Cancer Therapy: Preclinical

Influence of Human OATP1B1, OATP1B3, and OATP1A2 on the Pharmacokinetics of Methotrexate and Paclitaxel in Humanized Transgenic Mice

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Abstract

**Purpose:** Organic anion-transporting polypeptide (OATP) drug uptake transporters are thought to play an important role in drug pharmacokinetics and toxicokinetics. We aimed to determine the influence of the individual human OATP1B1, OATP1B3, and OATP1A2 transporters on the in vivo disposition of the anticancer drugs methotrexate and paclitaxel by using liver-specific humanized OATP1A/1B transgenic mice.

**Experimental Design:** Wild-type, Slco1a/1b⁻/⁻ (Oatp1a/1b knockout), Slco1a/1b⁻/⁻;1B1tg, Slco1a/1b⁻/⁻;1B3tg, and newly generated Slco1a/1b⁻/⁻;1A2tg (humanized OATP1B1, OATP1B3, and OATP1A2 transgenic) mice were characterized biochemically and physiologically, and subsequently intravenously dosed with methotrexate or paclitaxel (2 or 10 mg/kg each) for pharmacokinetic analyses.

**Results:** Humanized OATP1B1, OATP1B3, and OATP1A2 transgenic mice all showed partial or complete rescue of increased plasma bilirubin levels, but also of the increased plasma levels and decreased liver and small intestinal accumulation of methotrexate observed in Slco1a/1b⁻/⁻ mice. Furthermore, hepatic expression of OATP1B3 and OATP1A2, but not OATP1B1, resulted in increased liver uptake of paclitaxel (2 mg/kg). At 10 mg/kg, a modest effect of only OATP1A2 on paclitaxel liver uptake was observed.

**Conclusion:** Human OATP1A/1B transporters play an important role in plasma and tissue distribution of the structurally diverse chemotherapeutics methotrexate (organic anion) and paclitaxel (hydrophobic, bulky). Variation in OATP1A/1B activity due to genetic variation and pharmacologic inhibition, or differences in tumor-specific expression levels might therefore affect plasma, tissue, and tumor levels of these drugs in patients, and hence their therapeutic efficacy. Humanized transgenic OATP1A/1B mice will provide excellent tools to further study these aspects in vivo for many (anticancer) drugs. Clin Cancer Res; 1–12. ©2012 AACR.

Introduction

Organic anion-transporting polypeptides (OATP; gene name: SLCO) are transmembrane transporters that can mediate the cellular uptake of a broad range of substrates. Many are expressed in various tissues important for pharmacokinetics (1). OATPs are classified into 6 distinct families and currently 11 human and 15 mouse OATPs/Oatps have been identified (2, 3). Especially within the OATP family, based on amino acid homology, no clear and direct orthologs between individual human and mouse OATPs exist. While humans only have one OATP1A transporter (OATP1A2), mice have at least 4 (Oatp1a1, Oatp1a4, Oatp1a5, and Oatp1a6; refs. 2, 4). On the other hand, while humans have 2 OATP1B transporters (OATP1B1 and OATP1B3), mice only have one, Oatp1b2 (2).

Human OATP1B1 and OATP1B3 are highly and predominantly expressed at the basolateral membrane of the liver. They can therefore make an important contribution to the hepatic uptake and hence systemic clearance of drugs and toxins after intravenous and oral administration (5–7). OATP1A2, on the other hand, is more widely expressed in several tissues like brain (8), kidney (9), liver (in cholangiocytes, not hepatocytes; ref. 9), and in small intestine (10), where its effect on drug absorption and/or elimination needs further investigation. Besides their physiologic expression, many OATPs have been found expressed in certain malignancies like breast, gastrointestinal, and lung cancers (6, 11–14), where they may well be involved in the tumor uptake of anticancer drugs.

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Translational Relevance

Interindividual variation in drug response and drug toxicity and drug–drug interactions complicate anticancer drug treatment. Given their tissue distribution and broad substrate specificity, organic anion transporting polypeptide (OATP) drug uptake transporters may play a marked role in these processes. Using humanized OATP1B1, -1B3, and -1A2 transgenic mice, we found that all 3 human proteins transport the anticancer drug methotrexate in vivo, whereas only OATP1B3 and OATP1A2 transport paclitaxel. Methotrexate and paclitaxel are commonly used in cancer treatment, but they are also associated with severe and sometimes lethal toxicity in patients. Moreover, as many of the OATP1A/1B transporters occur in certain tumors, they might also directly affect tumor susceptibility to methotrexate or paclitaxel treatment. We speculate that variation in OATP1A/1B activity, because of genetic variation or drug–drug interactions, and tumor-specific expression, can be an important factor in determining treatment efficacy and/or toxicity of these and likely many other anticancer drugs.

