and the apical localization of ABCC2 in the MDCKII transfectants, thus confirming previous localization studies (Evers et al., 1998, König et al., 2000a, Cui et al., 2001, Sasaki et al., 2002). MDCKII cells stably expressing OATP2B1 are novel, and this protein was localized to the basolateral membrane both in the ABCC2/OATP2B1 double-transfectants and in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected cells.

By using BSP as a common substrate, we demonstrate that all recombinantly expressed transporters in the quadruple-transfected cells are functionally active in transcellular BSP transport (Fig. 5). The rates of transcellular BSP transport by the double-transfected MDCKII cell lines ABCC2/OATP1B1, ABCC2/OATP1B3, and ABCC2/OATP2B1 were relatively low at 100 nM BSP; however, an increased intracellular accumulation of BSP was measurable even at this low substrate concentration (Fig. 5), probably as a result of the lower affinity of BSP for ABCC2 (K_m 12 μM ; Cui et al., 2001) compared with the OATPs (K_m values $<5 \mu\text{M}$; Table 1).

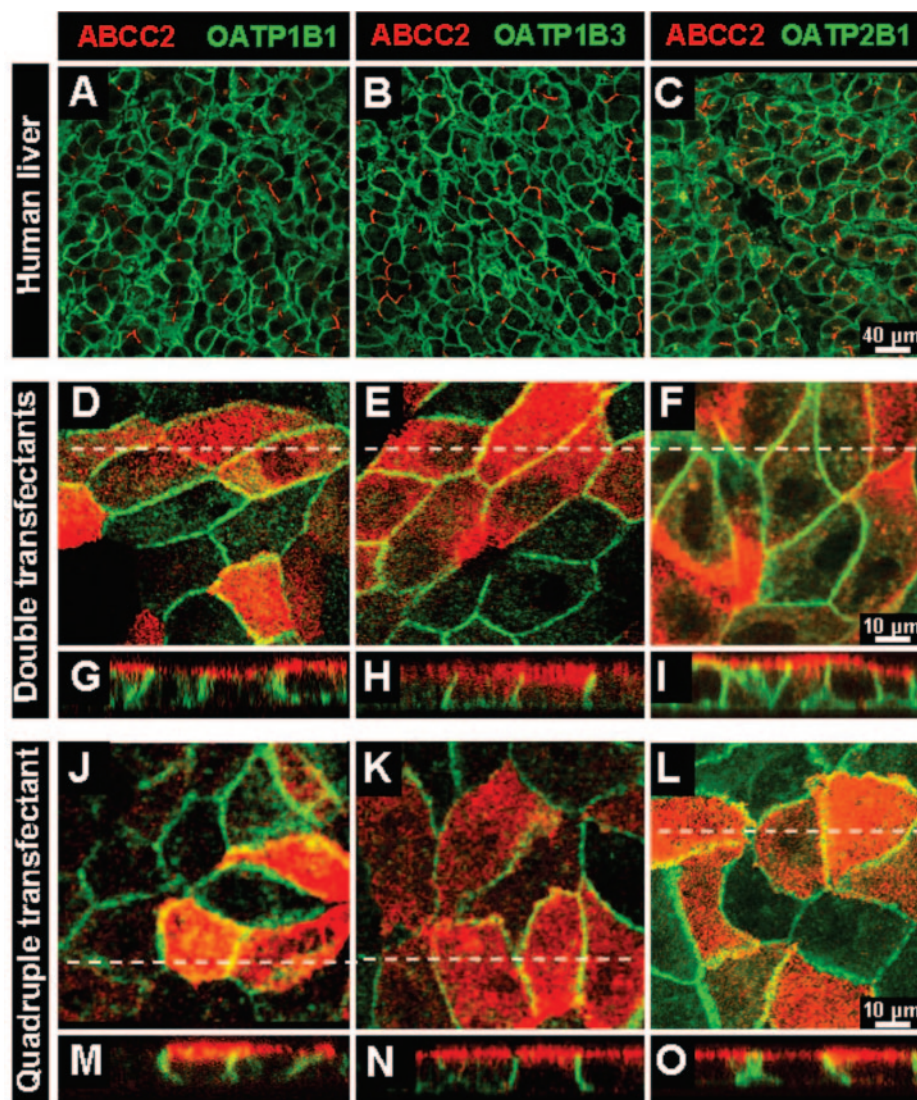


Fig. 3. Immunolocalization of ABCC2 and OATP proteins in human liver (A–C) and in transfected MDCKII cells (D–O) by confocal laser scanning immunofluorescence microscopy. Human liver cryosections as well as MDCKII transfectants grown on cell culture inserts were fixed with 10% and 2% formaldehyde solution, respectively. ABCC2 (red fluorescence) was detected with the monoclonal antibody M₂III-6. The OATP proteins are shown by the green fluorescence. OATP1B1 (A, D, G, J, and M) was detected by the antiserum ESL (König et al., 2000a). OATP1B3 (B, E, H, K, and N) was detected by the antiserum SKT (König et al., 2000b). OATP2B1 (C, F, I, L, and O) was detected by the antiserum SPA (see *Materials and Methods*). D, E, F, J, K, and L are en face images at the top of the cell monolayers. G, H, I, M, N, and O are vertical sections through the cell monolayers at positions indicated by the broken white lines. In human liver, ABCC2 is localized to the bile canaliculi of hepatocytes (A–C), whereas OATP1B1, OATP1B3, and OATP2B1 are localized to the basolateral membrane of hepatocytes. Likewise, in the MDCKII transfectants, ABCC2 is localized apically and the OATP proteins are localized basolaterally.

