Transporters involved in the hepatic uptake of (99m)Tc-mebrofenin and indocyanine green

de Graaf, W; Häusler, S; Heger, M; van Ginhoven, T M; van Cappellen, G; Bennink, R J; Kullak-Ublick, G A; Hesselmann, R; van Gulik, T M; Stieger, B

Postprint available at:
http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich.
http://www.zora.uzh.ch

Originally published at:
Transporters involved in the hepatic uptake of (99m)Tc-mebrofenin and indocyanine green

Abstract

The transporter specificity of (99m)Tc-mebrofenin and ICG partially overlaps as both compounds are transported by OATP1B3. (99m)Tc-mebrofenin is also taken up by OATP1B1, whereas ICG is additionally transported by NTCP.
Transporters involved in the hepatic uptake of $^{99m}$Tc-mebrofenin and indocyanine green

Wilmar de Graaf, Stephanie Häusler, Michal Heger, Tessa M. van Ginhoven, Gert van Cappellen, Roelof J. Bennink, Gerd A. Kullak-Ublick, Rolf Hesselmann, Thomas M. van Gulik, Bruno Stieger

PII: S0168-8278(10)00840-8
DOI: 10.1016/j.jhep.2010.07.047
Reference: JHEPAT 3505

To appear in: Journal of Hepatology

Received Date: 3 May 2010
Revised Date: 20 July 2010
Accepted Date: 28 July 2010


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Transporters involved in the hepatic uptake of $^{99m}$Tc-mebrofenin and indocyanine green

Wilmar de Graaf$^1$, Stephanie Häusler$^2$, Michal Heger$^1$, Tessa M. van Ginhoven$^3$, Gert van Cappellen$^4$, Roelof J. Bennink$^5$, Gerd A. Kullak-Ublick$^2$, Rolf Hesselmann$^6$, Thomas M. van Gulik$^1$, Bruno Stieger$^2$

1. Department of Experimental Surgery, Academic Medical Center, Amsterdam, the Netherlands.

2. Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich, Switzerland.

3. Department of Surgery, Erasmus MC, Rotterdam, the Netherlands.

4. Optical Imaging Center, Erasmus MC, Rotterdam, the Netherlands.

5. Department of Nuclear Medicine, Academic Medical Center, Amsterdam, the Netherlands.

6. Department of Nuclear Medicine, University Hospital, Zurich, Switzerland.

Keywords: liver function test, hepatobiliary scintigraphy, organic anion transporting polypeptide, Na+-taurocholate co-transporting polypeptide, transport kinetics, liver disease

Contact information:

Thomas M. van Gulik, MD, PhD
Department of Surgery
Academic Medical Center
Meibergdreef 9
1105 AZ Amsterdam
the Netherlands
Tel. +31 20 5665572
Fax +31 20 6976621
E-mail: t.m.vangulik@amc.uva.nl
Abstract

Background & aims: $^{99m}$Tc-mebrofenin hepatobiliary scintigraphy (HBS) and the indocyanine green (ICG) clearance test are used for the assessment of hepatic function before and after liver surgery. The hepatic uptake of $^{99m}$Tc-mebrofenin and ICG is considered similar to the uptake of organic anions such as bilirubin and bile acids. Little is known about hepatic uptake mechanisms of both compounds and recent evidence suggests that the hepatic transporters for ICG and $^{99m}$Tc-mebrofenin are distinct. The aim of this study was to identify the specific human hepatic transporters of $^{99m}$Tc-mebrofenin and ICG.

Methods: The uptake of $^{99m}$Tc-mebrofenin was investigated in cRNA-injected X. laevis oocytes expressing human OATP1B1, OATP1B3, OATP2B1, or NTCP. Chinese hamster ovary (CHO) cells stably expressing OATP1B1, OATP1B3, OATP2B1, or NTCP were used as a mammalian expression system. ICG transport into CHO cells was additionally imaged with confocal microscopy.

