Organic anion transporting polypeptide 1B transporters modulate hydroxyurea pharmacokinetics

Aisha L. Walker,1 Cynthia S. Lancaster,2 David Finkelstein,3 Russell E. Ware,4 and Alex Sparreboom1
1Department of Pharmaceutical Sciences, St. Jude Children’s Research Hospital, Memphis, Tennessee; 2Department of Information Sciences, St. Jude Children’s Research Hospital, Memphis, Tennessee; 3Department of Computational Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee; and 4Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio

Submitted 2 August 2013; accepted in final form 19 August 2013

Hydroxyurea is presently the only FDA-approved drug for the treatment of sickle cell anemia (SCA) that modifies the disease pathophysiology. The clinical benefits of hydroxyurea for SCA have been in large part attributed to the drug’s ability to increase fetal hemoglobin (3, 18, 23). Maximum benefits of the drug are thought to occur when the patient reaches a maximum tolerated dose, which is based on mild marrow suppression, typically neutropenia (31). Clinical studies have shown that the degree to which fetal hemoglobin is increased as well as the final maximum tolerated dose are highly variable among individuals with SCA (32). The etiology of this variation is poorly understood but is likely to be linked to the pharmacokinetics (PK) of hydroxyurea.

Hydroxyurea is a ribonucleotide reductase inhibitor that was initially developed and used as an antineoplastic agent. It is rapidly absorbed after oral administration with a reported bioavailability of 108% after an 80 mg/kg dose in patients with solid malignancies (26). Hydroxyurea is widely distributed and is excreted primarily in the urine as the parent compound (9). A fraction of hydroxyurea may undergo hepatic elimination. Possible metabolites that have been identified include carbon dioxide (CO2), nitric oxide (NO), and urea (1, 5, 11). However, the proportion of drug that is eliminated via the liver and extent of in vivo hydroxyurea metabolism are unclear (9). Great variation in hydroxyurea PK has been documented in populations of sickle cell patients showing 20–45% variation in area under the curve (AUC), maximum concentration, and clearance (8, 26, 32). Recent population PK analysis and modeling of hydroxyurea in adults with SCA reported that exposure varied by fivefold and the variation in hydroxyurea response was related in part to the PK (22). Because little is known about mechanisms that modulate the absorption, distribution, metabolism, and excretion of hydroxyurea, the potential sources of variation in hydroxyurea PK remain incompletely defined.

Various solute carrier (SLC) cell membrane transporters have been identified as key modulators of xenobiotic PK (12). Previously, an in vitro screen of hydroxyurea uptake by SLC transporter-overexpressing cells identified specific human SLC transporters that mediate the transmembrane movement of hydroxyurea (30). The human organic anion transporting polypeptide 1B (OATP1B) family of transporters was among the transporters identified. In humans, the OATP1B family of transporters consists of OATP1B1 and OATP1B3 transporters that mediate the transmembrane movement of various drugs such as methotrexate and various statins (14, 16, 19, 21, 27). The rodent ortholog of the human OATP1B transporters is Oatp1b (4). Substrates for OATP1B transporters identified specific human SLC transporters, have become useful models for predicting the impact of OATP1B transporters on the disposition of various drugs and substrates including methotrexate and bilirubin (13). In these models when the Oatp1b2 is absent, there is an increase in systemic docetaxel and bilirubin levels (7, 34). The estab-
lished role of OATP1B transporters in modulating PK of various drugs and the fact that hydroxyurea is substrate for these transporters led to the hypothesis that Oatp1b1 transporters may modulate hydroxyurea PK.

In the present study, the ability of OATP1B transporters to modulate hydroxyurea PK was evaluated in OATP transporter-deficient mice. Here we demonstrate for the first time that the absence of these drug transporters can significantly decrease systemic exposure, tissue accumulation, and elimination of hydroxyurea. This study highlights the importance of examining the role of transporters as sources of inter-patient variability in hydroxyurea PK.

**METHODS**

*In vitro analysis of hydroxyurea accumulation and protein binding assay.* Hydroxyurea accumulation was measured in oocytes injected with rodent Oatp1b2 transporter cRNA or water (BD Biosciences). Oocytes were incubated in transportocyte sodium buffer (pH 7.4; BD Biosciences) containing 50 μM [14C]hydroxyurea (American Radiolabeled Chemicals, St. Louis, MO) and washed four times in cold buffer. Individual oocytes were lysed in 10% SDS, and radioactivity was measured using liquid scintillation counter. For inhibition studies, 1 mM naringin (Sigma, St. Louis, MO) or rifampin (Sigma, St Louis, MO) dissolved in 1% DMSO solution or 1 mM methotrexate (Sigma, St Louis, MO) dissolved in water was added to hydroxyurea uptake medium. Inhibition is expressed as a percent of accumulation in the water-injected control oocytes.

