Organic anion transporting polypeptide 1B (OATP1B) transporters modulate hydroxyurea pharmacokinetics

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Abstract

Hydroxyurea is currently the only FDA-approved drug that ameliorates the pathophysiology of sickle cell anemia. Unfortunately, substantial inter-patient variability in the pharmacokinetics (PK) of hydroxyurea may result in variation of the drug’s efficacy. However, little is known about mechanisms that modulate hydroxyurea PK. Recent *in vitro* studies identifying hydroxyurea as a substrate for organic anion transporting polypeptide (OATP1B) transporters prompted the current investigation assessing the role of OATP1B transporters in modulating hydroxyurea PK. Using wild-type and Oatp1b knockout (Oatp1b-/-) mice, hydroxyurea PK was analyzed *in vivo* by measuring $^{14}$C-hydroxyurea distribution in plasma, kidney, liver, urine, or the exhaled $^{14}$CO$_2$ metabolite. Plasma levels were significantly reduced by 20% in Oatp1b-/- mice compared to wild-type (AUC of 38.64 μg-hr/ml or 48.45 μg-hr/ml, respectively) after oral administration; whereas no difference was observed between groups following intravenous administration. Accumulation in the kidney was significantly decreased by 2-fold in Oatp1b-/- mice (356.9 pmol/g vs. 748.1 pmol/g), which correlated with a significant decrease in urinary excretion. Hydroxyurea accumulation in the liver was also decreased (136.6 pmol/g vs. 107.3 pmol/g in wild-type or Oatp1b-/- mice, respectively) correlating with a decrease in exhaled $^{14}$CO$_2$. These findings illustrate that deficiency of Oatp1b transporters alters the absorption, distribution, and elimination of hydroxyurea thus providing the first *in vivo* evidence that cell membrane transporters may play a significant role in modulating hydroxyurea PK. Future studies to investigate other transporters and their role in hydroxyurea disposition are warranted for understanding the sources of variation in hydroxyurea’s PK.
Introduction

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 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 is 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 anti-neoplastic 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 (10). 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, 12). However, the proportion of drug that is eliminated via the liver and extent of in vivo hydroxyurea metabolism is unclear (10). Great variation in hydroxyurea PK has been documented in populations of sickle cell patients showing 25-45% variation in 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 5-fold 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 (9). Previously, an in vitro screen of hydroxyurea uptake by SLC transporter over-expressing cells identified specific human SLC transporters that mediate the transmembrane movement of hydroxyurea (30). The human Organic Anion Transporting Polypeptide 1B (OATP1) family of transporters was among the transporters identified. In humans, the OATP1B family of transporters consists of OATP1B1 and OATP1B3 transporters that are encoded by SLCO1B genes (11). Predominantly expressed in the liver on the sinusoidal membrane of hepatocytes(15), these transporters are important for regulating the elimination of endogenous substrates such as bilirubin and can impact the disposition
of drugs such as methotrexate and various statins (14, 16, 19, 21, 27). The rodent ortholog of the human OATP1B transporters is Oatp1b2 (4). Substrates for Oatp1b2 as well as the function of the Oatp1b2 are similar to those of the human transporters (6). Mouse models deficient for Oatp1b2, or deficient for both Oatp1b2 and Oatp1a 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 established 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 Oatp1b 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 HU 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 50µM 14C-Hydroxyurea (American Radiolabeled Chemicals, St. Louis, MO) and washed 4 times in cold buffer. Individual oocytes were lysed in 10% SDS and radioactivity was measured by liquid scintillation counter. For inhibition studies, 1mM of naringin (Sigma, St Louis, MO) or rifampin (Sigma, St Louis, MO) dissolved in 1% DMSO solution or 1mM 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. De-identified 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 at concentrations ranging from 1.5µM to 500µM was incubated at 37°C in an equilibrium dialysis plate containing a dialysis membrane with a molecular
weight cut off of 5KD (Harvard Apparatus, Holliston, MA). PBS was added to the opposite side of the
dialysis membrane. After a 6 hour incubation period, an aliquot was taken from each side of the dialysis
membrane, and the radioactivity was measured using a liquid scintillation counter. The unbound
fraction of hydroxyurea was calculated as the percent $^{14}$C-hydroxyurea measured in 1xPBS compartment
relative to the initial amount in the plasma compartment. The final reported unbound fraction is the
average percentage measured in 4 replicates of 3 different samples of mouse or human plasma.