Results: We demonstrated that OATP1B1 and OATP1B3 are involved in the transport of $^{99m}$Tc-mebrofenin. OATP1B1 showed an approximately 1.5-fold higher affinity for $^{99m}$Tc-mebrofenin compared to OATP1B3. ICG is transported by OATP1B3 and NTCP.

Conclusion: The transporter specificity of $^{99m}$Tc-mebrofenin and ICG partially overlaps as both compounds are transported by OATP1B3. $^{99m}$Tc-mebrofenin is also taken up by OATP1B1, whereas ICG is additionally transported by NTCP.
Introduction

Preoperative evaluation of future remnant liver function is crucial in order to select patients with an increased risk for postoperative liver failure before major liver surgery or living donor liver transplantation. The indocyanine green (ICG) clearance test and technetium-99m (99mTc)-mebrofenin hepatobiliary scintigraphy (HBS) are valuable quantitative dynamic liver function tests used for the assessment of liver function before and after liver surgery(1-3).

99mTc-mebrofenin is a 99mTc-labeled iminodiacetic (IDA) derivate originally used for the diagnosis of a multitude of biliary diseases(4) and since recently for the assessment of liver function(1). ICG is a tricarbocyanine dye that was first introduced to measure blood flow and later recognized as a useful compound for liver function testing(5). 99mTc-mebrofenin and ICG, both organic anions, are almost exclusively taken up by the liver and rapidly excreted into the bile without undergoing biotransformation and with no enterohepatic circulation(1,6). Although a similar hepatocellular uptake mechanism has been postulated, we recently demonstrated a significant difference between ICG and 99mTc-mebrofenin uptake profiles in a rat model of hepatic ischemia-reperfusion injury(7), suggesting that the specific hepatic transporters for ICG and 99mTc-mebrofenin are distinct. The mechanisms underlying hepatocellular uptake of either compound have not yet been completely elucidated.

The hepatic clearance of numerous organic anions, including drugs and xenobiotics, is mainly facilitated by the Na+-independent uptake by organic anion transporting polypeptides (OATPs)(8, 9) and organic anion transporters (OATs)(10). In human hepatocytes, OATP1B1, OATP1B3, and OATP2B1 isoforms are expressed at the basolateral plasma membrane of hepatocytes. In addition to OATs and OATPs, human Na+-taurocholate co-transporting polypeptide (NTCP), which constitutes the main uptake system for bile acids, has been shown to also transport some drugs in a Na+-dependent manner(11). The uptake of 99mTc-mebrofenin and ICG by any of these transporters is therefore probable. This is supported by the fact that bilirubin, sulfobromophthalein (BSP), and bile salts, which are OATP(12) and NTCP(13) substrates, inhibit the uptake of 99mTc-IDA agents(14, 15), suggesting that a common transport mechanism exists for these compounds and 99mTc-mebrofenin. Indirect evidence further suggests that ICG uptake is mediated by members of the OATP family, which is mainly based on the observation that ICG has the ability to inhibit the uptake of different OATP substrates(12).
From a clinical perspective, an understanding of the hepatic uptake mechanisms of $^{99m}$Tc-mebrofenin and ICG is necessary for proper interpretation of both liver function tests under various conditions of liver disease. The aim of this study was therefore to identify the specific human hepatocellular transporters of $^{99m}$Tc-mebrofenin and ICG. We hypothesized that specific members of the human OATP family or NTCP are involved in the uptake of both substances and that the specific transporters for $^{99m}$Tc-mebrofenin and ICG are, at least in part, distinct. While our experiments were ongoing, a report was published that identified OATP1B1 and OATP1B3 as specific $^{99m}$Tc-mebrofenin transporters(16).