Unbound fraction of hydroxyurea in mouse and human plasma was determined in vitro using an equilibrium dialysis procedure. Deidentified samples of human plasma were obtained from Blood Bank at St. Jude Children’s Research Hospital and mouse plasma was obtained from wild-type mice. Human or mouse plasma containing [14C]hydroxyurea (1 μCi/100 g: American Radiolabeled Chemicals) in saline. Mice were then placed in a water-sealed polyurethane breath chamber with air continuously drawn through a vapor trap (acetone and dry ice), bubbled through an acidic methanol solution, and finally through three gas-trapping washes containing 30 ml of gas-trapping solution composed of 27% (vol/vol) methanol, 41% toluene, 5% Emulsifier-Safe, and 27% phenylethylamine. Breath collection was performed at 5, 15, 30, 60, 90, and 120 min. Samples were analyzed using liquid scintillation counting. The values obtained were used to calculate 14CO2 exhaled during the collection period and are reported as the percentage of dose given to the mice. The experiments were performed in triplicate on six separate occasions, and statistical significance was determined by two-tailed paired t-test comparing the DBA WT to Oatp1b2−/− mice.

**RNA isolation and gene expression analysis.** Organs for RNA analysis were harvested from DBA-WT and Oatp1b1−/− mice following euthanasia, excision, and perfusion with 60 ml saline. The stomach and the duodenum (first 1.5 cm of small intestine) were excised and placed in RNA later (Invitrogen, Carlsbad, CA). RNA was isolated using an RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer protocol and quantified by Nanodrop (Thermo Scientific, Waltham, MA) spectrophotometry. The quality of the mRNA was determined using Affymetrix quality control methods, and gene expression was assessed using Affymetrix GeneChip Mouse Gene 1.0 ST. Gene expression was normalized across samples using Robust multiaarray analysis. Student’s t-test of unequal variance was performed to identify genes that were differentially expressed between DBA-WT and Oatp1b2−/− mice.

Real-time PCR analysis was performed to evaluate relative expression of Scl14A2 in the kidneys of wild-type and Oatp1b1−/− mice. Kidneys were harvested from untreated mice and RNA was isolated using an RNaseasy extraction kit (Qiagen). mRNA was reverse transcribed into cDNA using High Capacity RNA-to-cDNA Reverse Transcription Kit (Invitrogen). Taqman primers Assay no. Mm01261839_m1 (Invitrogen) were used in real-time PCR. For analysis, data were normalized to β-actin levels and relative quantity was determined by comparative CT method (ΔΔCT).

**RESULTS**

*In vitro hydroxyurea accumulation by Oatp1b2.* Our recent studies have shown that hydroxyurea is a substrate for the human OATP1B family of transporters (30). In vitro uptake studies were conducted to determine whether hydroxyurea is a substrate for rodent Oatp1b2 (rOatp1b2) transporter, the homolog of the human OATP1B1 and 1B3 transporters. In oocyte uptake experiments, hydroxyurea accumulation was significantly increased by fivefold in oocytes that overexpress rOatp1b2 transporter compared with control (P = 0.0001).
Hydroxyurea accumulation in Oatp1b2 oocytes was inhibited during coincubation with other substrates of the transporter, and the accumulation was not different from control oocytes that did not express the rOatp1b2 transporter (Fig. 1A). These data indicate that hydroxyurea is a strong substrate for Oatp1b2 transporter. Prior to conducting in vivo PK studies, protein binding of hydroxyurea in mouse and human serum was determined in vitro. Greater than 75% of hydroxyurea remains unbound to serum proteins in either mouse or human serum (Fig. 1B).

