Low molecular weight acids and OATP1B mediated hepatic clearance: In vitro and in vivo evaluation using novel hypoxia-inducible factor prolyl hydroxylase inhibitors (Dustats)

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ABBREVIATIONS: AUC, area under the plasma concentration-time curve; CKD, chronic kidney disease; CL, clearance; DDI, drug-drug interactions; ECCS, extended clearance classification system; \( f_{\text{OATP1B}} \), fraction transported by OATP1B; HEK, human embryonic kidney; HIF-PH, hypoxia-inducible factor prolyl hydroxylase; IC\(_{50}\), inhibition potency; MW, molecular weight; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; PK, pharmacokinetics; SCHH, sandwich culture human hepatocytes; VD\(_{ss}\), volume of distribution; \( t_{1/2} \), effective half-life;
ABSTRACT

Organic anion transporting polypeptide (OATP1B) plays a key role in the hepatic clearance of a majority of high molecular weight (MW) acids and zwitterions. Here, we evaluated the role of OATP1B-mediated uptake in the clearance of novel hypoxia-inducible factor prolyl hydroxylase inhibitors ("Dustats"), which are typically low MW (300-400 daltons) aliphatic carboxylic acids. Five acid dustats, namely daprodustat, desidustat, enarodustat, roxadustat and vadadustat, showed specific transport by OATP1B1/1B3 in transporter-transfected HEK293 cells. Neutral compound, molidustat, was not a substrate to OATP1B1/1B3. None of the dustats showed transport by other hepatic uptake transporters, including NTCP, OAT2 and OAT7. In the primary human hepatocytes, uptake of all acids was significantly reduced by rifampin (OATP1B inhibitor); with an estimated fraction transported by OATP1B ($f_{\text{OATP1B}}$) of up to >80% (daprodustat). Molidustat uptake was minimally inhibited by rifampin; and low permeability acids (desidustat and enarodustat) also showed biliary efflux in sandwich culture human hepatocytes. In vivo, intravenous pharmacokinetics of all 5 acids was significantly altered by a single-dose rifampin (30 mg/kg) in Cynomolgus monkey. Hepatic clearance (non-renal) was about 4-fold (vadadustat) to >11-fold (daprodustat and roxadustat) higher in control group compared to rifampin-treated subjects. In vivo $f_{\text{OATP1B}}$ was estimated to be ~70-90%. In the case of molidustat, rifampin had a minimal effect on overall clearance. Rifampin also considerably reduced volume of distribution of daprodustat and roxadustat. Overall, OATP1B significantly contribute to the hepatic clearance and pharmacokinetics of several dustats, which are low MW carboxylic acids. OATP1B activity should therefore be evaluated in this property space.
Significance statement

Our in vitro and in vivo results suggest that OATP1B-mediated hepatic uptake play a significant role in the pharmacokinetics of low MW acidic dustats, which are being developed or are approved for the treatment of anemia in chronic kidney disease. Significant active uptake mechanisms are not apparent for the neutral compound, molidustat. Characterization of uptake mechanisms is therefore important in predicting human pharmacokinetics and evaluating drug-drug interactions for low MW acids.
INTRODUCTION

Extended clearance classification system (ECCS) framework, based on drug properties such as ionization, permeability, and molecular weight (MW), suggest that OATP1B-mediated uptake acts as rate-determining step in the hepatic clearance of acids and zwitterions with MW >400 daltons (Da) (Eng et al., 2021; Varma et al., 2015; Varma, 2022). Much of the available data suggests an important role of OATP1B-mediated uptake in the pharmacokinetics (PK) of high MW acids and zwitterions (Varma, 2022). Significance of uptake is noted irrespective of passive permeability, wherein several lipophilic and high permeable acids were shown to be impacted by OATP1B inhibitors in preclinical and clinical studies (Eng et al., 2021). However, the role of OATP1B in the PK of acids of MW lower than 400 Da is not well studied. In previous studies, evaluating ~25 ECCS class 1A compounds (high permeable, low MW (<400 Da) acids and zwitterions), we found only four compounds showing OATP1B substrate activity in vitro (Kimoto et al., 2018). In vivo significance of uptake mechanisms for class 1A drugs, however, is not completely understood.