**Materials and Methods**

**Materials** Tritiated estrone-3-sulfate ([$^3$H]E3S) and tritiated taurocholic acid ([$^3$H]TC) were purchased from Perkin Elmer (Waltham, MA). Mebrofenin ((2,4,6-trimethyl-3-bromo)-iminodiacetic acid, Bridatec, GE Healthcare, Chalfont St. Giles, UK) was labeled with 300MBq $^{99m}$Tc in 1mL of 0.9% NaCl according to the manufacturer’s instructions, using the second eluate. Quality control was routinely performed to assure <5% free pertechnetate. ICG was purchased from PULSION Medical Systems (Munich, Germany). Cell culture media and antibiotics were obtained from Invitrogen (Carlsbad, CA) unless stated otherwise. L-proline and sodium butyrate were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell Lines** Chinese hamster ovary (CHO) cell lines stably expressing human OATP1B1, OATP1B3, and OATP2B1 have been described(17, 18). CHO wild type (CHO-WT) cells were used as control. Flip-In CHO cells stably expressing human NTCP were generated by ligating the coding sequence of NTCP into pcDNA5/FRT (Invitrogen, Carlsbad, CA) as described previously(19). Control cells were transfected with the empty pcDNA5/FRT vector (CHO-pcDNA5/FRT).

**Cell Culture** CHO-WT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 50mM L-proline, and 100U/mL penicillin/streptomycin. The OATP1B1-, OATP1B3-, and OATP2B1-transfected CHO-cells were grown in DMEM containing 10% fetal calf serum, 0.05mg/mL L-proline, 100U/mL penicillin/streptomycin, and 500μg/mL geneticin G-
Flp-In CHO cells (pcDNA5/FRT and NTCP) were grown in HAM F-12 medium containing 10% fetal calf serum, 1mM L-glutamine, 100U/mL penicillin/streptomycin, and 500μg/mL hygromycin. Cells were incubated at 37°C in an atmosphere containing 5% CO₂ and 95% humidity. For transport experiments, cells were seeded on 3.5-cm dishes. Transport experiments were performed 3-4d after seeding after confluence had been reached. To enhance the expression of the recombinant proteins, cells were induced with 5mM sodium butyrate 24h before the transport experiment.

**99mTc-Mebrofenin Uptake in X. laevis Oocytes**  X. laevis oocytes were prepared as described before(20). In brief, 30 healthy oocytes were injected with 50nL of water or with 5ng of cRNA encoding human OATP1B1, OATP1B3, OATP2B1(21), or NTCP(22) in 50nL of water. After a 3-d culture, 10 oocytes were pre-washed in uptake buffer and 99mTc-mebrofenin uptake was measured at 25°C in 100μL of uptake solution (100mM NaCl, 2mM KCl, 1mM MgCl₂, 1mM CaCl₂, and 10mM HEPES, pH=7.5) for 30 and 60min. The functional expression of the OATPs and NTCP was verified with [³H]E3S and [³H]TC, respectively.

**Transport of 99mTc-Mebrofenin and ICG in Stably Transfected CHO Cells**  Transport experiments were performed in OATP-expressing CHO cells and NTCP-expressing Flp-In CHO cells as described before(17, 20). Briefly, cells were washed 3× with pre-warmed (37°C) sodium buffer or choline buffer. The uptake of 99mTc-mebrofenin in sodium or choline buffer was assayed at various predefined 99mTc-mebrofenin concentrations in a 1-mL final volume. After incubation for 0-2min, the cells were washed 4× with 2mL of ice-cold choline buffer and lysed with 1mL of 1% Triton-X100 (Fluka, Buchs, Switzerland) in PBS. A 500-μL aliquot was used for liquid scintillation counting. Protein concentrations were determined with the BCA assay (Thermo Fisher Scientific, Waltham, MA) using bovine serum albumin (BSA) as standard.