Oatp1b2 dependent absorption and systemic exposure in vivo. To examine the impact of the Oatp1b2 transporter on the disposition of hydroxyurea in vivo, systemic exposure (AUC) of hydroxyurea was evaluated in Oatp1a/1b−/− and Oatp1b−/− mice. Because OATP transporters are highly expressed in the GI tract and may have a role in absorption, we examined hydroxyurea PK in fasted FVB mice that were deficient in all Oatp1a and Oatp1b transporters. Plasma concentration of hydroxyurea was measured in mice after administration of 50 mg/kg hydroxyurea following a 3-h fast. In this model, AUC of hydroxyurea was significantly decreased by 22% in the Oatp1a/1b−/− mice (50.45 μg·h⁻¹·ml⁻¹) compared with the FVB WT mice (64.43 μg·h⁻¹·ml⁻¹) (P = 0.0185; Fig. 2A). To determine the impact of the Oatp1b transporters specifically, similar experiments were performed in mice with a targeted deletion of only the Oatp1b2. Following oral administration, AUC of hydroxyurea was significantly decreased by 20% in Oatp1b−/− mice (38.64 μg·h⁻¹·ml⁻¹) compared with DBA WT (48.45 μg·h⁻¹·ml⁻¹; P = 0.04), and there was a signifi-
significant reduction in maximum concentration in Oatp1b2−/− mice (25.06 μg/ml) compared with DBA WT (33.02 μg/ml; P = 0.021; Fig. 2B). Interestingly, when mice were not fasted prior to the PK studies, the differences in the systemic exposure between DBA WT and Oatp1b2−/− mice were negated (Fig. 2C). The AUC of hydroxyurea in DBA WT decreased to 31.17 μg·h·ml−1 in nonfasted DBA WT mice and was comparable to AUC measured in Oatp1b2−/− mice (Fig. 2D). Following intravenous injection of 50 mg/kg hydroxyurea, plasma levels detected in Oatp1b2−/− mice were no different from plasma levels in DBA WT (Fig. 2E).

Microarray analysis was conducted to evaluate possible compensatory expression of other SLC or ATP-binding cassette (ABC) transporter genes as well as cytochrome-P450 (CYP) and UDP glucuronosyltransferases (UGT) families of metabolic enzyme genes. Analysis of this gene subset demonstrated a near perfect linear correlation between gene expression for SLC14A2, with mean relative expression of 9.937 and 9.908 in DBA WT and Oatp1b2−/− mice, respectively (Fig. 3, A and B). This means that gene expression for SLC, ABC, CYP, and UGT families of genes was not different between the Oatp1b2−/− and DBA WT mice. Real-time PCR analysis of gene expression in the kidney of the mice confirmed similar expression of Slc14A2, with mean relative expression of 9.937 and 9.908 in DBA WT and Oatp1b2−/− mice, respectively. Together, these results indicate that the Oatp1b2 transporter may play an important role in the disposition of hydroxyurea and suggest that the absence of Oatp1b2 transporters may impact hydroxyurea absorption.

** Elimination of hydroxyurea in the Oatp1b2 knockout mouse model.** The Oatp1b2 transporter is expressed in both the kidney and the liver of mice and may play a role in the elimination of hydroxyurea. Therefore, the contribution of Oatp1b2 to renal and hepatic elimination of hydroxyurea was evaluated in Oatp1b2−/− mice. Reports that a substantial fraction of orally absorbed hydroxyurea is excreted primarily through the urine prompted the study of renal elimination, in which the accumulation of hydroxyurea in kidney and urine of mice was measured. Two hours after oral administration, renal accumulation of hydroxyurea was significantly less in Oatp1b2−/− mice compared with DBA WT mice (P = 0.0006), with mean levels measuring 356.9 and 748.1 pmol/g, respectively (Fig. 4A).

Calculation of kidney-to-plasma ratio resulted in Oatp1b2−/− mice having a mean ratio of 2.2 while DBA WT mice had a significantly higher ratio of 3.0 (P = 0.04; Fig. 4B). While both ratios are greater than 1, which suggests that a large portion of hydroxyurea accumulates in the kidney compared with plasma, the difference in ratios of Oatp1b2−/− compared with DBA WT mice indicates that decreased renal accumulation is not merely a result of decreased circulating levels of hydroxyurea. There may also be Oatp1b2-dependent mechanisms in the kidney that accounts for the twofold decrease of renal hydroxyurea accumulation. As expected based on renal accumulation differences, a significant decrease in hydroxyurea accumulation in the urine of Oatp1b2−/− mice was detected (P = 0.02; Fig. 4C).