By using Oatp1a/1b knockout mice, we recently established an important role of mouse Oatp1a/1b transporters in the in vivo pharmacokinetics and tissue distribution of methotrexate and paclitaxel. Briefly, in the absence of Oatp1a/1b transporters, plasma levels of methotrexate and paclitaxel were markedly increased, whereas hepatic uptake and biliary excretion were significantly decreased (15, 16). Methotrexate and paclitaxel are structurally diverse anticancer drugs, with methotrexate being a charged organic anion and paclitaxel a highly hydrophobic drug. These results illustrate the broad substrate specificity of OATP1A/1B transporters. However, due to the lack of direct orthology between mouse and human OATPs, together with the current lack of knowledge about species differences concerning substrate specificity, extrapolation of results obtained from Oatp1a/1b knockout mice to the human situation may at times be difficult. Moreover, in vitro to in vivo extrapolation of human transporter protein data can be challenging for various reasons (plasma protein binding of substrates, redundancy with or counteraction by other transporters, tissue- or cell-specific modification of proteins, and/or composition of the plasma membranes). To overcome these limitations, we have now generated humanized OATP1B1, OATP1B3, and OATP1A2 transgenic mice (Slco1a/1b<sup>−/−</sup>;1B1<sup>tg</sup>, Slco1a/1b<sup>−/−</sup>;1B3<sup>tg</sup>, and Slco1a/1b<sup>−/−</sup>;1A2<sup>tg</sup>, respectively) with liver-specific expression of each of the transgenic cDNAs in an Oatp1a/1b knockout background. Transgenic expression in liver parenchyma cells (hepatocytes) was chosen in view of the exclusive liver expression and basolateral (sinusoidal) hepatocyte localization of OATP1B1 and OATP1B3 in humans, and the previously established prominent pharmacokinetic role of mouse Oatp1a/1b transporters in the liver (15, 16). Preliminary partial physiologic analysis of 2 of these 3 mouse models has shown the importance of human OATP1B1 and OATP1B3 in bilirubin detoxification by the liver. This resulted in the discovery that complete and simultaneous absence of OATP1B1 and OATP1B3 in humans underlies Rotor syndrome, a rare, benign hereditary conjugated hyperbilirubinemia (17). That study also revealed the existence of individuals with a complete deficiency of either OATP1B1 or OATP1B3, which do not display obvious jaundice, and likely occur at much higher frequencies in various human populations than Rotor subjects.

We note that the Slco1a/1b<sup>−/−</sup>;1A2<sup>tg</sup> mice do not present a physiologic model for uptake of compounds into the liver, as, due to the transgenic promoter used (ApoE), OATP1A2 is expressed in hepatocytes in these mice, whereas in humans, hepatic OATP1A2 is only found in cholangiocytes (9). Slco1a/1b<sup>−/−</sup>;1A2<sup>tg</sup> mice, therefore, do not mimic the normal function of OATP1A2 in the liver. However, they can be readily used to study the in vivo transport capacity of human OATP1A2 for various compounds, which is of importance given the possible role of OATP1A2 in drug uptake among others in the intestine, blood–brain barrier, and tumor cells.

In the present study, we conducted a basic characterization of the humanized strains. We then used these strains to analyze the in vivo influence of the 3 individual human OATP1A/1B transporters on the disposition of the anticancer drugs methotrexate and paclitaxel, to better understand their possible relevance in human cancer chemotherapy.

Materials and Methods

Animals

Mice were housed and handled according to the Institutional guidelines complying with the Dutch legislation. Animals used in this study were wild-type, Slco1a/1b<sup>−/−</sup> (Oatp1a/1b knockout; ref. 15), Slco1a/1b<sup>−/−</sup>;1B1<sup>tg</sup>, Slco1a/1b<sup>−/−</sup>;1B3<sup>tg</sup>, and Slco1a/1b<sup>−/−</sup>;1A2<sup>tg</sup> mice (OATP1B1, OATP1B3, and OATP1A2 humanized transgenic mice, respectively) of comparable genetic background (>99% FVB) between 9 and 14 weeks of age. Generation of OATP1B1 transgenic mice has been described (18), and OATP1B3 and OATP1A2 transgenic mice were generated as described below. Slco1a/1b<sup>−/−</sup>;1B1<sup>tg</sup>, Slco1a/1b<sup>−/−</sup>;1B3<sup>tg</sup>, and Slco1a/1b<sup>−/−</sup>;1A2<sup>tg</sup> humanized mice were generated by cross-breeding and by breeding the transgenes to homozygosity. Animals were kept in a temperature-controlled environment with a 12-hour light/dark cycle. They received a standard diet (AM-III; Hope Farms) and acidified water <i>ad libitum</i>.

Chemicals and reagents

Paclitaxel was from Sequoia Research Products, methotrexate (100 mg/mL Emthexate PF) from Pharmachemie, isofurane (Forane) from Abbott Laboratories, and heparin (5,000 IE/mL) from Leo Pharma BV.
Transgene construction and generation of transgenic mice