Considerable interest was placed on the development of the oral hypoxia-inducible factor (HIF) prolyl hydroxylase (HIF-PH) inhibitors (referred as “dustats”), as a potential alternative approach to the erythropoiesis-stimulating agents (ESAs), for the treatment of anemia in patients with chronic kidney disease (CKD) (Locatelli and Del Vecchio, 2022; Sugahara et al., 2022). ESAs need parenteral administration, intravenous or subcutaneous, and additionally carry many safety issues including increased occurrences of cardiovascular events, venous thromboembolism, and death (Babitt and Lin, 2012; Batchelor et al., 2020). Dustats stimulate endogenous erythropoietin production even in patients with end-stage kidney disease by stabilizing the HIF-α subunit, allowing it to dimerize with the HIF-β subunit and to stimulate genes involved in protection
against hypoxia, including the erythropoietin gene (Kaplan et al., 2018; Sugahara et al., 2017). Many dustats have reached clinical development with some achieving successful regulatory approvals. For instance, daprodustat has been approved by USFDA recently for treatment of anemia caused by CKD in adults who have been receiving dialysis. Roxadustat is currently approved in a number of countries, including China and Japan for the treatment of anaemia in CKD in non-dialysis dependent and dialysis-dependent adult patients. Moreover, preclinical studies indicated that the members of this drug class (e.g. roxadustat and molidustat) also promote kidney function and may have additional clinical benefits (Burmakin et al., 2021). Many dustats are low MW acids and are suggested to be primarily eliminated via liver, however, their hepatobiliary transport mechanisms are not well understood. Using the candidates of this drug class, we aim to understand the significance of OATP1B in the PK of acids with MW below 400 Da (ECCS class 1A).

Here, we evaluated hepatic uptake mechanisms of novel hypoxia-inducible factor prolyl hydroxylase inhibitors (“Dustats”), which are low MW (300-400 Da) acids, except for molidustat (neutral) (Figure 1). Five of the six dustats evaluated have a common structural feature of carbonyl amino acetic acid with acid pKa values in the range of 2.8 to 5.8. Compounds were evaluated in vitro using transporter-transfected cells. Subsequently, uptake transport phenotype was studied using primary human and monkey hepatocytes and biliary clearance was evaluated in sandwich culture human hepatocytes. Finally, intravenous PK of dustats were studied in the presence and absence of single oral dose rifampin, an OATP1B inhibitor, in Cynomolgus monkeys.
MATERIALS AND METHODS

Materials

Daprodustat, enarodustat, molidustat, roxadustat, vadadustat were purchased from MedChemExpress (Monmouth Junction, NJ). Desidustat was purchased from AmBeed (Arlington Heights, IL). Rosuvastatin was purchased from Sequoia Research Products Ltd. (Oxford, UK). Rifampin, rifamycin SV, quinidine, ketoprofen were purchased from Sigma-Aldrich (St. Louis, MO). Cryopreserved human plateable hepatocytes (Hu8246, Caucasian, female 37 years old and HH1062, female 33 years old) were purchased from Life Technologies (Carlebad CA) and In Vitro ADMET Laboratories (Columbia, MD). Cryopreserved cynomolgus hepatocytes (10106012) was purchased from In Vitro ADMET Laboratories (Columbia, MD). InVitroGro-HT, CP and HI hepatocyte media were purchased from Celsis (Baltimore, MD). MDCKII-LE cells were from Pfizer (Groton, CT). MEMα, FBS, 100x NEAA, 100x GlutaMAX® and 1% of 10,000 U/mL penicillin-10,000 µg/mL streptomycin were purchased from Gibco Life Technology (Waltham, MA). Millicell-96 insert plates and BSA were purchased from MilliporeSigma (Burlington, MA).

