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Transfected Rat cMOAT Is Functionally Expressed on the Apical Membrane in Madin-Darby Canine Kidney (MDCK) Cells

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Purpose. The purpose of the present study is to investigate the expression of canalicular multispecific organic anion transporter (cMOAT) by its cDNA transfection in polarized Madin-Darby canine kidney cells (MDCK).

Methods. MDCK cells were transfected with an expression vector (pCXN2) containing the rat cMOAT cDNA with lipofectamine to obtain the stable transfectant under G418. Cells from a single colony whose cMOAT expression was the highest were seeded to form a tight epithelial monolayer on microporous membrane filters. Export of glutathione S-bimane (GS-B) from monolayers was determined after preloading its precursor, monochloro bimane (MCB).

Results. A comparable amount of GS-B was excreted to the apical and basal compartments in the vector-transfected cells. In contrast, in cMOAT-transfected cells, the amount apically excreted was approximately twice that excreted into the basal compartment. Cyclosporin A (CsA) (30μM), an inhibitor of cMOAT at higher concentrations, inhibited the preferential apical export of GS-B from cMOAT-transfected cells.

Conclusions. Rat cMOAT is functionally expressed on the apical membrane of MDCK cells after transfection.

KEY WORDS: cMOAT; MRP; GS-X pump; multidrug resistance; glutathione S-bimane; polarized monolayer.

INTRODUCTION

It is well established that the excretion of organic anions from hepatocytes into bile is mediated by a primary active transporter, referred to as the canalicular multispecific organic anion transporter (cMOAT) (1–4). The substrate specificity of cMOAT has been clarified by examining the transport properties across the bile canalicular membrane in normal and mutant rats (e.g., GY, TR^- and Eisai hyperbilirubinemic rats (EHBR)) whose cMOAT function is hereditarily defective; the substrates for cMOAT include glutathione conjugates (e.g., leukotriene C_4 (LTC_4), glutathione disulfide and 2,4-dinitrophenyl-S-glutathione (DNP-SG)), glucuronide conjugates (e.g., bilirubin glucuronide) and anionic xenobiotics including a number of clinically important drugs (e.g., CPT11 and its metabolites (5,6), methotrexate (7) and temocaprilat (8)). In addition, these mutant rats exhibit hyperbilirubinemia due to defective biliary excretion of bilirubin glucuronide and they have been used as animal models to study the pathogenesis of Dubin-Johnson syndrome found in humans (1–4). Recently, cDNA for cMOAT has been cloned in rats and humans as a homologue of multidrug resistance associated protein (MRP1), a primary active transporter whose substrate specificity is similar to that of cMOAT (9–13). Because the cloned gene product is expressed on the bile canalicular membrane and its expression is defective in mutant rats (TR^- and EHBR) and in patients suffering from Dubin-Johnson syndrome, it has been concluded that this cDNA encodes the cMOAT (3,9–10,12,14–16). The function of this cloned cDNA product has been clarified by examining the export of DNP-SG from cRNA-injected Xenopus *laevis* oocytes and cDNA transfected COS-7 cells (13). We have also demonstrated that the membrane vesicles isolated from NIH/3T3 cells stably expressing rat cMOAT have the ability to transport glutathione conjugates (e.g., LTC_4 and DNP-SG) in an ATP-dependent manner (17). Moreover, the function of cMOAT has been examined by transfecting the antisense cDNA to the cells stably expressing cMOAT (18). Since similarity in the substrate specificity and in molecular structure have been clarified between cDNA products of cMOAT and MRP1, cMOAT is also referred to as MRP2 (3).