Compared with the double-transfectants, the ABCC2/OATP1B1/1B3/2B1 quadruple-transfectants showed a significantly increased intracellular accumulation and transcellular transport even at 100 nM BSP. The transcellular transport was also highest in the quadruple-transfected cells at 5 μ M BSP compared with any of the double-transfected MDCKII cells (Fig. 5). The export of BSP via ABCC2 may depend on a threshold concentration of intracellularly accumulated BSP after uptake via the OATP proteins. If three OATP proteins act together in one cell to accumulate BSP intracellularly, as is the case in the quadruple-transfectants, this threshold concentration of intracellular BSP is achieved earlier. Moreover, transcellular transport of CCK-8 was examined in the MDCKII transfectants. Both ABCC2/OATP1B3 double-transfected MDCKII cells and ABCC2/OATP1B1/1B3/2B1 quadruple-transfectants transported CCK-8 (Fig. 6) but not the ABCC2/OATP1B1 and ABCC2/OATP2B1 double-transfectants. Our data confirm that CCK-8 is a selective substrate for OATP1B3 with a K_m value of 11.1 μ M (Ismair et al., 2001) and a substrate for ABCC2 with a K_m value of 8.1 μ M (Letschert et al., 2005). These results also demonstrate that the transcellular transport of a compound that is a selective substrate for only one of the OATP proteins will be detected by the ABCC2/OATP1B1/1B3/2B1 quadruple-transfectants, whereas only one of the

three double-transfectants would show a significant transcellular transport. Because the affinities of OATP1B3 and ABCC2 for CCK-8 are in a similar range, the cellular uptake of CCK-8 and export via ABCC2 proceeded equally well (Letschert et al., 2005). We observed that estrone 3-sulfate, an established substrate for OATP1B1, OATP1B3, and OATP2B1 (Kullak-Ublick et al., 2001), is transported transcellularly by the double-transfectants and by the quadruple-transfectants (Fig. 6). The affinity to estrone 3-sulfate is higher for OATP1B1 than for OATP2B1 (Tamai et al., 2000). This is in line with the observation that the uptake rate of 1 μ M estrone 3-sulfate in the ABCC2/OATP1B1 double-transfectants is higher than in the ABCC2/OATP1B3 or the ABCC2/OATP2B1 double-transfectants (Fig. 6). Estrone 3-sulfate is also a substrate for ABCC2, as evidenced by its vectorial transport (Fig. 6) and by recent data on transcellular estrone 3-sulfate transport in LLC-PK1 cells double-transfected with OATP1B1 and ABCC2 (Spears et al., 2005). Moreover, we demonstrated directly by membrane vesicle studies that estrone 3-sulfate is a substrate for ABCC2 (Fig. 7).