To achieve liver-specific expression of human OATP1B3 and OATP1A2, we generated transgene constructs as follows (Fig. 1A). pLIV-LE6 (kindly provided by Dr. J. Taylor, Gladstone Institute, University of California, San Francisco, CA; ref. 19) was completely digested with Clal followed by a partial digestion with Asp718. The 7.6 kb fragment of the vector was isolated, and 5′- and 3′-ends of the vector were filled up with Klenow enzyme to create blunt ends. After blunt end ligation, the vector was digested with XhoI. PCR was used to generate the SLCO1B3 and SLCO1A2 constructs with XhoI recognition sites at the 5′-ends, respectively. The templates, human SLCO1B3 or SLCO1A2 cDNA in pBluescript SK+ (Stratagene, La Jolla, CA) were a kind gift by Dr. T. Abe (University Graduate School of Medical Science, Sendai, Japan). Inserted cDNA ranged from 5′-CTGTTGGTATCTGTA-3′ (position –23–9) to 5′-AATGCTGCTGCCAAC-3′ (position 2092–2106) for SLCO1B3 and 5′-CACCTGGAAGAGCAA-3′ (position –16–9) to 5′-TGTTATTGCCAACAG-3′ (position 1996–2010) for SLCO1A2 (positions correspond to OATP1B3 and OATP1A2 cDNA (accession NM_019844 and NM_134431, respectively), with the first base of the ATG start codon set to 1). Except for 1833G>A in SLCO1B3 (a previously described silent mutation (Gly611Gly); ref. 20), no other variations were detected. PCR products were digested with XhoI and ligated into the dephosphorylated 7.6 kb pLIV-LE6 fragment, yielding pLIV-LE6-SLCO1B3 and pLIV-LE6-SLCO1A2. Plasmid DNA was linearized, and irrelevant plasmid DNA was removed by restriction digestion with SalI and DdrI, followed by pronuclear injection into fertilized oocytes of FVB mice. Two-cell stage embryos were implanted into oviducts of pseudopregnant F1 fosters and carried to term. Positive founders were bred to homozygosity of the transgene locus, also in Slco1a/1b backgrounds.

PCR and Southern blot analysis

OATP1B3 and OATP1A2 transgenic founder lines were initially detected by PCR screen using the following primers: forward 5′-GAAGGCTAACCTGGGGTGAG-3′ and reverse 5′-CAGTTCCCATAAGGAGACAACC-3′ for OATP1B3 transgenics (yielding a 457 bp fragment), and forward 5′-GAAGGCTAACCTGGGGTGAG-3′ and reverse 5′-GGCTTGAATTCA-3′ for OATP1A2 transgenics (yielding a 217 bp fragment; Fig. 1A). Southern blot analysis was used for the definitive identification of transgenic founders, as well as for the differentiation between heterozygous and homozygous individuals. DNA was extracted from the tail tips of mice, digested with Ncol, and probed with an approximately 1.5-kb DraI pLIV-LE6-SLCO1B3 fragment or approximately 1.9 kb DraI/ScaI pLIV-LE6-SLCO1A2 fragment (Fig. 1B). Total protein staining (Ponceau S) confirmed equal loading across the lanes (Supplementary Fig. S1).

Figure 1. Generation and characterization of Slco1a/1b−/−;B1B1, Slco1a/1b−/−;B3B3, and Slco1a/1b−/−;A2A2 mice. A, structure of the ApoE promoter-HCR1–driven expression cassette, containing human SLCO1B1, SLCO1B3, or SLCO1A2 cDNA. Functional elements are presented approximately to scale. Translational start (ATG) and stop (TAA) codons, and reading frame direction (bold arrow) for the transgenes are indicated. Small arrows indicate the primers used for PCR detection (F, forward; R, reverse). Probes used for Southern blot analysis (1.9 kb for SLCO1B3 detection and 1.5 kb for SLCO1A2 detection). B, expression of human OATP1B1 (B1), -1B3 (B3), and -1A2 (A2) in liver, kidney, and small intestine of male (m) or female (f) wild-type, Slco1a/1b−/−;B1B1, Slco1a/1b−/−;B3B3, and Slco1a/1b−/−;A2A2 mice was detected by Western blotting. HCLM, human crude liver membrane; WT, wild-type; B1B1, Slco1a/1b−/−;B1B1; B3B3, Slco1a/1b−/−;B3B3; A2A2, Slco1a/1b−/−;A2A2. Crude membrane protein (20 μg) was analyzed for all fractions. A molecular mass marker of 85 kDa is indicated.
RNA isolation, cDNA synthesis, and real-time PCR

RNA isolation from mouse liver, kidney, and small intestine and subsequent cDNA synthesis and real-time PCR (RT-PCR) were conducted as described (21). Specific primers (Qiagen) were used to detect expression levels of the following mouse genes: Slco2b1, Slc10a1, Abcc2-4, Abcb1a, Abcb1b, Abcb1l, Abcg2, Aox1, Aox2, and Ugt1a1.

Western blot analysis

Isolation of crude membrane fractions from liver, kidney, and small intestine, and Western blot analysis were conducted as described previously (18). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. Human OATP1B1 and OATP1B3 were detected with the polyclonal ECL and SKT antibodies, respectively, kindly provided by Prof. Dr. D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany; refs. 7, 22). Human OATP1A2 was detected with antibody OATP1 (sc-18428; dilution 1:1,000) from Santa Cruz.

Immunohistochemical staining of OATP1B3

Livers from wild-type and Slco1a1b−/−;1B3tg mice were fixed in 4% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, and incubated with the rabbit polyclonal SKT antibody (1:250 in PBS) followed by horseradish peroxidase-labeled secondary antibody (Dako Denmark A/S). Nuclei were stained with hematoxylin and eosin according to the standard procedures.