However, until now there has been no information on the expression of transfected cMOAT product in polarized cells. Under physiological conditions, cMOAT and MRP1 are located on the apical and basal membrane of hepatocytes, respectively (3), so the expression of these transporters in the polarized epithelium needs to be compared. In the present study, we examined the expression of cMOAT in polarized renal epithelial cell lines in order to compare its localization with that of MRP1 which has been shown to be localized in the basolateral membrane of a porcine kidney epithelial cell line ($LLC\text{-}PK_1$) after transfection (19). Although one of the best methods to examine the role of transfected cMOAT on polarized cells is to examine the transcellular transport of its substrates, it may be that the cellular uptake of cMOAT substrates is restricted. In order to circumvent this problem, Evers *et al.* (19) had examined the export of 2,4-dinitrophenyl-S-glutathione, a typical MRP1/cMOAT substrate, after preloading MRP1-transfected $LLC\text{-}PK_1$ cells with its hydrophobic precursor ([^{14}C]1-chloro-2,4-dinitrobenzene). In the present study, we examined the export of glutathione S-bimane (GS-B), a fluorescent cMOAT substrate, after preloading the cells with its non-fluorescent hydrophobic precursor (monochloro bimane; MCB) (20). Due to its high hydrophobicity, MCB is easily transported across the plasma membrane via passive diffusion and converted intracellularly to GS-B with the aid of glutathione-S-transferase (20).

METHODS

Materials

pCXN2 mammalian expression vector (21) was donated by Dr. J. Miyazaki, Osaka University School of Medicine. cMOAT cDNA with the shortest 3′-UTR length in the pBluescript II SK(-) vector described previously (12) was excised with EcoR I and inserted into the EcoR I site in pCXN2 (17). Polyethylene terephthalate cell culture inserts (0.31 cm^2 and 3.0 μm pore size) were purchased from Becton Dickinson

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Labware (Franklin Lakes, NJ). MCB was purchased from Molecular Probes, Inc. (Eugene, OR). [glycine-2-^{3}H]Glutathione was purchased from New England Nuclear (Boston, MA). Glutathione-S-transferase was purchased from Sigma Chemical (St. Louis, MO). All other chemicals were commercially available, of reagent grade and used without further purification.

Preparation of the Transfected Cell Line

MDCK and LLC-PK$_1$ cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown routinely at 37°C in Dulbecco's modified Eagle's medium (low glucose) containing 5% fetal bovine serum under 5% CO_2/95% air atmosphere. The MDCK and LLC-PK$_1$ cells, transfected with pCXN2 vector with or without cMOAT cDNA by Lipofectamine (Life Technologies, Rockville, MD), were maintained in the presence of 800 μg/ml Geneticin (Life Technologies) to obtain the colonies. The significant expression of cMOAT was observed in five and six colonies from twenty MDCK and eighteen LLC-PK$_1$ colonies, respectively. In the transport experiments, the clone with the highest cMOAT expression was used.

Northern Blot Analysis

Northern hybridization was performed as described previously (12). Briefly, RNA isolated from the MDCK cell lines was separated on 0.7% agarose gel containing 3.7% formaldehyde and transferred to a nylon membrane (Biodyne, Pall Corporation, Glen Cove, NY), prior to fixation by baking for 2 h at 80°C. Blots were prehybridized in hybridization buffer containing 4 × SSC, 5 × Denhardt's solution, 0.2% sodium dodecyl sulfate (SDS), 0.1 mg/ml sonicated salmon sperm DNA and 50% formamide at 42°C for 2 h. Hybridization was performed overnight in the same buffer containing 10^6 cpm/ml [^{32}P]labeled cDNA probes including the carboxy-terminal ABC region of rat cMOAT (22) prepared by a random primed labeling method (Rediprime, Amersham International, Ltd.). The hybridized membrane was washed in 2 × SSC and 0.1% SDS at room temperature for 20 min. Filters were exposed to autoradiography film (Eastman Kodak Company, Rochester, NY) for 48 h at room temperature.

Transport Studies

Cells were seeded on Cell Culture Inserts at a density of 2.8 × 10^4 cells per well and cultured for 3 days to allow them to form a polarized monolayer. The medium used in the transport study was composed of 122 mM NaCl, 3 mM KCl, 0.4 mM K_2HPO_4, 25 mM $NaHCO_3$, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesufonic acid (HEPES), 10 mM glucose, pH 7.4. Then 250 μl and 950 μl of this medium were added to the apical and basal compartments of the monolayer, respectively. In order to determine the time-dependent export of GS-B, the cells were preincubated for 20 min with the medium containing 20 μM MCB. At time zero, the medium was replaced by fresh medium containing 20 μM MCB. To determine the effect of CsA, cells were preincubated for 20 min with medium containing 30 μM CsA. At time zero, the medium was replaced by fresh medium containing 20 μM MCB and 30 μM CsA. At designated times, 100 μl aliquots of medium were collected from the apical and basal compartments and replaced by equivalent volumes of fresh medium with the same composition. At the end of the experiment, the cells were solubilized by adding 500 μl 0.1% SDS. The previously described specimens were diluted with 2 ml 10 mM phosphate buffer (pH 6.5) to determine the concentration of GS-B by measuring the fluorescence (Ex 386 nm; Em 476 nm). Since the fluorescence of MCB is much lower than that of GS-B, MCB in the specimens did not interfere with the determination of GS-B. The amount of cellular protein was determined by the Lowry method (23) using bovine serum albumin as a standard.