Kinetic analysis for the uptake of 99mTc-mebrofenin was performed for a 0.25-5mM substrate concentration range. Cellular uptake rates were determined after normalization for incubation time and total protein content. Net uptake rates by OATPs and NTCP were calculated as the difference in the uptake rate of the transporter and controls, namely CHO-WT and CHO-pcDNA5/FRT, respectively, for each concentration. Kinetic parameters, $K_m$ and $V_{max}$, were determined with nonlinear regression analysis using the Michaelis-Menten equation.
Transport experiments with ICG were performed as described above with the modification that, after incubation (0-5min) in ICG-containing sodium or choline buffer (0-200μM), cells were washed 4× with 2mL of ice-cold choline buffer containing 1% BSA followed by 4× washing with 2mL of ice-cold choline buffer. The cells were subsequently lysed with 300μL of 1% Triton-X100 in PBS. ICG concentrations were determined spectrophotometrically (Varioskan Flash, Thermo Fisher Scientific) at 805nm with the use of a standard curve.

To confirm the antagonistic effect of ICG (ranging from 0-100μM) on OATP-mediated transport, [³H]E3S (5μM) was used as substrate for OATP1B1, OATP1B3, and OATP2B1 and [³H]TC (2.5μM) for NTCP. The uptake of [³H]E3S or [³H]TC was measured following 1min incubation. IC₅₀ values were determined by plotting the log inhibitor concentration against the net uptake rate with nonlinear regression of the data set.

Confocal Laser Scanning Microscopy (CLSM) CHO cells were grown on glass cover slips (Ø=25mm, Menzel-Gläzer, Braunschweig, Germany) for 3d until 50-60% confluence was reached. Cells were induced with 5mM sodium butyrate 24h before CLSM. The cells were washed 3× with pre-warmed (37°C) sodium buffer and incubated with 1mL of ICG solution for 0, 2, or 5min. The cells were subsequently washed 4× with 2mL of ice-cold choline buffer containing 1% BSA, followed by 4× washing with ice-cold choline buffer. Cells were labeled with 1μM of carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 5min, followed by 3× washing with ice-cold choline buffer. The cell nucleus was stained by incubating the cells with 0.01mg/mL 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 10min, followed by 3× washing with ice-cold choline buffer. Cells were incubated for 2min with propidium iodide (PI) to confirm cell integrity.

CLSM was performed with an LSM 510 NLO confocal microscope (Carl Zeiss, Jena, Germany). Glass cover slips containing the cells were mounted in a metal ring system that was fitted in the temperature-controlled (37°C) translator stage of the microscope. PBS (1mL) was applied on the glass cover slip to prevent desiccation. ICG was excited with the 632.8-nm HeNe laser line and imaged through a 650-nm long pass emission filter. The 488-nm argon laser line was used in combination with a 525±25-nm band pass emission filter to visualize CFSE. DAPI was visualized in two-photon excitation mode using an 800-nm Ti:sapphire laser with a 425±35-nm band pass emission filter. PI was excited at 543nm and visualised using a 590±25-nm band pass emission filter.
Data and Statistical Analysis  Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA) and Statistical Package for Social Sciences (SPSS, Chicago, IL). IC₅₀ and kinetic parameters were calculated using non-linear regression analysis in GraphPad Prism. Mann-Whitney U tests were performed to compare two ordinal variables. Data are expressed as mean±SD. All statistical tests were two-tailed and differences were considered significant at a P-value of ≤0.05.

Results

Transport of ⁹⁹ᵐTc-Mebrofenin in X. laevis Oocytes  The uptake of ⁹⁹ᵐTc-mebrofenin (25μM) was first investigated in cRNA-injected X. laevis oocytes expressing human OATP1B1, OATP1B3, OATP2B1, or NTCP. As shown in Fig. 1, OATP1B1 and OATP1B3 transported ⁹⁹ᵐTc-mebrofenin whereas no transport was observed in OATP2B1 and NTCP. Uptake into control (water-injected) oocytes was negligible, indicating that no endogenous transport or diffusion of ⁹⁹ᵐTc-mebrofenin took place across the oocyte membrane.