Analysis of bilirubin in mouse plasma and urine

For the detection of bilirubin levels in wild-type, Slco1a1b−/−;1B3tg, Slco1a1b−/−;1B1tg, Slco1a1b−/−;1B3tg, and Slco1a1b−/−;1A2tg mice, we isolated hepatic plasma by cardiac puncture and urine by spot-collection. Ten microliters of ascorbate (100 mg/mL) was added to all samples to prevent the oxidation of bilirubin. All samples were immediately protected from the light, snap-frozen, and stored at −80°C until further analysis. Concentrations of bilirubin monoglucononides (BMG), bilirubin digluconuride (BDG), and unconjugated bilirubin (UCB) in plasma, bile, and urine were determined as described (23).

Pharmacokinetic experiments

For intravenous administration of methotrexate, the stock solution (100 mg/mL) was diluted with saline for administration of dose levels of 2 and 10 mg/kg, yielding final drug concentrations of 0.4 (2 mg/kg) and 2 mg/mL (10 mg/kg). A total of 5 μL/g body weight was injected as single bolus into the tail vein of mice (n = 3–6 for each group). Animals were sacrificed at indicated time points (15 or 30 minutes after methotrexate and paclitaxel dosage, respectively) by terminal bleeding through cardiac puncture under isoflurane anesthesia and tissues were isolated. Blood samples were centrifuged at 5,000 rpm for 5 minutes at 4°C, and plasma was collected and stored at −20°C until analysis.

Drug analysis

Amounts of methotrexate and its hydroxylated metabolite 7-hydroxymethotrexate (7OH-MTX) in plasma and organs [homogenized in ice-cold 4% (w/v) BSA] were determined by high-performance liquid chromatography (HPLC) analysis as described (24). Amounts of paclitaxel in plasma and organs [homogenized in ice-cold 4% (w/v) BSA] were determined using liquid–liquid and solid-phase extraction followed by reversed-phase HPLC with UV detection, as described before (25).

Statistical analysis

Statistical significance of differences between wild-type and Slco1a1b−/−;1B1tg, Slco1a1b−/−;1B3tg, Slco1a1b−/−;1B3tg, or Slco1a1b−/−;1A2tg mice, or between Slco1a1b−/− mice and Slco1a1b−/−;1B1tg, Slco1a1b−/−;1B3tg, or Slco1a1b−/−;1A2tg mice was assessed by one-way ANOVA followed by Dunnett multiple comparison test (or as indicated otherwise). Results are presented as the mean ± SD. Differences were considered to be statistically significant when P<0.05.

Study approval

All mouse studies were ethically reviewed and carried out in accordance with Dutch legislation, European Directive 86/609/EEC, and the GlaxoSmithKline policy on the Care, Welfare, and Treatment of Laboratory Animals. Mouse experiments were approved by the Animal Experimentation Committee (DEC) of the Netherlands Cancer Institute (Amsterdam, the Netherlands).

Results

Generation and characterization of humanized Oatp1a1b−/− knockout mice with liver-specific expression of human OATP1B1, OATP1B3, or OATP1A2

OATP1B3 and OATP1A2 transgenic mice were generated using an ApoE promoter-driven expression cassette, as described before for OATP1B1 liver-specific transgenic mice (18), and crossed back with Slco1a1b−/− mice to obtain homozygous humanized strains with predominant liver-specific expression of the transgenes (Fig. 1A). A partial physiologic characterization of the OATP1B1- and OATP1B3- (but not OATP1A2-) humanized mice was published recently (17). Lifespan, body weight, and liver weight of Slco1a1b−/−;1B1tg, Slco1a1b−/−;1B3tg, or Slco1a1b−/−;1A2tg mice were not different from those of wild-type mice. The expression levels of various endogenous transporters and metabolic enzymes (see Materials and Methods) in livers of the different mouse strains were analyzed by RT-PCR (Supplementary Methods and Supplementary Table
Only Abcc3/Mrp3 mRNA was substantially (2- to 4-fold) downregulated in all the humanized strains relative to the wild-type and Slco1a1b\(^{-/-}\) mice, but subsequent protein immunoblot analyses failed to reveal differences at the protein level (not shown).

Crude membrane fractions of liver, kidney, and small intestine of the humanized strains were analyzed for protein expression of the transgenes on the Western blots (Fig. 1B). Results have shown specific expression of OATP1B1 and OATP1A2 in livers of Slco1a1b\(^{-/-}\);B1\(^{15}\) and Slco1a1b\(^{-/-}\);A2\(^{15}\) mice, respectively. Only after very long exposure, minimal amounts of OATP1B1 were detected in kidneys of Slco1a1b\(^{-/-}\);B1\(^{15}\) mice (not shown). Slco1a1b\(^{-/-}\);B3\(^{15}\) mice showed abundant expression of OATP1B3 in the liver, whereas some expression of the transgene was detected in the kidney as well (Fig. 1C). Expression of the transgenic proteins was similar in male and female mice. Expression of OATP1B1 and OATP1B3 in livers of humanized mice was roughly comparable with that in a pooled human crude liver membrane fraction, but OATP1A2 expression in humanized mice was much higher than that in human liver (Fig. 1). Low levels of OATP1A2 in a total human liver preparation reflect the fact that this protein is restricted to cholangiocytes, which only form a small fraction of the total liver cell pool (9). In contrast, the transgenic promoter used by us (ApoE) drives expression of OATP1A2 in Slco1a1b\(^{-/-}\);A2\(^{15}\) mice in the abundant hepatocytes.