Preparation of Labeled or Unlabeled GS-B

[^{3}H]GS-B was synthesized enzymatically from [glycine-2-^{3}H]glutathione and MCB using glutathione S-transferase from equine liver. Dithiothreitol was removed from the [glycine-2-^{3}H]glutathione solution by extraction with a 10-fold excess of ethyl acetate. This specimen was then adjusted to pH 2 by the addition of 2 M HCl. Extracted [glycine-2-^{3}H]glutathione was reacted with excess MCB in 50 mM potassium phosphate buffer, pH 6.5, at 37°C in the presence of 30 μg/ml of glutathione S-transferase for 60 min. [^{3}H]GS-B was purified from residues using high performance liquid chromatography (HPLC) with an ODS2 column (LiChrosorb RP-18, ø 4.6 × 250 mm, GL Science, Tokyo, Japan). Mobile phase consisted of CH_3CN and H_2O containing 0.1% CF_3COOH (13:87) was pumored through the column. [^{3}H]GS-B elution was detected by UV detection, at 386 nm (purity > 99.5%). Unlabeled GS-B was synthesized using the same principle and also purified using HPLC. Fluorescence quantum yield of unlabeled GS-B was determined from the fluorescence intensity associated with [^{3}H]GS-B solution whose concentration was determined based on its specific radioactivity.

RESULTS

Northern Blot Analysis of Rat cMOAT

The expression of rat cMOAT in the cells was confirmed by Northern blot analysis. As shown in Figure 1, rat cMOAT probe hybridized to the MDCK cells transfected with a vector

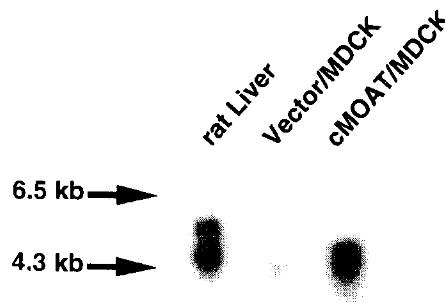

Fig. 1. Northern blot analysis of the expression of cMOAT. Expression of cMOAT in the transfected MDCK cells was studied by northern blot analysis. Four micrograms of poly A$^+$ RNA from SD rat liver, vector- and cMOAT-transfected MDCK cells were subjected to the analysis. The autoradiographs probed with [^{32}P]labeled cDNA including the carboxy-terminal ABC region of rat cMOAT had 48 h exposure at room temperature to a autoradiography film.

containing rat cMOAT cDNA. However the probe hybridized weakly to those transfected with the vector. The weak band may represent the endogenous cMOAT in MDCK cells. The length of the transcript in cMOAT-transfected MDCK cells was comparable with the shortest band (5 kb) observed in SD rat liver (12).

Export of GS-B from Transfected MDCK Monolayer

The export of GS-B from monolayers of vector- and cMOAT-transfected MDCK cells was examined by incubating them with MCB. The amount of GS-B excreted to the apical and basal compartments of the vector-transfected cell monolayer was comparable (Fig. 2A). In contrast, in the cMOAT-transfected MDCK cell monolayer, the amount of GS-B excreted to the apical compartment was 2.2-fold higher than that to the basal compartment (Fig. 2A). Although the export of GS-B into the basal compartment was reduced by cMOAT-transfection, this reduction could be accounted for by the reduced intracellular concentration of GS-B as a result of cMOAT transfection; at 12 min, the amount of GS-B associated with the cells was 1.32 ± 0.09 and 0.37 ± 0.03 nmol/mg protein for the vector- and cMOAT-transfected cells, respectively. Although the amount of intracellulary formed GS-B, calculated as the sum of the amount of GS-B excreted into the medium and that remaining in the cells, was reduced somewhat by cMOAT transfection (3.03 ± 0.13 vs 2.05 ± 0.09 nmol/mg protein up to 12 min), the reduced intracellular GS-B in cMOAT-transfected cells can largely be accounted for by the increased export. The intrinsic activity of the penetration of GS-B across the apical and basal membranes was estimated by normalizing the amount of GS-B excreted into the medium with respect to that remaining in the cells (Fig. 2B). The normalization revealed that cMOAT transfection resulted in an increase in the export of GS-B across the apical membrane with a much lesser effect on the penetration across the basolateral membrane (Fig. 2B).