Cell monolayer permeability study

MDCKII-low efflux cells were plated in MEMα containing 10% FBS, 1% of 100x NEAA, 1% of 100x GlutaMAX® and 1% of 10,000 U/mL penicillin-10,000 µg/mL streptomycin, at a seeding density of 26,400 cells/well on Millicell-96 cell culture insert plates and cultured for 4 days. Cells were maintained at 37°C, 5% CO₂, 90% relative humidity. Permeability studies were performed in transport buffer (HBSS with 20 mM HEPES, pH 7.4) at 37°C. The transport was initiated by adding 6 µM of test compound in donor (apical) wells at a volume of 0.1 mL/well
and placing the insert plates into receiver (basolateral) wells preloaded with 0.3 mL of transport buffer containing 0.4% BSA. Samples were collected from donor and receiver compartments at time 0 min and 90 min for analysis. Transcellular permeability ($P_{\text{app}}$) was calculated as described previously (Varma et al., 2012).

**Transport studies in transporter-transfected cells**

All studies were conducted using transporter-transfected (OATP1B1, OATP1B3, OAT2, OAT7 and NTCP) and un-transfected HEK293 cells using methodology similar to that previously reported (Bi et al., 2019; Bi et al., 2017). Briefly, cells were thawed and seeded in DMEM high glucose supplemented with 10% fetal bovine serum, 1% 10 mg/mL gentamicin, 1% 100X NEAA, 1% 100X GlutaMAX®, and 1% 100 mM sodium pyruvate, at a density of 6 - 7.5×10$^4$ cells/well on BioCoat™ 96-well poly-D-lysine coated plates, and cultured for 2 - 3 days. Cells were maintained at 37°C, 5% CO$_2$, and 90% relative humidity. Transport buffer was prepared at pH 7.4 using HBSS supplemented with 20 mM HEPES. Stock solutions of all compounds were diluted in transport buffer. All cell plates were washed three times with transport buffer and pre-incubated for 2 – 5 min with blank transport buffer at 37°C. To assess the transporter-mediated uptake of dustats, the cells were incubated with 0.05 - 0.1 mL of compound (1 µM dustats or 0.5 µM rosuvastatin) with and without 200 µM rifamycin SV (OATP1B inhibitor) at 37°C with timepoints of 1 and 3 min. The uptake was stopped by the removal of the dosing solution followed by three quick washes with ice-cold HBSS.

Cellular concentrations of dustats and rosuvastatin were extracted with 0.15 – 0.23 mL/well of 100% methanol containing internal standard. Cells were shaken for 30 – 60 minutes at room temperature. Cell extracts were transferred to 96-well plates, centrifuged, and 0.1 or 0.2 mL/well was transferred to new 96-well plates and dried down under nitrogen. Samples were
reconstituted with 50:50 methanol:water and analyzed by LC-MS/MS. Total cell protein per well per cell type was determined using a bicinchoninic acid protein assay following the manufacturer’s recommended protocol (Pierce, Thermo Fisher Scientific, Rockland, IL).

Uptake studies in primary human and monkey hepatocytes

The cell culture and uptake study conditions using monkey and human hepatocytes were identical. The hepatic uptake assay was performed using short-term culture format as described previously with some modification (Bi et al., 2019; Bi et al., 2017). Briefly, monkey hepatocytes (10106012, IVAL) and human hepatocytes (lot 1, Hu8246; lot 2, HH1062) were thawed at 37°C and seeded at a density of 0.35×10^6 cells/well on 24-well collagen I coated plates. After cultured in InVitro-CP medium overnight (~18h), the cells were preincubated with HBSS buffer in the presence or absence of OATP1B (rifampin 20 µM) and pan-SLC inhibitor (rifamycin SV 1 mM) for 10 min at 37°C. After preincubation, the buffer was aspirated, and the uptake reactions were initiated by adding prewarmed buffer containing test compounds with or without inhibitors. The reactions were terminated at designated time points by adding ice-cold HBSS immediately after removal of the incubation buffer. The cells were washed three times with ice cold HBSS and lysed with 100% methanol containing internal standard and the samples were analysed by LC-MS/MS. Uptake clearance (PS_{inf}) was estimated from the initial time-course (0.5-2 minutes) by linear regression (Bi et al., 2017; Bi et al., 2019). The in vitro fraction transported by OATP1B (f_{t,OATP1B}) was calculated from PS_{inf} measured in the absence and presence of 20 µM rifampin. At this concentration, rifampin completely inhibits OATP1B1 and OATP1B3 in vitro (Bi et al., 2017; Bi et al., 2019).