Hepatic elimination was examined by measuring hydroxyurea accumulation in the liver as well as the production of 14CO2 metabolite. Accumulation in the livers of the Oatp1b2−/− mice was slightly less than accumulation in DBA WT mice, which measured 107.3 and 136.6 pmol/g, respectively (Fig. 5A). This modest difference was statistically significant (P = 0.05), but the liver-to-plasma ratio was not different between the two groups, with mean ratios <1 (Fig. 5B). Because hydroxyurea may be metabolized by liver enzymes to form CO2 metabolite (1), the amount of exhaled 14CO2 was measured after [14C]hydroxyurea administration using a breath test assay. Less than 5% of the administered dose of hydroxyurea was exhaled as the 14CO2 metabolite over
porters are involved in the renal and hepatic processes of excretion. Studies of hydroxyurea elimination indicate that Oatp1b2 transporters are important for determining hydroxyurea PK in vivo. Results of the present study support this hypothesis, showing that mice deficient in the Oatp1b transporter have an altered disposition of hydroxyurea, specifically differences in systemic exposure and excretion. These PK studies provide the first evidence that transmembrane transporters may be important modulators of hydroxyurea PK in vivo and could help explain interindividual PK differences.

The modulators of hydroxyurea PK have not been elucidated to date, but the present study provides compelling evidence that OATP1B transporters may be involved. OATP1A and OATP1B transporters were found to mediate hydroxyurea intracellular accumulation in previous studies. Here, hydroxyurea PK was also evaluated in both Oatp1a/1b−/− and Oatp1b−/− mice. In both mice genotypes, a significant decrease in systemic exposure was observed compared with wild-type mice (Fig. 2). In contrast to the Oatp1b−/−, larger differences in hydroxyurea concentrations between Oatp1a/1b−/− and wild-type mice were noted at the earlier time points. The exaggerated differences at the early time points suggest a contribution of the Oatp1a transporters in addition to the Oatp1b transporters for affecting hydroxyurea PK. Because of the differences noted at early time points and the fact that Oatp1a transporters are predominantly found in the small intestine, it is most likely these transporters contribute to absorption of hydroxyurea (13). During in vivo experiments to study a specific transporter, compensatory transporters can sometimes mask the effects of the transporter of interests. In Oatp1b−/− mice, after confirming the lack of compensatory expression of Oatp1a and other SLC transporters in the stomach and duodenum, we conclude that the decrease in hydroxyurea AUC in Oatp1b−/− is at least partially mediated by Oatp1b2 transporters. Despite the possibility of increased function or affinity of other transporters, a 20% decrease in hydroxyurea AUC in the absence of Oatp1b2 was observed, suggesting that this transporter has an influential role in determining the PK of hydroxyurea.

The findings of this study are likely relevant in identifying modulators of hydroxyurea disposition in humans. Because of the functional homology that has been demonstrated between the rodent Oatp1b2 transporters and human OATP1B1 and 1B3 transporters (29), the Oatp1b knockout mouse provides a suitable model for investigating the PK of hydroxyurea. Based on what is currently known, this mouse model recapitulates the PK of hydroxyurea. Our data confirm that hydroxyurea is rapidly absorbed and excreted primarily through the kidneys, with some evidence of hepatic elimination including the excretion of CO2 metabolite (1, 9). In this study, the PK profiles of the mice demonstrated rapid absorbance with detectable levels of hydroxyurea in plasma as early as 5 min and concentrations peaking around 60 min after an oral dose (Fig. 2). It was also observed that the primary route of excretion was through renal elimination as indicated by the high kidney-to-plasma ratio and the high percentage of hydroxyurea measured in the urine (Fig. 4). Furthermore, a small degree of hepatic elimination of hydroxyurea was measured in these murine models as noted by liver accumulation and excretion of the CO2 metabolite (Fig. 5). The absence of the Oatp1b2 created a notable change in the distribution and elimination of hydroxyurea in this model. Because the model recapitulates key aspects of human hydroxyurea disposition, the results presented here suggest that Oatp1b transporters may be modulators of hydroxyurea PK in humans.