In contrast, cMOAT transfection did not affect the export of GS-B from LLC-PK_1 cells; up to 12 min, 50.1 ± 1.6 and 69.9 ± 8.2 pmol/mg protein of GS-B were excreted to the apical and basal compartments of vector-transfected LLC-PK_1 cells, and 47.4 ± 2.1 and 46.4 ± 3.9 pmol/mg protein of GS-B were excreted to the respective compartments of cMOAT-transfected LLC-PK_1 cells.

Effect of CsA on the Export of GS-B

To characterize the export of GS-B, the effect of CsA was examined. In this experiment, the amount of GS-B excreted into the basal compartment was not affected by cMOAT transfection (Fig. 3A); this differed from the observations illustrated in Figure 2. This difference may be accounted for by a difference in the disposition of GS-B among the cell populations used in the present study. The amount of GS-B remaining in the cMOAT-transfected cells was significantly increased by CsA (Fig. 3B), suggesting that the cMOAT-mediated efflux of GS-B was inhibited by CsA. CsA (30 μM) reduced the preferential apical efflux of GS-B from cMOAT-transfected cells (Fig. 3C). CsA did not affect the intracellular formation of GS-B in the cMOAT-transfected cells, since the sum of the amount remaining in the cells and that released into the medium was comparable for control and CsA-treated cells (1.52 ± 0.05 vs 1.64 ± 0.07 nmol/mg protein/12 min).

DISCUSSION

In the present study, we investigated the function of rat cMOAT by examining the transport of GS-B, a typical substrate for cMOAT, in cDNA transfected MDCK cells. The CsA-sensitive export of GS-B to the apical compartment was enhanced by transfection of cMOAT cDNA, suggesting that the function of transfected cMOAT is expressed on the apical membrane in MDCK cells (Fig. 2.). Although genistein, probenecid and

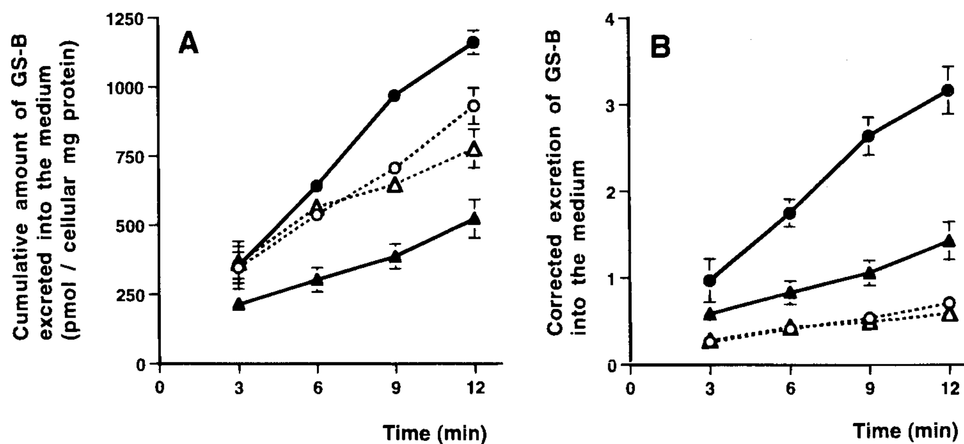

Fig. 2. Export of GS-B from transfected MDCK monolayer. The vector- and cMOAT-transfected MDCK monolayer was preincubated with 20 μM MCB for 20 min. At time zero, the medium was replaced with fresh medium containing the same concentration of MCB. The amount of GS-B in the medium at the designated times is shown in Panel A. The amount of GS-B excreted into the medium divided by the amount of intracellular GS-B at 12 min is shown in Panel B. The results of one representative experiment performed in triplicate are shown as the mean ± S.E. Where vertical bars are not shown, the S.E. is contained within the limits of the symbol. Open symbol, export of GS-B from vector-transfected cells; closed symbol, export of GS-B from cMOAT-transfected cells; circle, export of GS-B into the apical compartment; triangle, export of GS-B into the basal compartment.