Immunohistochemical staining confirmed basolateral (sinusoidal) localization and disperse distribution of human OATP1B3 throughout the liver lobules of Slco1a1b\(^{-/-}\);B3\(^{15}\) mice (Fig. 2). This is similar to human liver, although, often a more zonal lobular distribution of OATP1B3 with higher expression around the central vein has been observed (22, 26, 27). In the kidney, we mainly observed cytoplasmic staining of OATP1B3 toward the basolateral side of the proximal tubular epithelium of Slco1a1b\(^{-/-}\);B3\(^{15}\) mice, with possibly some protein expression in the basolateral membrane, but not in the apical (luminal) membrane (Supplementary Fig. S2). We previously showed that the expression of human OATP1B1 in the livers of Slco1b1 transgenic mice was basolateral and throughout the liver lobule, albeit with stronger staining of OATP1B1 around the portal vein (18). Immunohistochemical staining confirmed a similar basolateral localization and lobular distribution of OATP1B1 in the liver of Slco1a1b\(^{-/-}\);B1\(^{15}\) mice (not shown). The basolateral localization of these OATPs throughout the liver lobule supports the physiologic relevance of these humanized models. OATP1B2 antibodies available to us did not work well in immunohistochemistry, but subsequent physiologic and pharmacologic analyses (see below) were in line with the basolateral expression and transport activity of transgenic OATP1A2 in hepatocytes, analogous to OATP1B1 and OATP1B3.

Analysis of plasma and urine levels of unconjugated and conjugated bilirubin

Preliminary partial physiologic characterization of Slco1a1b\(^{-/-}\);B1\(^{15}\) and Slco1a1b\(^{-/-}\);B3\(^{15}\) mice has shown almost complete reversal of the increased plasma and urine levels of bilirubin glucuronide seen in Slco1a1b\(^{-/-}\) mice (15, 17). Measurement of these parameters in Slco1a1b\(^{-/-}\);A2\(^{15}\) mice and comparison with the known results for Slco1a1b\(^{-/-}\);B1\(^{15}\) and Slco1a1b\(^{-/-}\);B3\(^{15}\) mice showed that the transgenic OATP1A2 also reversed the increased plasma and urine levels of bilirubin monogluconide, but not as strongly as OATP1B1 and OATP1B3 (Fig. 3). Interestingly, however, the modestly but reproducibly increased plasma levels of UCB in Slco1a1b\(^{-/-}\);A2\(^{15}\) mice were reversed at least as efficiently by OATP1A2 as by OATP1B1 or OATP1B3, and highly significantly (Fig. 3D). These results suggest that transgenic OATP1A2 transports bilirubin glucuronides into hepatocytes in vivo, but less efficiently than OATP1B1 and OATP1B3. In contrast, the clear decrease in plasma UCB suggests that OATP1A2 is a more efficient uptake transporter of UCB than OATP1B1 or OATP1B3 in the livers of the transgenic mice.

Role of OATP1A and 1B transporters in MTX disposition in vivo

To study the effect of human OATP1B1, OATP1B3, and OATP1A2 on methotrexate disposition in vivo, we intravenously administered 2 or 10 mg/kg of this drug to wild-type, Slco1a1b\(^{-/-}\), Slco1a1b\(^{-/-}\);B1\(^{15}\), Slco1a1b\(^{-/-}\);B3\(^{15}\), and Slco1a1b\(^{-/-}\);A2\(^{15}\) mice, and analyzed plasma, liver, and

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**Figure 2.** Immunolocalization of human OATP1B3 in the liver of Slco1a1b\(^{-/-}\);B3\(^{15}\) mice. Paraffin-embedded livers were sectioned (4 μm) and stained with a rabbit polyclonal antibody against human OATP1B3 (brown). Nuclei were stained with hematoxylin/eosin (blue). The picture shows a basolateral staining pattern throughout the liver lobule of Slco1a1b\(^{-/-}\);B3\(^{15}\) mice (A and B). Wild-type liver did not show significant background staining with the OATP1B3 antibody (data not shown). Scale bars are indicated.
intestinal (tissue plus contents) levels. The effect of removal of mouse Oatp1a/1b transporters on methotrexate disposition 15 minutes after dosing to \textit{Slco1a/1b/C0/C0} mice was very clear at both 2 and 10 mg/kg: plasma levels were 3.9- and 5.0-fold increased, liver accumulation was 17.6- and 23.8-fold decreased, and small intestinal accumulation was 7.5- and 20.3-fold decreased, respectively, compared with wild-type mice (Fig. 4A–F). In general, hepatic expression of OATP1B1, OATP1B3, or OATP1A2 in the transgenic strains resulted in substantial and highly significant, albeit not complete, rescue of the impaired liver uptake of methotrexate in \textit{Slco1a/1b/C0/C0} mice after intravenous administration of 2 and 10 mg/kg methotrexate, with liver levels 2.7- to 9.2-fold increased. Plasma levels of methotrexate were concomitantly 1.6- to 2.2-fold decreased in the transgenic strains, whereas small intestinal amounts of methotrexate were 1.6- to 5.6-fold increased (Fig. 4A–F). Reversal of liver and small intestinal methotrexate levels was more pronounced in the OATP1B3 and OATP1A2 transgenic strains than in the OATP1B1-humanized mice. The increased small intestinal methotrexate levels likely reflect higher hepatobiliary excretion due to the higher liver methotrexate levels, as intestinal excretion of methotrexate directly from blood is negligible in mice (28, 29). Very similar effects of transgenic OATP1B1, OATP1B3, and OATP1A2 were observed for the reversal of the strongly reduced liver and small intestinal levels of the main hepatic (and toxic) metabolite of methotrexate, 7-hydroxymethotrexate (7OH-MTX) in \textit{Slco1a/1b/C0/C0} mice after dosing of methotrexate at 10 mg/kg (Fig. 5). Tissue and plasma levels of 7OH-MTX at 2 mg/kg methotrexate and plasma levels at 10 mg/kg methotrexate were too low for quantification in all strains. Collectively, the bilirubin and methotrexate data described above support functional activity of each of the 3 human OATPs in the basolateral membrane of the transgenic hepatocytes.