The results from this study also indicate a potential role for OATP1B transporters beyond their function in hepatic uptake and elimination. OATP1B transporters are highly expressed on the sinusoidal membrane of hepatocytes and are generally thought to be liver specific. These transporters have been implicated in the hepatic elimination of bilirubin and various xenobiotics. The absence of the Oatp1a/1b transporters in a mouse model resulted in the increase in plasma and urinary concentrations of hydroxyurea.
levels of bilirubin. There is also a significant increase in the plasma and decrease in the liver and intestine of methotrexate and paclitaxel and (28, 29). In the present study, hepatic accumulation of hydroxyurea was found to be minimal, yet there was a significant change in systemic exposure. Analysis of mRNA expression resulted in the detection of the transcript at low levels in both the duodenum and the stomach, which is consistent with other studies (20). Microarray analysis verified that no other transporter transcripts were increased or decreased in Oatp1b<sup>−/−</sup> compared with the DBA WT mice in the stomach and duodenum (Fig. 3). However, the change occurred only after oral administration and not after intravenous injection, indicating a potential role in for the Oatp1b transporters in gastrointestinal drug absorption.

A low level of Slco1b2 transcript has been previously detected in the kidney of DBA mice (20). Previous microarray analysis showed that expression of transporter genes in the kidneys of wild-type and Oatp1b<sup>−/−</sup> mice is similar with the exception of Slc14a2, which was increased in Oatp1b<sup>−/−</sup> mice (17). Because Slc14a2 transcript encodes urea transporter A (UTA), a protein that has been shown to mediate cellular entry of hydroxyurea (30), real-time analysis was conducted to evaluate the relative expression of this gene in wild-type and Oatp1b<sup>−/−</sup> mice. In these studies, differential expression of Slc14a2 or UTA protein was not detected by real-time PCR, suggesting that there was no compensatory upregulation of UTA in Oatp1b<sup>−/−</sup> mice. Since no changes in transporter or enzyme expression in the kidney were detected, the significant decrease in renal accumulation and urinary excretion observed in Oatp1b<sup>−/−</sup/>mice indicate a potential role for Oatp1b2 transporters in the kidney. Taken together, these results suggest a potential role for the OATP1B transporter separate from its documented role in the liver. This finding is supported by studies by Ramsey and colleagues, which showed that the presence of functional variants in the OATP1B1 transporter was associated with changes in the clearance of methotrexate (24, 25), a drug that mostly is eliminated via urinary excretion (2).

The clinical translation of these results could potentially involve in-depth pharmacogenetic studies. Here, the OATP1B family of transporters was tested, yielding results that provide proof-of-concept that transporters may be important modulators of hydroxyurea PK. If these transporters and others are important for determining PK properties of hydroxyurea, then pharmacogenetic analysis of mutations and polymorphisms could help explain interpatient variability. It is important to investigate the role of relevant transporters and their transporter functional variants in modulating hydroxyurea PK. Pharmacogenetic studies related to cancer therapies have identified specific variants in OATP1B transporters that are associated with drug disposition. In addition, although urea transporter function in hydroxyurea PK has not been evaluated in vivo, hydroxyurea pharmacogenetic analysis in pediatric sickle cell patients has identified significant associations between genetic variants of the urea transporters and AUC and maximum concentration of hydroxyurea (32). Additional pharmacogenetic studies to investigate PK changes related to transporter variants are needed for evaluation of hydroxyurea use in patients with sickle cell anemia.

In conclusion, increased knowledge about mechanisms driving pharmacologic efficacy of hydroxyurea is needed to increase the effectiveness of hydroxyurea therapy for individuals with SCA. Because drug efficacy may be directly related to drug disposition, identification of mechanisms that modulate hydroxyurea PK will play an important role in understanding and improving hydroxyurea efficacy. Results from the present study suggest an influential role of transporters in mediating hydroxyurea PK. Though these studies do not fully elucidate how OATP1B transporters impact the specific processes of absorption, distribution, and renal excretion of hydroxyurea, these studies clearly demonstrate that the absence of functional OATP1B transporters can decrease systemic exposure of hydroxyurea. This knowledge is important for future studies seeking to understand the interindividual variability of hydroxyurea PK and efficacy, and it may contribute to strategies for improving hydroxyurea treatment for sickle cell patients.

**ACKNOWLEDGMENTS**

The authors acknowledge the Animal Resource Center and Hartwell Center for Bioinformatics at St. Jude Children’s Research Hospital for assistance in these research studies. We thank Richard Kim and Jeffrey Stock for providing the Oatp1b<sup>−/−</sup> Oatp1b<sup>−/−</sup> mice.

**GRANTS**

This study was supported by the American Lebanese Syrian Associated Charities (ALSAC), National Cancer Institute Cancer Center Support Grant P30 CA-021765, and the Digsg Cell Sickle Cell Scholar Fellowship.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

C1229


25. Rolled ..........................