\[
\text{f}_{t,OATP1B} = \frac{\text{PS}_{\text{inf, control}} - \text{PS}_{\text{inf, +Rifampin}}}{\text{PS}_{\text{inf, control}}} \quad \text{Eq. 1}
\]
Sandwich culture human hepatocyte (SCHH) studies

For SCHH studies, cell thawing and seeding procedures were similar to described above. After 18-24 h of incubation at 37°C, cells were overlaid with ice-cold 0.25 mg/ml Matrigel in incubation medium at 0.5ml/well (Kimoto et al., 2017). Cultures were maintained in incubation media, which was replaced every 24 h. SCHH were rinsed twice with 0.5 ml of 37°C regular HBSS buffer or Ca2+/Mg2+-free HBSS containing 1 mM EGTA, and then replaced with fresh regular HBSS buffer or Ca2+/Mg2+-free HBSS containing 1 mM EGTA. The disruption of the bile canalicular network was achieved by pre-incubation with Ca2+/Mg2+-free HBSS containing 1 mM EGTA buffer for 10 minutes. Substrates in regular HBSS were then added to both sets of cultures. Reactions were terminated at designated time points by quickly washing the hepatocytes three times with ice-cold HBSS buffer. The sample processing and analysis were the same as described above.

Pharmacokinetic studies in Cynomolgus Monkey (NHP)

All procedures performed on NHPs were conducted in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee through an ethical review process. NHP studies were conducted at Pfizer (Groton, CT). Three male cynomolgus monkeys (7.3-9.2 kg, age 7-8 yr) were fasted overnight then fed 1 hour and 4 hours post-dose and water was provided freely. In this crossover design, subjects received an intravenous bolus dose of six dustats (vadadustat, daprodustat, molidustat, roxadustat, enarodustat, and desidustat) in a cassette format via the cephalic vein (0.2 mg/kg each compound, 1 mL/kg, in 5% polyethylene glycol 400:95% (5% w/v Hydroxypropyl-beta-cyclodextrin in 25 mM phosphate buffered saline, pH adjusted to ~7.4 and sterile filtered). At
predetermined timepoints, blood was collected serially from the femoral vein, and plasma was separated and kept cold before storing at −20 °C until LC-MS/MS analysis. Urine was also collected and analyzed. After 1 week washout period, subjects received rifampin orally via gavage (30 mg/kg, 4 mL/kg, in 0.5% (w/v) methylcellulose in water), and 1 hour later received the same intravenous dose of dustats in a cassette and study proceeded as described above.

**LCMS analysis of samples from in vitro and in vivo studies and pharmacokinetic analysis**

*In vitro* samples were dried down and reconstituted in 150 µL of 80:20 water:acetonitrile containing 0.1% formic acid. Samples were vortexed and analyzed by LC-MS/MS. Plasma samples from *in vivo* studies in NHP were prepared as follow. Analytical standards were prepared in blank NHP matrix across the linear range of the assay. Standards, blanks and unknowns were prepared by protein precipitation. 30 µL of each sample, standard or blank was added to a clean plate and precipitated with 150 µL of acetonitrile containing the internal standard tolbutamide at 5 ng/mL. Plates were vortexed and centrifuged and 100 µL of supernatant was transferred to a clean plate and diluted with 200 µL of 0.1% formic acid and analyzed by LC-MS/MS.

Analyte separation and detection was achieved by LC-MS/MS using a Waters Acquity LC system (Waters Corporation, Milford, MA) coupled to a Sciex API-6500 mass spectrometer (Sciex Corporation, Framingham, MA). Chromatographic separation was achieved using a Waters HSS T3 1.8uM 2.1x50mm column and a binary mobile phase system consisting of 0.1% formic acid and 0.1% formic acid in Acetonitrile, as mobile phase A and B respectively. The column temperature was held at 60°C and the flow rate was 0.6 ml/min. The gradient program had initial conditions of 5% B which was held for 0.3 min, followed by a linear ramp to 95% B
over 2 min, a hold for 0.3 min and a return to starting conditions, for a total run time of 3.0 min. Detection was achieved using a triple quadrupole mass spectrometer with an ion-drive source with electrospray ionization (ESI). The mass spectrometer was operated in negative mode using multiple reaction monitoring (MRM) with the specific precursor→product ion pairs for each analyte and internal standard as follows: 392.1→291.2 for daprodustat, 331.1→131.1 for desidustat, 313.1→188.1 for molidustat, 339.1→295.1 for enarodustat, 351.4→307.1 for roxadustat, 305.1→261.1 for vadadustat, 339.1→295 for enarodustat, and 269.0→170.0 for the IS tolbutamide. Data was processed using analyte/IS peak area ratios with Sciex Analyst software using a linear least square regression with 1/X² weighting.