Uptake of ⁹⁹ᵐTc-Mebrofenin by Transfected CHO Cells  To exclude effects of the heterologous amphibian expression system on substrate specificity, transport experiments were repeated in a mammalian expression system. The OATP1B1- and OATP1B3-mediated transport of ⁹⁹ᵐTc-mebrofenin was confirmed in OATP-expressing CHO cells (Fig. 2). At a concentration of 25μM, the uptake of ⁹⁹ᵐTc-mebrofenin into the OATP1B1-transfected cells was enhanced by more than 30-fold vs. uptake into CHO-WT cells (P<0.05), whereas the uptake by OATP1B3 was enhanced more than 20-fold compared to the CHO-WT cells (P<0.05). Negligible transport was seen in the CHO-WT, OATP2B1-, and NTCP-transfected cells. Therefore, different expression systems do not affect the substrate specificity of the investigated transporters.

Next, we investigated the time-dependence of ⁹⁹ᵐTc-mebrofenin (100μM) uptake into CHO cells expressing OATP1B1 and OATP1B3. The uptake of ⁹⁹ᵐTc-mebrofenin by OATP1B1 (Fig. 3A) and OATP1B3 (Fig. 3B) was linear up to 40 and 60s, respectively. The kinetic experiments were therefore performed using a 40-s incubation time.

Fig. 4 depicts exemplary uptake kinetics for ⁹⁹ᵐTc-mebrofenin. The uptake was saturable with increasing concentrations of substrate for both OATP1B1- and OATP1B3-expressing cells. The
following Michaelis-Menten constants \( (K_m) \) with associated maximum transport rates \( (V_{max}) \) were calculated (derived from 3 independent experiments for each transporter): OATP1B1: \( K_m = 1.68 \pm 0.29 \) mM, \( V_{max} = 386 \text{nmol/mg protein/min} \) and OATP1B3: \( K_m = 2.57 \pm 0.85 \) mM, \( V_{max} = 372 \text{nmol/mg protein/min} \). OATP1B1 exhibited a slightly higher affinity for \( ^{99}\text{Tc-mebrofenin} \) than OATP1B3 with similar maximal transport rates.

To correct for unknown transporter expression levels and to obtain an idea on individual transporter efficiency, the intrinsic clearance \( \left( V_{max}/K_m \right) \) was calculated. OATP1B1 had a 1.5-fold higher intrinsic clearance compared to OATP1B3 (0.23 vs. 0.14 ìL/min/ìg protein).

**Uptake of ICG by Transfected CHO Cells** CLSM demonstrated that OATP1B3 and NTCP were able to transport ICG (Fig. 5A, B). ICG was also taken up at low amounts by the CHO-WT cells as well as by OATP1B1, OATP2B1, and CHO-pcDNA5/FRT cells, which is suggested by the relatively low fluorescence intensity in the cytoplasm and the higher fluorescence intensity in perinuclear granules/vesicles (Fig. 6). In OATP1B3- and NTCP-transfected cells, the cytoplasmic ICG fluorescence was considerably higher (Fig. 5A, B) than in CHO-WT cells and cells expressing the other OATP isoforms. The absence of PI staining confirmed that the cells remained intact during the experiment (data not shown).

Next, the extent of ICG uptake by OATPs and NTCP was quantified spectrophotometrically in transfected CHO cells (Fig. 7A, B). At a concentration of 50 ìM, the uptake of ICG after 1-min incubation increased twofold in OATP1B1-transfected cells compared to the CHO-WT cells. Considerable background fluorescence was observed in the CHO-WT samples that was attributable to ICG adhering to the culture dish (not shown). OATP1B1- and OATP2B1-transfected cells exhibited a similar uptake to CHO-WT cells. The abovementioned background fluorescence made it difficult to determine transporter-specific kinetic parameters for ICG, as a result of which these data are not presented. The ICG uptake by NTCP-transfected Flp-In CHO cells was enhanced threefold after 1-min incubation compared to control (CHO-pcDNA5/FRT cells). The uptake of ICG by NTCP was significantly reduced when incubated in the absence of Na\(^+\), providing corroborative evidence for the involvement of NTCP in the Na\(^+\)-dependent transport of ICG.
Inhibition of OATP-Mediated Transport by ICG  Earlier studies had demonstrated that ICG is an inhibitor of OATP-mediated transport(21,24). We therefore performed experiments to investigate the inhibitory potential of ICG on the uptake of $[^3]$H]E3S by OATPs and $[^3]$H]TC by NTCP. As shown in Fig. 8, ICG was able to inhibit the uptake of $[^3]$H]E3S by all OATP isoforms. In addition, ICG inhibited the uptake of $[^3]$H]TC by NTCP.