Hydroxyurea PK in mouse models

Hydroxyurea PK were assessed in 8-12 week old female Oatp1b2-knockout mice (Oatp1b2-/-) (34) and
age matched wild-type mice on a DBA1/lacJ background (DBA WT), and 8-12 week old female
Oatp1a/1b knockout mice (Oatp1a/1b-/-) and age matched wild-type mice on an FVB background (FVB
WT) (Taconic Farms Inc, Germantown, NY). All mice were housed in a temperature-controlled
environment with a 12-hour light cycle and given a standard diet and water ad libitum. Experiments
were approved by the Institutional Animal Care and Use Committee of St. Jude Children’s Research
Hospital. For PK experiments all mice received a dose of 50mg/kg hydroxyurea spiked with $^{14}$C-
hydroxyurea via tail vein injection or oral gavage. Blood samples were collected at 5, 15, 30, 60, 90, and
120 minutes after drug administration from each animal. Approximately 50μl blood samples were
obtained from the facial vein, retro orbital plexus, and terminal cardiac puncture. At the terminal 120
minute time point, liver and kidneys were excised from each mouse. Plasma from the blood samples
was isolated and radioactivity in each sample was measured using a liquid scintillation counter. Tissues
were homogenized in homogenization buffer containing 100mM Tris-Base, 100mM potassium Chloride,
1mM EDTA and 20μM butylated hydroxytoluene. Radioactivity was then measured in the tissue samples
by liquid scintillation counter. To determine urinary concentration of hydroxyurea, mice were given a
single 50mg/kg oral dose of hydroxyurea and place in a metabolic cage for 72 hours to collect the urine.
Cumulative urine concentration was calculated based on radioactivity measurements taken at 5, 24, 48,
and 72 hours after drug administration. Analytical analysis confirmed that radioactivity measured in
tissues and plasma represented the parent compound and not the metabolites of hydroxyurea.
Student’s t-tests comparing hydroxyurea levels in knockout mice and wild-type mice were performed to
detect statistically significant differences.
**14C-Hydroxyurea Breath Test**

The amount of exhaled CO₂ metabolite of hydroxyurea was measured using a breath test as described elsewhere (16, 33). Briefly, the mice received a tail vein injection of 50mg/kg hydroxyurea containing 14C-hydroxyurea (American Radiolabeled Chemicals) (1 μCi/100 g) 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% phenethylamine. Breath collection was performed at 5, 15, 30, 60, 90, and 120 minutes. Samples were analyzed using liquid scintillation counting. The values obtained were used to calculate 14CO₂ exhaled during the collection period and reported as the percent of dose given to the mice. The experiments were performed in triplicate on 6 separate occasions and statistical significance was determined by 2-tailed paired t-test comparing the DBA WT to Oatp1b2-/-.

**RNA isolation and gene expression analysis**

Organs for RNA analysis were harvested from DBA-WT and Oatp1b2-/- mice following euthanasia, exsanguination, 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 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 by 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 multi-array analysis. Student’s t-test of unequal variance was performed to identify genes that were differentially expressed between DBA-WT and Oatp1b2-/-.