Data were imported into Watson LIMS™ version 7.6.1 (Thermo Fisher Scientific Inc, Waltham, MA) for standard curve regression and non-compartmental pharmacokinetic parameter calculations – area under the plasma concentration-time curve (AUC), intravenous clearance (CL_\text{total}), volume of distribution (VD_\text{SS}) and effective half-life (t_{1/2}). The fraction of unchanged parent excreted into urine (f_e) was calculated as follows: Urine concentration x total urine volume / dose. Non-renal clearance was assumed as hepatic clearance (CL_h).

\[
CL_h = CL_{\text{total}} \cdot (1 - f_e) \quad \text{; where, } CL_{\text{total}} = \frac{Dose_{\text{IV}}}{AUC_{\text{inf}}} \quad \text{Eq. 2}
\]

VD_\text{SS} and t_{1/2} were calculated using the following expressions:

\[
VD_{\text{SS}} = Dose \cdot \frac{AUMC}{(AUC)^2} \quad \text{Eq. 3}
\]

\[
t_{1/2} = \ln(2) \cdot \frac{VD_{\text{SS}}}{CL_{\text{total}}} \quad \text{Eq. 4}
\]
where AUMC is the total area under the first moment of the drug concentration–time curve from time zero to infinity.

Changes in clearance and \( V_{Dss} \) on rifampin treatment were presented as CL ratio and \( V_{Dss} \) ratio.

\[
\text{CL ratio} = \frac{CL_{\text{control}}}{CL_{+\text{Rifampin}}}, \quad \text{and} \quad \text{VDss ratio} = \frac{VD_{SS,\text{control}}}{VD_{SS,+\text{Rifampin}}} \quad \text{Eq. 5}
\]

The in vivo fraction transported by OATP1B \( (f_{t,OATP1B}) \) was calculated from intravenous clearance in the absence and presence of rifampin.

\[
f_{t,OATP1B} = \frac{CL_{h,\text{control}} - CL_{h,+\text{Rifampin}}}{CL_{h,\text{control}}} \quad \text{Eq. 6}
\]

Here, subscripts ‘\( \text{control} \)’ and ‘\( +\text{Rifampin} \)’ represent PK parameters in control and treatment groups, respectively. Our previous studies showed complete inhibition of OATP1B-mediated hepatic clearance of probe drugs (pitavastatin and rosuvastatin) in monkey following rifampin 30mg oral dose (Eng et al., 2021; Ufuk et al., 2018).

**Statistical analysis**

Unpaired \( t \)-test was used to assess significance of inhibitor effects in vitro. NHP PK parameters (control versus rifampin-treatment groups) were compared using paired \( t \)-test. *\( P < 0.05 \) was considered statistically significant. All statistical analyses were performed using Graphpad Prism V 9.5.1 (Graphpad Software, LLC, La Jolla, CA).
RESULTS

OATP1B1 and OATP1B3 transport activity

Transporter substrate activity was evaluated for the six dustats using HEK293 cells un-transfected or transfected with hepatic uptake transporters including OATP1B1/1B3, NTCP and OAT2/7 (Table 1). OATP1B1 and OATP1B3 transport activity were noted for daprodustat, desidustat, enarodustat, roxadustat and vadadustat; wherein, uptake by transfected cells is significantly higher than that by un-transfected cells. OATP1B1/1B3 uptake ratios ranged from ~1.7 (roxadustat) to ~7 (desidustat). Rifamycin SV, an OATP1B inhibitor, significantly reduced (p<0.05) transport via OATP1B1 and OATP1B3 for all 5 acid dustats (Figure 2A-F). Molidustat did not show transport via OATP1B, nor did rifamycin SV modulated its cell accumulation in transfected cells (Figure 2D). Significant uptake by NTCP was observed for daprodustat and desidustat, but not for the other 4 compounds (Table 1). None of the compounds showed substrate activity via other hepatic uptake transporters evaluated, OAT2 and OAT7.