Discussion

Although ICG and $^{99m}$Tc-mebrofenin are frequently used in clinical practice for the assessment of liver function, little is known about the hepatic uptake mechanisms of both agents. In this study we demonstrated that $^{99m}$Tc-mebrofenin, an IDA derivative, is taken up into hepatocytes by OATP1B1 and OATP1B3 and that ICG is taken up by OATP1B3 and NTCP.

Studies on the uptake of IDA derivates in cultured rat hepatocytes have evinced that IDA uptake is Na$^+${-independent, temperature- and pH-dependent, and reduced in the presence of increasing bilirubin concentrations(15, 14). In addition, BSP, taurocholate, ICG, and Rose Bengal have been implicated as inhibitors of IDA uptake(14, 15), whereby the antagonistic effect of these compounds is highly dependent on the structure of the IDA agent. For instance, of all IDA agents, $^{99m}$Tc-mebrofenin uptake is inhibited the least by bilirubin(14). Based on these findings it was concluded that, at least in part, the uptake of IDA derivates was similar to the uptake mechanism of bilirubin and BSP(4). A possible involvement of OATPs in the uptake of IDA derivates became evident after Jacquemin et al.(23) found that BSP and TC are taken up by OATP. The specific involvement of the OATP1B1 and OATP1B3 isoforms in the uptake of $^{99m}$Tc-mebrofenin has recently been confirmed(16), but the possible role of the OATP2B1 isoform and NTCP were not considered and no kinetics were calculated.

Our transport experiments in cRNA-injected X. laevis oocytes and transfected CHO cells corroborated that $^{99m}$Tc-mebrofenin is transported by OATP1B1 and OATP1B3 and showed that OATP2B1 and NTCP are not involved in the uptake of $^{99m}$Tc-mebrofenin. OATP1B1 exhibited an approximately 1.5-fold higher affinity and intrinsic clearance ($V_{\text{max}}/K_m$) for $^{99m}$Tc-mebrofenin compared to OATP1B3, suggesting that OATP1B1 may be the preferential transporter for $^{99m}$Tc-mebrofenin. Although the mM-range of $K_m$ implies a relatively low affinity for $^{99m}$Tc-mebrofenin in comparison to
other OATP1B1 and OATP1B3 substrates(18, 24), the intrinsic transport capacities ($V_{\text{max}}/K_m$) of OATP1B1 and OATP1B3 indicate that the transport of $^{99m}\text{Tc}$-mebrofenin is highly effective.

Similarly, indirect evidence that ICG is transported by members of the organic OATP family is mainly based on the observation that ICG is a well-known inhibitor of OATP-mediated transport(12). Our study confirmed that ICG is a potent inhibitor of all investigated OATP isoforms and of NTCP. We also demonstrated that only OATP1B3 and NTCP are involved in uptake of ICG, clearly indicating that the capacity of a compound to inhibit OATP-mediated transport does not necessarily coincide with its uptake by the respective OATP isoform, as was shown previously for drugs(17).

CLSM confirmed that OATP1B3 and NTCP facilitate ICG transport. Surprisingly, ICG was also taken up, albeit in low quantities, by CHO-WT cells, OATP1B1- and OATP2B1-transfected CHO cells, as well as by CHO-pcDNA5/FRT cells. Although ICG was diffusely present in the cytoplasm at low concentrations, it was enriched in perinuclear granules/vesicles. It is known that ICG is not exclusively taken up by hepatocytes, but also by reticulocytes, keratinocytes, and retinal pigment epithelial cells(25-27), whereby the intracellular localization of ICG in CHO-WT cells exhibited a similar granular pattern as reported for the other cell types. The significance of the vesicular accumulation and perinuclear localization of ICG in CHO-WT cells is presently unclear, but suggests that additional mechanisms are involved.