Real-time PCR analysis was performed to evaluate relative expression of Slc14A2 in the kidneys of wild-type and Oatp1b2-/- mice. Kidneys were harvested from untreated mice and RNA was isolated using RNeasy extraction kit (Qiagen). mRNA was reverse transcribed into cDNA using High Capacity RNA-to-cDNA Reverse Transcription Kit (Invitrogen). Taqman primers Assay # Mm01261839_m1 (Invitrogen) were used in real-time PCR. For analysis, data was normalized to β-actin levels and relative quantity was determined by Comparative CT (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 if 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 5-fold in oocytes that over-express rOatp1b2 transporter compared to control (p = 0.0001). Hydroxyurea accumulation in Oatp1b2 oocytes was inhibited during co-incubation with other substrates of the transporter and the accumulation was not different from control oocytes that did not express the rOatp1b2 transporter (Figure 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 (Figure 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 Oatp1b2-/- 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 administering 50 mg/kg hydroxyurea following a 3 hour fast. In this model, AUC of hydroxyurea was significantly decreased by 22% in the Oatp1a/1b-/- mice (50.45 μg-hr/ml) compared to the FVB WT mice (64.43 μg-hr/ml) (p = 0.0185; Figure 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 is significantly decreased by 20% in Oatp1b2-/- mice (38.64 μg-hr/ml) compared to DBA WT (48.45 μg-hr/ml; p = 0.04), and there was a significant reduction in maximum concentration (C_{max}) in Oatp1b2-/- mice (25.06 μg/ml) compared to DBA WT (33.02 μg/ml; p = 0.021; Figure 2B). Interestingly, when mice were not fasted prior to the PK studies, the differences in the systematic exposure between DBA WT and Oatp1b2-/- mice were negated (Figure 2C). The AUC of hydroxyurea in DBA WT decreased to 31.17 μg-hr/ml in non-fasted DBA WT mice and was comparable to AUC measured in Oatp1b2-/- mice.
Following intravenous injection of 50 mg/kg hydroxyurea, plasma levels detected in Oatp1b2-/- mice were no different from plasma levels in DBA WT (Figure 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 of Oatp1b2-/- and DBA WT mice in both the stomach and duodenum with r-squared values of greater than 0.99 in both tissues (Figure 3A-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 oat1b2 transporter may play an important role in the disposition of hydroxyurea and suggest that the absence of Oatp1b transporters may impact hydroxyurea absorption.

Elimination of hydroxyurea in the Oatp1b2 knockout mouse model

The Oatp1b2 transporter is expressed in both the kidney and 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 to DBA WT mice (p=0.0006) with mean levels measuring 356.9 pmol/g and 748.1 pmol/g, respectively (Figure 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; Figure 4B). While both ratios are greater than 1, which suggests that a large portion of hydroxyurea accumulates in the kidney compared to plasma, the difference in ratios of Oatp1b2-/- compared to 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 2-fold 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; Figure 4C).
Hepatic elimination was examined by measuring hydroxyurea accumulation in the liver as well as the production of $^{14}$CO$_2$ metabolite. Accumulation in the livers of the Oatp1b2-/- mice was slightly less than accumulation in DBA WT which measured 107.3 pmol/g and 136.6 pmol/g, respectively (Figure 5A). This modest difference was statistically significant (p=0.05), but the liver to plasma ratio is not different between the two groups with mean ratios less than 1 (Figure 5B). Because hydroxyurea may be metabolized by liver enzymes to form CO$_2$ metabolite (1), the amount of exhaled $^{14}$CO$_2$ was measured after $^{14}$C-hydroxyurea administration using a breath test assay. Less than 5% of the administered dose of hydroxyurea was exhaled as the $^{14}$CO$_2$ metabolite over a 2 hour period in the DBA WT mice and this amount metabolite was decreased in the Oatp1b2-/- mice (P=0.035; Figure 5C). These studies of hydroxyurea elimination indicate that Oatp1b2 transporters are involved in the renal and hepatic processes of excretion and/or metabolism of hydroxyurea.