![Figure 3. Analysis of plasma and urine from male wild-type, \textit{Slco1a/1b/C0/C0}, \textit{Slco1a/1b/C0/C0;1B1tg}, \textit{Slco1a/1b/C0/C0;1B3tg}, and \textit{Slco1a/1b/C0/C0;1A2tg} mice. Levels of total bilirubin (A), BMG (B), BDG (C), and UCB (D) in plasma and levels of BMG (E) and BDG (F) in urine are shown. Plasma was collected by cardiac puncture, whereas urine was collected by spot-sampling from nonanesthetized mice. Data are presented as mean ± SD (n = 5–8). One-way ANOVA followed by Tukey multiple comparison test was used to determine statistical significance (**, P < 0.01; ***, P < 0.001 when compared with wild-type mice; †††, P < 0.001 when compared with \textit{Slco1a/1b/C0/C0} mice). ND, not detectable; detection limit was 0.1 μmol/L. For statistical comparison of bars with values below the detection limit, we used values of 0.1 ± 0.1 μmol/L. For very low bars, the measured value is also presented above the bar. Note that part of these data included for comparison has been published before (17).]
Role of OATP1A and 1B transporters in PTX disposition in vivo

To study the possible independent effects of OATP1B1, OATP1B3, and OATP1A2 on paclitaxel disposition in vivo, OATP1B1, OATP1B3, or OATP1A2 humanized transgenic mouse strains were intravenously treated with 2 or 10 mg/kg paclitaxel. As we found before (16), liver levels of paclitaxel 30 minutes after administration were about 2-fold reduced in \( \text{Slco1a/1b}^{-/-} / \text{C0}/ \text{C0} \) mice compared with wild-type mice (Fig. 6B and E). Concurrently, plasma levels at this time point were modestly, but significantly, increased in the absence of Oatp1a/1b transporters (Fig. 6A and D), although differences with wild-type were somewhat smaller than observed before. The expression of transgenic OATP1A/1B transporters caused either no or small and rather erratic changes in plasma paclitaxel levels at both 2 and 10 mg/kg paclitaxel compared with the levels in \( \text{Slco1a/1b}^{-/-} / \text{C0} \) mice (Fig. 6A and D). However, at 2 mg/kg paclitaxel, the clearly reduced hepatic uptake of paclitaxel in \( \text{Slco1a/1b}^{-/-} / \text{C0} \) mice was restored to wild-type levels in \( \text{Slco1a/1b}^{-/-} / \text{1B1tg} \), \( \text{Slco1a/1b}^{-/-} / \text{1B3tg} \), and \( \text{Slco1a/1b}^{-/-} / \text{1A2tg} \) mice, but not in \( \text{Slco1a/1b}^{-/-} / \text{1B1tg} \) mice (Fig. 6B). At 10 mg/kg paclitaxel, only the OATP1A2 transgenic mice showed partial reversal of the reduced hepatic paclitaxel uptake, but not the OATP1B1- or OATP1B3-humanized mice, suggesting saturation of transgenic OATP1B3, but not OATP1A2 at this dosage. Modest alterations in the levels of paclitaxel in the small intestine of the various strains were, like the plasma levels, quite erratic and variable (Fig. 6C and F). This may reflect a combination of biliary excretion and the substantial direct intestinal

Figure 4. Influence of human OATP1B1, OATP1B3, and OATP1A2 transporters on methotrexate (MTX) disposition in vivo. Female wild-type, \( \text{Slco1a/1b}^{-/-} / \text{Slco1a/1b}^{-/-} / \text{1B1tg} \), \( \text{Slco1a/1b}^{-/-} / \text{1B3tg} \), and \( \text{Slco1a/1b}^{-/-} / \text{1A2tg} \) mice were i.v. dosed with methotrexate [2 mg/kg (A–C) or 10 mg/kg (D–F)] and drug concentrations in plasma (A, D), liver (B, E), and small intestine (including contents; C, F) 15 minutes after administration were determined. All data are presented as mean ± SD (\( n = 3–5; \), \( P < 0.05; \); \( P < 0.01; \); \( P < 0.001 \) when compared with wild-type mice; †, \( P < 0.05; \); ††, \( P < 0.01; \); †††, \( P < 0.001 \) when compared with \( \text{Slco1a/1b}^{-/-} / \text{C0} \) mice).
excretion of paclitaxel (from blood) by P-glycoprotein (16, 30). Nonetheless, especially the liver accumulation of anticancer drugs, directly affecting plasma and tissue pharmacokinetics. These observations also imply that the expression of one or more of the human OATP1A/1B transporters in various tumors in vivo may directly affect the drug uptake, and hence susceptibility of the tumor to the wide range of anticancer drugs that are substrates of these transporters (31). Moreover, genetic polymorphisms that affect the activity of these transporters may alter both tumor susceptibility and the plasma disposition, tissue distribution, and elimination of substrate anticancer drugs, and hence their overall therapeutic efficacy and risk of toxicity (32). The same applies to coadministered drugs that substantially inhibit activity of OATP1A/1B transporters through drug–drug interactions, or induce or repress the expression of these transporters.