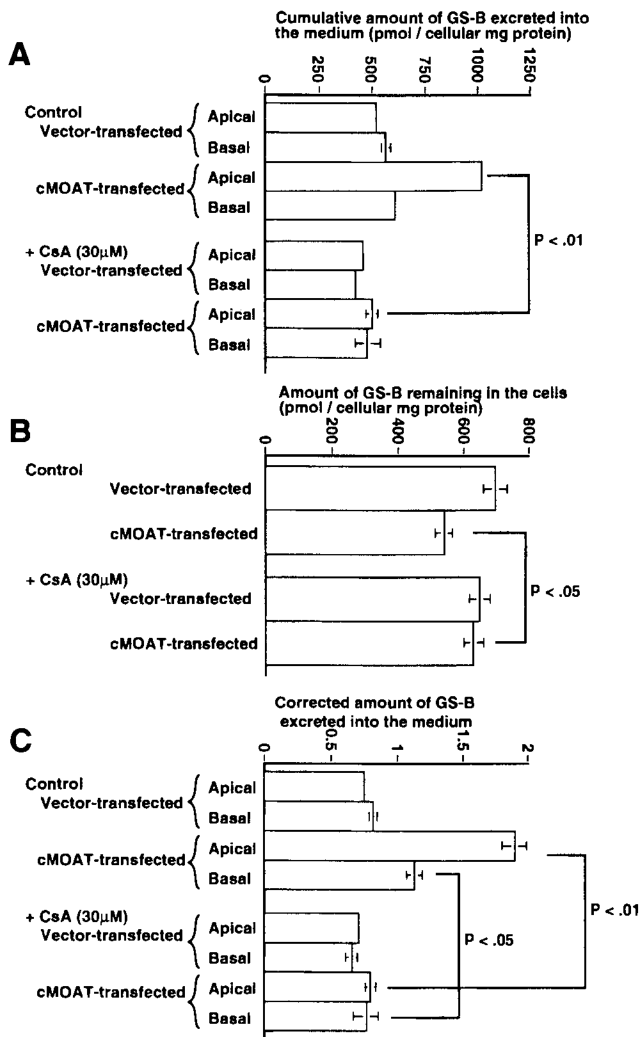

Fig. 3. Effect of CsA on the export of GS-B from transfected MDCK monolayer. After preincubation for 5 min, the vector- and cMOAT-transfected MDCK monolayer was incubated with CsA (30 μM) for an additional 10 min. At time zero, the medium was replaced with fresh medium containing MCB (20 μM) and CsA (30 μM). The cumulative amount of GS-B excreted into the medium up to 12 min is shown in Panel A. Panel B shows the amount of GS-B remaining in the cells at the end of the experiment. The amount of GS-B excreted into the medium divided by the amount of intracellular GS-B at 12 min is shown in Panel C. The results of one representative experiment performed in triplicate are shown as the mean ± S.E.

sulfinpyrazone were used as inhibitors for the transport of S-(2, 4-dinitrophenyl)-glutathione in MRP1-transfected LLC-PK_1 cells, our preliminary experiments indicated that these inhibitors also reduced the intracellular formation of GS-B. In order to circumvent the difficulties associated with the reduced formation of GS-B by these inhibitors, CsA, which did not affect the conversion of MCB to GS-B, was used as a cMOAT inhibitor in the present study (Fig. 3). Although CsA is a substrate and an inhibitor of P-gp, this compound can actually inhibit cMOAT function at higher concentrations; Böhme *et al.* (24) examined the ATP-dependent uptake of daunorubicin and LTC_4 into bile canalicular membrane vesicles and found that the uptake of these ligands was competitively inhibited by CsA with K_i values of 1.5 and 3.4 μM, respectively. In addition, Böhme *et al.* (25) demonstrated that the administration of CsA to rats results in hyperbilirubinemia due to inhibition of the biliary excretion of bilirubin glucuronide mediated by cMOAT.