In the present study, fluvastatin was identified as a substrate for OATP1B1, OATP1B3, and OATP2B1. The highest affinity was determined with the ABCC2/OATP2B1 double-transfectants (K_m 0.7 μ M), suggesting that OATP2B1 plays an important role in statin uptake into hepatocytes. The ABCC2/OATP1B1/1B3/2B1 quadruple-transfectants trans-

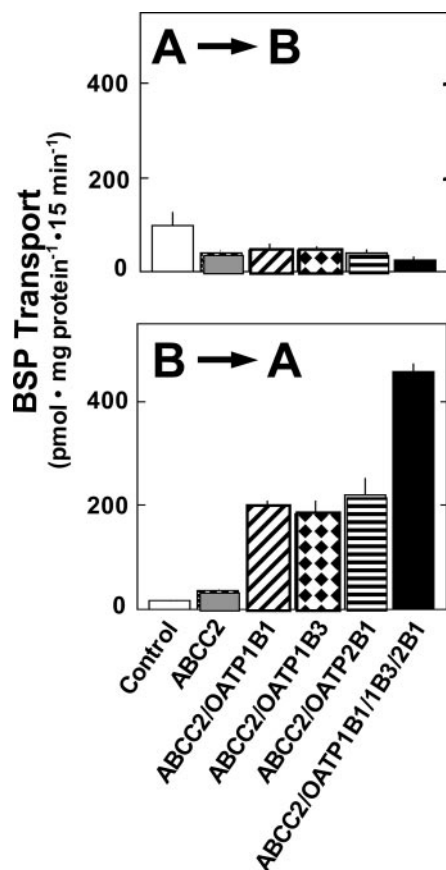


Fig. 4. Vectorial transport of [3 H]BSP. The vector-transfected MDCKII control cells and all transporter-transfected cells listed were grown on cell culture inserts (see *Materials and Methods*). [3 H]BSP (5 μ M) was either added to the apical compartment (A) or to the basolateral compartment (B). After 15 min, the radioactivity in the opposite compartment was measured. Data represent means \pm S.D. determined from three experiments, each performed in triplicate.

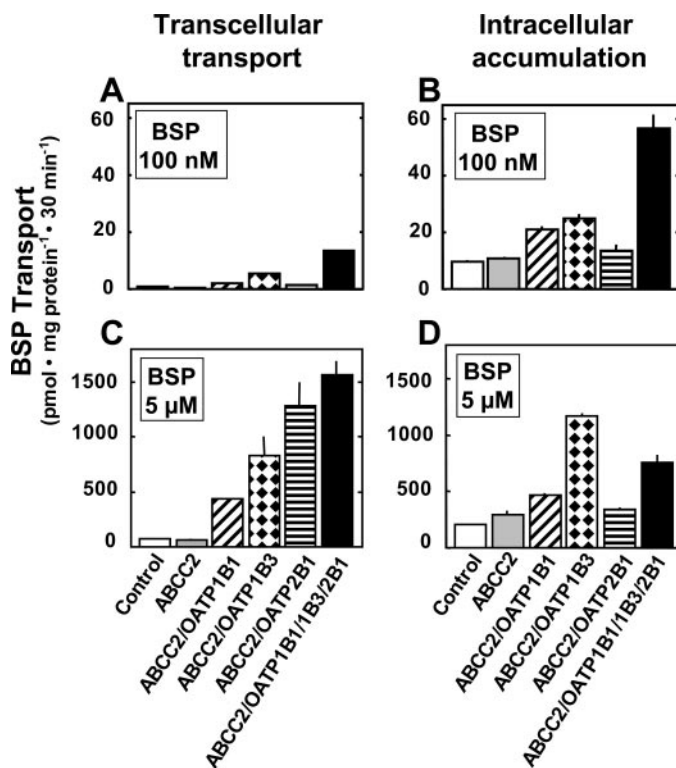


Fig. 5. Transport of [3 H]BSP. The vector-transfected MDCKII control cells and the transporter-transfected cells listed were grown as described under *Materials and Methods*, and incubated with 100 nM [3 H]BSP (A and B) or 5 μ M [3 H]BSP (C and D) in the basolateral chamber. After incubation for 30 min, radioactivity determined in the apical chamber was used to calculate the transcellular transport (A and C). Intracellular substrate accumulation was calculated after lysing the cells (B and D). Data represent means \pm S.D. determined from three experiments, each performed in triplicate.