We show that all 3 human OATP1A/1B transporters can substantially transport methotrexate in vivo, in line with, and extending previous in vitro results (6, 33, 34). Plasma levels were similarly decreased by the 3 transgenic transporters, but at both dose levels the effect of OATP1B1 on methotrexate liver and intestinal disposition was about 2- to 3-fold lower than that of OATP1B3 and OATP1A2 (Fig. 4). Although absolute quantification of the expression of the transgenes at the plasma membrane has not been carried out, our data suggest that in human liver both OATP1B1 and OATP1B3 can contribute to methotrexate pharmacokinetics. Note that the observed partial rescue in liver uptake of methotrexate by singly expressed OATP1B1 or OATP1B3 in the humanized transgenic mice probably underestimates the actual OATP1B1-mediated liver uptake in humans, as in human liver both proteins function at the same time, likely resulting in additive effects. Presumably, this would also apply to the effects of these transporters on plasma and small intestinal (content) levels of methotrexate in humans.

Paclitaxel is used in the treatment of various types of malignancies (e.g., breast, ovarian, and lung cancer; refs. 35, 36). Our results with the humanized OATP1A/1B transgenic mice indicate that OATP1B3 and OATP1A2, but not OATP1B1, can substantially transport paclitaxel in vivo into the liver, and that both transporters can at least partly compensate for the loss of the murine Oatp1a/1b transporters.

**Discussion**

We show here that human OATP1B1, OATP1B3, and OATP1A2 are functionally expressed in the basolateral membrane of hepatocytes of the 3 liver-specific humanized mouse strains we generated from Scoloa/1b<sup>−/−</sup> mice. Each could, to varying extents, rescue the markedly reduced hepatic uptake of bilirubin glucuronides and in part of UCB in Scoloa/1b<sup>−/−</sup> mice, thus affecting the plasma and tissue levels of these compounds. The same applies to hepatic uptake and plasma levels of methotrexate, whereas OATP1B3 and OATP1A2, but not OATP1B1, could rescue the reduced liver uptake of paclitaxel. These data show the substantial in vivo activity of the human OATP1A/1B transporters in hepatocellular uptake of 2 structurally diverse anticancer drugs, directly affecting plasma and tissue pharmacokinetics. These observations also imply that the expression of one or more of the human OATP1A/1B transporters in various tumors in vivo may directly affect the drug uptake, and hence susceptibility of the tumor to the wide range of anticancer drugs that are substrates of these transporters (31). Moreover, genetic polymorphisms that affect the activity of these transporters may alter both tumor susceptibility and the plasma disposition, tissue distribution, and elimination of substrate anticancer drugs, and hence their overall therapeutic efficacy and risk of toxicity (32). The same applies to coadministered drugs that substantially inhibit activity of OATP1A/1B transporters through drug–drug interactions, or induce or repress the expression of these transporters.

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approximately 7 μmol/L (~6 μg/mL) for paclitaxel transport by OATP1B3 (37), it is not surprising that OATP1B3 activity may have been saturated over the time period before 15 minutes after intravenous administration, when plasma levels must have been (far) higher than the approximately 7 μg/mL observed at 15 minutes (Fig. 6D). In the clinic, patients are generally treated with 5 to 6 doses of 135 or 175 mg/m² paclitaxel as a 3- or 24-hour infusion, every 3 weeks (40). Peak plasma concentrations after high-dose treatment (175 mg/m²) are about 5 or 0.4 μg/mL in patients receiving 3- or 24-hour infusions, respectively (41). In addition, despite extensive binding to plasma proteins (95%–98%), paclitaxel is readily cleared from the plasma. Plasma levels of paclitaxel in patients are thus generally well below the $K_m$ value of paclitaxel transport by OATP1B3 (and presumably also by OATP1A2), implying that these transport proteins could be significant determinants of paclitaxel pharmacokinetics and toxicokinetics (note that neutropenia in patients is related to paclitaxel plasma levels of more than 0.08 μg/mL; refs. 41, 42).

OATP1B1 and OATP1B3 are, in addition to OATP2B1, highly expressed at the basolateral membrane of hepatocytes and are, therefore, thought to be some of the most important hepatic (drug) uptake transporters (3, 32). Results from the present study indicate that human OATP1B1 and OATP1B3 are together involved in the hepatic uptake of methotrexate, whereas transporter-mediated liver uptake of paclitaxel may be primarily mediated by OATP1B3. Letschert and colleagues (2006) found that paclitaxel is not a substrate for human OATP2B1 in vitro.