**Discussion**

Numerous recent studies have demonstrated that transporters play an integral role in modulating the PK of endogenous substrates as well as xenobiotics. Previously, our *in vitro* studies were the first to identify specific SLC transporters that mediate the transmembrane movement of hydroxyurea (30). Here, it was hypothesized that SLC transporters may be 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 inter-individual 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 Oatp1b2-/- mice. In both mice genotypes, a significant decrease in systemic exposure was observed compared to wild-type (Figure 2). In contrast to the Oatp1b2-/-, larger differences in hydroxyurea concentrations between Oatp1a/1b-/- and wild-type 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 Oatp1b2 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 Oatp1b2-/ 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 Oatp1b2/- 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, 10). In this study, the PK profiles of the mice demonstrated rapid absorbance with detectable levels of hydroxyurea in plasma as early as 5 minutes, and concentrations peaking around 60 minutes after an oral dose (Figure 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 percent of hydroxyurea measured in the urine (Figure 4). Further, 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 (Figure 5). The absence of the oatp1a/1b transporters in a mouse model resulted in the increase in plasma and urinary 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 stomach which is consistent
with other studies (20). Microarray analysis verified that no other transporter transcripts were increased or decreased in Oatp1b2-/- compared to the DBA WT mice in the stomach and duodenum (Figure 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 transporters genes in the kidneys of wild-type and Oatp1b2-/- mice are similar with the exception of Slc14a2 which was increased in Oatp1b2-/- mice (17). Because Slc14a2 transcript encodes urea transporter 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 Oatp1b2-/- 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 Oatp1b2-/- 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 Oatp1b2-/- 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 et al, which showed that the presence of functional variants of 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 inter-patient 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 pharmacogenetics 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 inter-individual variability of hydroxyurea PK and efficacy, and may contribute to strategies for improving hydroxyurea treatment for sickle cell patients.

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Figure Legends

Figure 1. Hydroxyurea cellular accumulation and protein binding in vitro. (A) Intracellular accumulation of hydroxyurea in oocytes that over-express rodent Oatp1b2 transporters or water-injected controls after 30 minutes incubation in the presence or absence of Oatp1b2 inhibitors. (n= 8-12; * P= 0.0001 compared to all other conditions) (B) Fraction of unbound hydroxyurea in mouse or human plasma as determined by equilibrium dialysis (n= 3).

Figure 2. Plasma PK of hydroxyurea in vivo. (A) Plasma concentration of hydroxyurea in Oatp1a/1b-/- mice (black open circle) compared to FVB wild-type (gray diamond) mice following oral administration (n= 4). (B) plasma concentration of hydroxyurea in Oatp1b2-/- mice (black triangle) compared to DBA wild-type mice (gray square) following oral administration after a 3 hour food fast (n= 9-12) and (C) after non-fasting conditions. (D) Systemic exposure in DBA wild-type and Oatp1b2-/- with and without fasting (*P= 0.0009 compared to all other conditions). (E) Plasma concentration of hydroxyurea in DBA wild-type and Oatp1b2-/- mice following intravenous administration.

Figure 3. Microarray analysis of mRNA expression in stomach and duodenum of mice. Comparison of SLC, ABC, CYP, and UGT gene expression in the stomach (A) and duodenum (B) of DBA wild-type and Oatp1b2-/- mice shows near perfect correlation in all genes (r^2 > .99).

Figure 4. Renal accumulation and urinary excretion of hydroxyurea. (A) Hydroxyurea accumulation in the kidneys of DBA wild-type and Oatp1b2-/- mice (*** P= 0.0006). (B) Kidney to plasma ratio of hydroxyurea levels in DBA wild-type and Oatp1b2-/- mice (* P= 0.04). (C) Hydroxyurea accumulation in the urine of DBA wild-type (gray squares) and Oatp1b2-/- (black triangles) mice (n=5; **P=0.02)

Figure 5. Hepatic accumulation and exhaled CO2 metabolite of hydroxyurea. (A) hydroxyurea accumulation in liver of DBA wild-type and Oatp1b2-/- mice (P=0.05). (B) Liver to plasma ratio of hydroxyurea in DBA wild-type and Oatp1b2-/- mice. (C) Cumulative amount of ^14CO2 exhaled by DBA wild-type (gray squares) and Oatp1b2-/- (black triangles) mice measured by breath test (n=5-10; *P= 0.035)
Figure 1

A

HU accumulation (% of Control)

B

Fraction of Unbound Hydroxyurea (%)
Figure 2

A

B

C

D

E
Figure 4

(A) HU accumulation (pmol/kg tissue) for DBA WT and Oatp1b2−/− mice.

(B) Kidney plasma ratio for DBA WT and Oatp1b2−/− mice.

(C) Hydroxyurea in urine (% of initial dose) over time (hours) for DBA WT and Oatp1b2−/− mice.
Figure 5