ported fluvastatin with a K_m value of 2.3 μM (Table 1). In addition, the most efficient uptake and intracellular accumulation were observed in the quadruple transfectants. A vectorial transport of fluvastatin into the apical compartment could not be detected, suggesting that fluvastatin is not a substrate for ABCC2. This conclusion was supported by studies using inside-out membrane vesicles from MDCKII-

ABCC2 single-transfected cells (data not shown). Most of the statins acting as inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, including fluvastatin, act in hepatocytes and are metabolized there in part, followed by excretion into bile (Tse et al., 1995). Because we did not observe a significant transcellular transport of fluvastatin itself in the ABCC2/OATP1B1/1B3/2B1 quadruple-transfectants, either one of its metabolites may be transported by ABCC2 or other ATP-dependent efflux pumps in the apical membrane may be responsible for the in vivo efflux of fluvastatin into bile.

Kinetic analyses of the 5-min uptake transport of BSP by ABCC2/OATP1B1, ABCC2/OATP1B3, and ABCC2/OATP2B1 double-transfectants indicated K_m values for BSP of 2.4, 2.2, and 3.4 μM , respectively (Table 1). This confirms that these three OATP proteins have a relatively high affinity for BSP. These K_m values determined in the double-transfectants were somewhat higher than the values reported previously for OATP1B1-, OATP1B3-, and OATP2B1-mediated transport of BSP in cRNA-injected *Xenopus laevis* oocytes (0.3, 0.4, and 0.7 μM) (Kullak-Ublick et al., 2001). In the ABCC2/OATP1B1/1B3/2B1 quadruple-transfectants, BSP was transported with a K_m value of 4.8 μM , but the maximal velocity was elevated to 197.8 pmol/mg protein/min which is markedly above the maximal velocity of the double-transfectants (Table 1).

The novel quadruple-transfected MDCKII cells are thus useful in studies on the transport properties of ABCC2, OATP1B1, OATP1B3, and OATP2B1. Common substrates of the three uptake transporters, such as BSP and fluvastatin, are efficiently taken up into the quadruple-transfectants. Specific substrates for one of the uptake transporters, as demonstrated for CCK-8 and OATP1B3, are easily detected