Figure 6. Influence of human OATP1B1, OATP1B3, and OATP1A2 transporters on paclitaxel (PTX) disposition in vivo. Female wild-type, Slco1a1/sco1b1+/−, Slco1a1/sco1b1/−:1B1−/−;1B3−/−, and Slco1a1/sco1b1/−:1B1+/−:1A2−/− mice were intravenously dosed with paclitaxel [2 mg/kg (A–C) or 10 mg/kg (D–F)] and drug concentrations in plasma (A, D), liver (B, E), and small intestine (including contents; C, F) 30 minutes after administration were determined. All data are presented as mean ± SD (n = 3–6; *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type mice; †, P < 0.05; ††, P < 0.01; †††, P < 0.001 when compared with Slco1a1/sco1b1−/− mice).
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is expressed in hepatocytes in Because of the transgenic promoter used (ApoE), OATP1A2 uptake. murine Oatp2b1 is unlikely to contribute much to this uptake. Expression of OATP1A2 in the human liver is restricted to the cholangiocytes, the epithelial cells of the bile duct (9). Because of the transgenic promoter used (ApoE), OATP1A2 is expressed in hepatocytes in Sko1a1b−/−;1A2K mice and this model can therefore only be used to qualitatively study the in vivo transport capacity of compounds by human OATP1A2. Such information is still highly relevant, as abundant expression of OATP1A2 is found at the blood–brain barrier and at the apical membranes of the kidney tubules and small intestine (8–10), suggesting a possible role of this transporter in brain penetration and the reabsorption of urinary and intestinal compounds and/or drugs (including, e.g., methotrexate and paclitaxel).

Importantly, expression of OATPs has been detected in tumor cells as well. For example, OATP1B3 was found expressed in gastrointestinal cancers (gastric, pancreatic, and colon cancer; ref. 6), hepatocellular carcinomas (11), breast carcinomas (13), and lung cancer (12). In addition, expression of OATP1B1 and OATP1A2 was found in malignant breast cell lines (14, 43), and OATP1A2 (and OATP2B1) have also been localized in the blood–tumor barrier of gliomas (44). Recently, Takano and colleagues (2009) have shown in vitro that hepatoma cells made resistant to paclitaxel treatment showed, besides increased P-glycoprotein expression, drastically decreased mRNA expression of SLCO1B3 (45). Moreover, the immunoreactivity of OATP1B3 in breast carcinomas was inversely correlated with the tumor size and directly associated with improved effectiveness of tamoxifen therapy (46). These results suggest a potential link between expression of OATPs and tumor development and/or susceptibility to chemotherapy. The presence or absence of OATPs in tumor cells, therefore, might codetermine the sensitivity of these cells to methotrexate, paclitaxel, and many other anticancer drugs that are OATP substrates, as postulated earlier for methotrexate sensitivity of gastrointestinal cancers (6). Further studies with our humanized OATP1A1/1B mice may help to assess the in vivo importance of human OATP1A1/1B transporters in pharmacokinetics of many other anticancer drugs and their implications for therapeutic efficacy.

Many genetic polymorphisms affecting the functionality of OATP transporters have been identified (32). A recent genome-wide study showed that several SNPs in SLCO1B1 associated with reduced transport activity were linked with decreased plasma clearance and decreased gastrointestinal toxicity in children treated with methotrexate (47). Moreover, we recently discovered that individuals who suffer from the Rotor syndrome are fully deficient of both OATP1B1 and OATP1B3 activity (17). While the incidence of Rotor syndrome is very low (roughly estimated around 1 in 10⁶ individuals, depending on the population analyzed), we also found indications for a much higher incidence of single full OATP1B1 or OATP1B3 deficiencies in various populations (17). Again, roughly estimated, it would not surprise us if at least 1 in 10,000 individuals, if not more, in many human populations would be fully deficient for either OATP1B1 or OATP1B3 alone. Such full deficiencies may substantially affect the plasma, tissue, and tumor cell pharmacokinetics and toxicokinetics of administered OATP substrate drugs. Note also that such individuals, unlike Rotor subjects, will not be immediately recognizable from obvious jaundice (17).

The present study illustrates that human OATP1B1, OATP1B3, and/or OATP1A2 affect the in vivo pharmacokinetic behavior of 2 anticancer drugs that are near the extremes of the OATP1A/1B substrate spectrum: methotrexate as a polar, constitutively charged anion, and paclitaxel as a highly lipophilic, uncharged compound. Very likely, many drugs in the vast array of OATP1A/1B substrates with intermediate physicochemical properties will be similarly affected. The OATP1A/1B-humanized mice will therefore present important models to study such in vivo functions of OATP1A/1B proteins.

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Disclosure of Potential Conflicts of Interest
A.H. Schinkel has a commercial research grant from GlaxoSmithKline (S2918) and his research group receives revenue from the commercial distribution of some of the mouse strains used in this study. No potential conflicts of interest were disclosed by the other authors.

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Influence of Human OATP1B1, OATP1B3, and OATP1A2 on the Pharmacokinetics of Methotrexate and Paclitaxel in Humanized Transgenic Mice

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