Hepatic ICG and $^{99m}\text{Tc}$-mebrofenin uptake are impaired in cholestatic patients. OATPs have been implicated in the uptake of bilirubin(12), which might explain the competitive inhibition of $^{99m}\text{Tc}$-mebrofenin by high bilirubin concentrations(14). Nevertheless, the involvement of OATP1B1 and OATP1B3 in the uptake of bilirubin is controversial. Transport of bilirubin by OATP1B1 and OATP1B3 has been demonstrated in cRNA-injected X. laevis oocytes, in which bilirubin uptake was significantly less efficient in OATP1B3 compared to OATP1B1(28). Similarly, OATP1B1 but not OATP1B3 was able to transport bilirubin in stably transfected human embryonic kidney cells (HEK293)(12). Contrastingly, Wang et al.(29) precluded a physiological role for OATP1B1 in bilirubin transport. Therefore, the specific effect and exact mechanism of bilirubin on OATP-mediated transport remains elusive and warrants closer investigation. Besides increased plasma bilirubin levels, conjugated bile acids accumulate in plasma during cholestasis. Conjugated bile acids are predominantly transported by NTCP and it is therefore probable that bile salts inhibit the NTCP-mediated uptake of ICG during cholestasis. In addition, downregulation of uptake transporters during cholestasis may contribute to the
impaired uptake of ICG and $^{99m}$Tc-mebrofenin(30). The specific effect of bilirubin and bile acids on OATP and NTCP-mediated transport of ICG and $^{99m}$Tc-mebrofenin requires further investigation in light of the utility of $^{99m}$Tc-mebrofenin HBS and the ICG clearance test as dynamic liver function tests in liver diseases where bilirubin and bile acid levels become aberrant.

Besides cholestasis, inflammation and drugs are known to influence the hepatic transport of several organic anions, including ICG and $^{99m}$Tc-mebrofenin(31-34). Identification of the transport mechanisms of $^{99m}$Tc-mebrofenin and ICG aids in the interpretation of both liver function tests under conditions of liver disease and liver regeneration. Cytokines such as TNF-α and IL-6 released by Kupffer cells in several liver diseases (e.g., steatosis and hepatitis) possibly affect the uptake of $^{99m}$Tc-mebrofenin in a different manner than ICG, as cytokines have a differential influence on the expression of different OATP isoforms and NTCP(30, 35-37). This differential response was confirmed in an in vivo rat model, which demonstrated that different stages of non-alcoholic fatty liver disease resulted in a differential decrease in several OATP and NTCP protein levels(38). Vetelainen et al. showed that the hepatic uptake of $^{99m}$Tc-mebrofenin decreases with the severity of liver steatosis(39). Furthermore, the abovementioned cytokines play an important role in ischemia-reperfusion injury as well as liver regeneration. We recently compared the ICG clearance test with $^{99m}$Tc-mebrofenin HBS in rats after ischemia-reperfusion injury combined with a partial hepatectomy(7) and during liver regeneration after major hepatectomy(40). In both models a differential decrease in OATP isoforms was observed, contributing to the difference in hepatic uptake between ICG and $^{99m}$Tc-mebrofenin after ischemia-reperfusion injury and during regeneration.