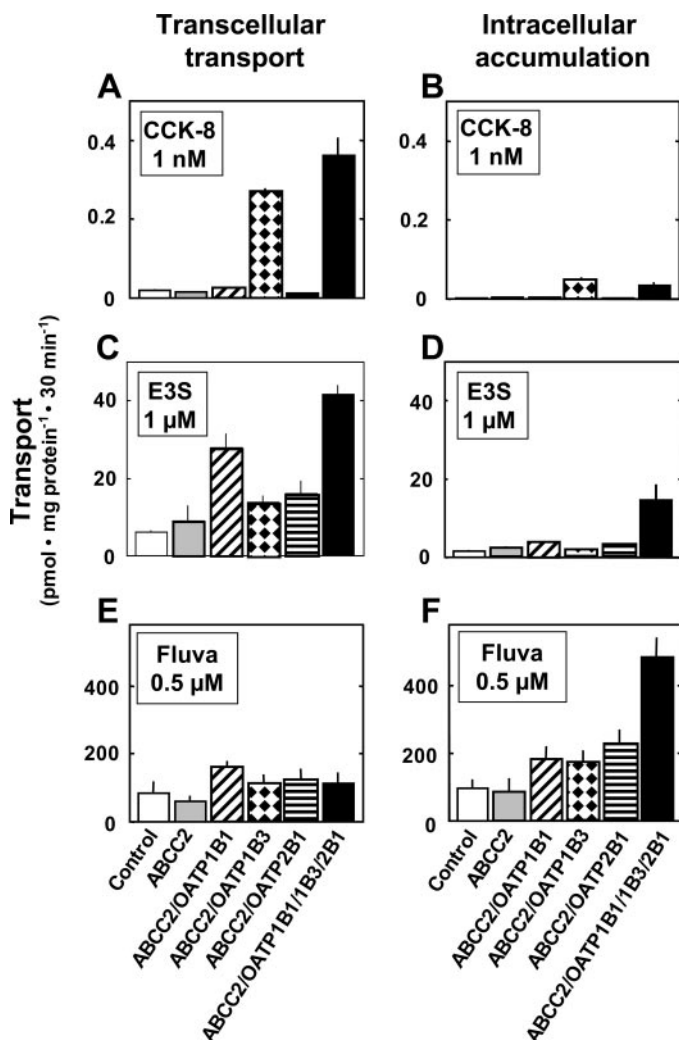


Fig. 6. Transport of [^3H]CCK-8, [^3H]estrone 3-sulfate (E3S) and [^3H]fluvastatin (Fluva). Cells were grown as described under *Materials and Methods* and incubated with 1 nM [^3H]CCK-8 (A and B), 1 μM [^3H]E3S (C and D) or 0.5 μM [^3H]Fluva (E and F). Transcellular transport (A, C, and E) and intracellular substrate accumulation (B, D, and F) were calculated as means \pm S.D. determined from three to seven experiments, each performed in triplicate.

TABLE 1

Kinetic constants for uptake of [^3H]BSP and [^3H]fluvastatin into cells stably expressing ABCC2 together with OATP1B1 or OATP1B3 or OATP2B1 and by the quadruple-transfectant expressing ABCC2 together with OATP1B1, OATP1B3, and OATP2B1

Uptake transport was measured over a 5-min period (see *Materials and Methods*) and based on the sum of intracellular radioactivity and the small amount, in the case of BSP, of radioactivity in the apical chamber. Data represent means \pm S.E. determined from 5 to 15 experiments, each performed in triplicate.

Cell line	Bromosulphophthalein		Fluvastatin	
	K_m	V_{max}	K_m	V_{max}
	μM	pmol/mg protein/min	μM	pmol/mg protein/min
ABCC2-OATP1B1	2.4 ± 0.7	41.5 ± 5.2	2.4 ± 0.9	43.4 ± 6.7
ABCC2-OATP1B3	2.2 ± 0.3	82.7 ± 4.0	7.0 ± 2.5	53.6 ± 11.0
ABCC2-OATP2B1	3.4 ± 1.2	52.8 ± 8.6	0.7 ± 0.3	23.0 ± 2.6
ABCC2-OATP1B1/1B3/2B1	4.8 ± 0.2	197.8 ± 3.0	2.3 ± 0.4	93.9 ± 7.4

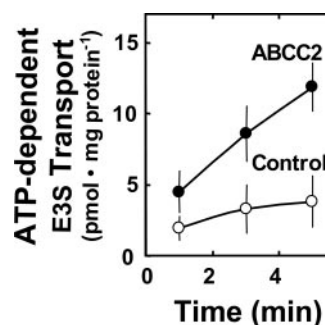


Fig. 7. Transport of [^3H]estrone 3-sulfate (E3S) by ABCC2. ATP-dependent transport into inside-out membrane vesicles prepared from MDCKII-ABCC2 single-transfectants (ABCC2, \bullet) or vector-transfected MDCKII cells (Control, \circ) was measured for 1, 3, and 5 min as described under *Materials and Methods*. Data are means \pm S.D. determined from three measurements.

by the ABCC2/OATP1B1/1B3/2B1 quadruple-transfected cells. These cells may be useful in the screening of drugs and drug candidates with respect to their substrate and inhibitor properties in hepatocellular uptake and ABCC2-mediated efflux into bile.

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