After submission of the present manuscript, Evers *et al.* reported the apical localization of transfected human cMOAT cDNA product in MDCK cells (26). They identified the apical localization of transfected cMOAT by an immuno-histochemical technique and also demonstrated the apical preferential efflux of DNP-SG after preloading the cells with [^{14}C]CDNB (27). Collectively, the results obtained in the transfection experiments in MDCK cells *in vitro* are consistent with the *in vivo* finding that cMOAT is localized on the apical (bile canalicular) membrane of hepatocytes under physiological conditions (3). In addition, the apical localization of the transfected cMOAT in MDCK cells is consistent with the recent *in vivo* histochemical finding that cMOAT is expressed on the apical membrane of S_1, S_2 and S_3 segments of the proximal renal tubule epithelium (27), although the expression of cMOAT in the kidney is much less marked than in the liver (9,12). The localization of cMOAT in the transfected cells was in marked contrast to that of MRP1; Evers *et al.* (19) transfected human MRP1 to LLC-PK_1 cells and, using immunohistochemical techniques, found that the transfected MRP1 is localized on the basolateral membrane. In addition, they reported the preferential export of DNP-SG into the basal compartment after preloading the MRP1-transfected LLC-PK_1 cells with its precursor, [^{14}C]CDNB (19). The results in the transfected cells are consistent with the *in vivo* finding that MRP1 is localized on the basolateral membrane of hepatocytes (3), although the hepatic expression of MRP1 is much less marked compared with that of cMOAT (3). Overall, the *in vivo* localization of these transporters is reproducible in polarized cell lines *in vitro*, suggesting that these cell lines can be used to study the mechanism involved in the sorting of these membrane proteins.

MDCK cells have been used to analyse the intracellular sorting of proteins; indeed, a large number of proteins (such as glucose transporters (28), Na-P_i and Na-SO_4 cotransporters (29) and so on) are sorted to the physiologically expected side, although some proteins (some neurotransmitter transporters (30), tissue factor (TF) (31) and β-amyloid precursor protein (βAPP) (32)) are sorted to the physiologically unexpected side. Gu *et al.* (30) reported a difference in the sorting of dopamine transporter (DAT) between LLC-PK_1 and MDCK cells. Since it has been hypothesized that the mechanism for the axonal targeting of membrane proteins in neurons is the same as that for the apical sorting in epithelial cells, the transporters located on the axonal membrane (transporters for serotonin (SERT), norepinephrine (NET) and dopamine (DAT)) would be expected to be expressed on the apical membrane of epithelial cells after transfection (30). However, SERT and NET are sorted to the basolateral membrane of MDCK and LLC-PK_1 cells whereas DAT is sorted to the apical and basolateral membrane of MDCK and LLC-PK_1 cells, respectively (30). Camerer *et al.* showed that TF is sorted to the apical surface of endothelial cells, whereas, in MDCK cells, TF is sorted to the basolateral surface (31). Haass *et al.* produced βAPP to be sorted to and secreted from the basolateral membranes of MDCK cells despite its axonal localization in neurons (32).

These results indicate that the mechanism for the sorting of membrane proteins may differ between MDCK and LLC-

PK_1 cells, suggesting that the localization of transfected MRP1 and cMOAT should be determined in the same cell line. Based on these considerations, we also transfected cMOAT to LLC-PK_1 cells. Although we were able to obtain colonies in which cMOAT cDNA is stably expressed, no significant increase in the release of GS-B from the transfected LLC-PK_1 cells was observed (see Results), which is consistent with the account given in the recent review article by Borst *et al.* (33).

In conclusion, the results of the present study suggest that the transfected rat cMOAT is functionally expressed on the apical membrane of MDCK cells. This polarized cell line may be useful for examining the mechanism involved in the sorting of cMOAT. In addition, if the transporters responsible for the cellular uptake of organic anions can be further expressed on the basolateral membrane after transfection, the monolayer formed by the transfected cells may be used as an *in vitro* model to study the transcellular transport of organic anions across hepatocytes.

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