In the final analysis, under normal conditions ICG and $^{99m}$Tc-mebrofenin are considered to have high hepatic extraction ratios. The total hepatic clearance from the blood is therefore rate-limited by blood flow. It is now evident that several key uptake transporters are downregulated during liver disease, affecting the transport capacity and hepatic extraction. Hence the ICG clearance test and $^{99m}$Tc-mebrofenin HBS monitor not only blood flow but also the transport capacity. Given the high extraction of both compounds, it is likely that not the basolateral uptake systems but the canalicular export systems will become the clearance determining transporters under pathophysiological conditions(41). Despite the relatively underexplored dynamics of blood flow and transport capacity under various pathophysiological conditions, the uptake and intracellular transit of $^{99m}$Tc-mebrofenin and ICG represent clinically relevant hepatic function parameters insofar as their transport is similar to
various endogenous and exogenous substances such as bilirubin, bile acids, hormones, drugs, and toxins, whose uptake also relies on blood flow(31, 41).

**Conclusion**

The transporter specificity of $^{99m}$Tc-mebrofenin and ICG partially overlap with both substances being transported by OATP1B3. $^{99m}$Tc-mebrofenin is also transported by OATP1B1, whereas ICG is additionally taken up by NTCP.

**Legends**

**Figure 1:** Transport of $^{99m}$Tc-mebrofenin in *X. laevis* oocytes. Uptake of 25µM $^{99m}$Tc-mebrofenin was measured for 60 min. Uptake was observed only in cells expressing OATP1B1 and OATP1B3. Results are presented as mean±SD for n=10 oocytes per experiment.

**Figure 2:** Transport of $^{99m}$Tc-mebrofenin in transfected CHO cells. Uptake of 25µM $^{99m}$Tc-mebrofenin was measured after 1-min incubation, confirming the involvement of OATP1B1 and OATP1B3 in the uptake of $^{99m}$Tc-mebrofenin. Results are presented as mean±SD for n=3 per experiment.

**Figure 3:** Time-dependent transport of $^{99m}$Tc-mebrofenin by OATP1B1- (A) and OATP1B3-transfected CHO cells (B). A $^{99m}$Tc-mebrofenin concentration of 100µM was used. Results are presented as mean±SD for n=3 per experiment.

**Figure 4:** Michaelis-Menten plots of $^{99m}$Tc-mebrofenin uptake into OATP1B1- (A) and OATP1B3-expressing CHO cells (B) measured at 37°C for 40s. Following baseline subtraction (CHO-WT), the $K_m$ and $V_{max}$ were derived from the Michaelis–Menten equation. Results are expressed as mean±SD of triplicate determinations from 3 independently performed transport experiments.

**Figure 5:** Confocal microscopy images of CHO cells incubated for 2min with 50µM ICG. ICG was taken up by OATP1B3- (A) and NTCP-expressing cells (B). Intracellular ICG was predominantly located in the cytoplasm with occasional nuclear colocalization.
**Figure 6:** Confocal microscopy image of ICG (20µM, 2-min incubation) localization in perinuclear granules or vesicles of CHO-WT cells.

**Figure 7:** The uptake of ICG by transfected CHO cells. After incubation for 1min, ICG (50µM) transport was enhanced twofold in OATP1B3-transfected cells compared to CHO-WT (A). ICG (50µM) transport into NTCP-transfected Flp-In CHO cells was enhanced threefold after 2min incubation compared to control cells (pcDNA5/FRT) (B). No transport of ICG by NTCP occurred in the absence of Na⁺ (choline buffer). Results are presented as mean±SD for n=3 per experiment.

**Figure 8:** ICG concentration-dependent inhibition of [³H]E3S uptake in OATP-expressing cells and [³H]TC in NTCP-expressing cells. ICG inhibited the uptake of competitive substrates in all transporter expressing cells at differential IC₅₀ values. Note the disproportionally low IC₅₀ value for OATP2B1 – a transporter that did not facilitate ICG transport. Results are presented as mean±SD for n=3 per experiment.
Reference List


A. OATP 1B1

\[ V_{\text{max}} = 385.9 \pm 67.3 \]
\[ K_{\text{n}} = 1.68 \pm 0.29 \]

B. OATP 1B3

\[ V_{\text{max}} = 371.5 \pm 114.6 \]
\[ K_{\text{n}} = 2.57 \pm 0.85 \]