Uptake phenotype and biliary secretion in primary human and monkey hepatocytes

Uptake of dustats was measured in short-term hepatocyte cultures using 2 independent cryopreserved human hepatocyte lots (Table 2). Initial hepatocyte uptake clearance ranged from ~1 µL/min/mg-protein (molidustat) to about 20 µL/min/mg-protein (daprodustat). Presence of rifampin and rifamycin SV in the incubations led to a significant reduction in cellular accumulation of all five acids, with maximum inhibition noted for daprodustat. In vitro fraction transported by OATP1B (f_{\text{i,OATP1B}}) and passive uptake contribution were discerned based on the uptake inhibition by OATP1B selective inhibitor (rifampin, 20 µM) and pan-SLC inhibitor (rifamycin SV, 1 mM). As described by Bi et al. (Bi et al., 2019), residual uptake in the presence of pan-SLC inhibitor, rifamycin SV, was assumed as passive uptake, while the fraction inhibited
by OATP1B inhibitor, rifampin, was assumed to be OATP1B1/1B3 contribution (Eq. 1). Daprodustat $f_{i,OATP1B}$ was estimated to be about 60-85%, while desidustat showed $f_{i,OATP1B}$ of about 60% in both hepatocyte lots (Figure 3). OATP1B contribution to hepatocyte uptake clearance of enarodustat, roxadustat and vadadustat was estimated to be in the range of 40-50%. Molidustat did not show measurable OATP1B activity and passive diffusion was found to be the predominant process in its uptake. There were some differences in the estimated percent contribution between the two hepatocyte lots, however, consistent OATP1B-mediated uptake was noted for the five acid dustats and rosuvastatin, an OATP1B probe substrate. A lack of clear difference between the effect of rifampin versus rifamycin SV suggest limited role of other hepatic uptake transporters including OATP2B1, OAT2 and NTCP (Table 2) (Bi et al., 2019). Uptake phenotyping studies using monkey hepatocytes also yielded similar outcome with significant OATP1B-mediated transport contribution to uptake of acid dustats, but not molidustat (Table 2, Figure 4A). Biliary secretion was detected in vitro using SCHH studies for desidustat, enarodustat and vadadustat with biliary excretion index estimated in the range of 20-50% (Table 2).

**Effect of rifampin on pharmacokinetics of dustats in Cynomolgus monkey**

A single oral dose rifampin (30 mg/kg) significantly altered plasma exposure of the five low MW acids dosed intravenously in Cynomolgus monkey (Figure 5, Table 3). Intravenous clearance ($CL_{total}$) was reduced by about 2- to 3-fold for desidustat, enarodustat and vadadustat. However, the change was much larger for daprodustat (~10 fold) and roxadustat (~11 fold). The amount excreted in the urine was less than 10% of intravenous dose for all compounds in control group. Rifampin treatment increased the percentage of dose recovered in the urine to as high as 30-50% in case of desidustat, enarodustat and vadadustat. Renal clearance in the control arm is
generally negligible-to-low (<0.2 mL/min/kg) and the mean renal clearance increased in rifampin treatment group by about 2-3-fold, although a statistical significance was not achieved. Notably, change in non-renal clearance (i.e., apparent hepatic clearance, CLh) on rifampin treatment was larger for desidustat (~5 fold), enarodustat (~8 fold) and vadadustat (~4 fold) than the observed change in total clearance (2-3 fold) (Table 3). Neutral compound, molidustat, showed minimal PK modulation by rifampin (clearance ratio ~1.3). There is generally a good agreement between the in vitro $f_{i,OATP1B}$ obtained using monkey hepatocytes and the in vivo $f_{i,OATP1B}$ estimated from change in non-renal clearance in NHP studies (Eq. 6); although in vitro activity was found to be relatively lower than that estimated in vivo, particularly for roxadustat and vadadustat (Figure 4).

VDss also decreased considerably following rifampin dosing, compared to control, particularly for daprodustat and roxadustat where the clearance change was large (Table 3). Effective $t_{1/2}$ was decreased significantly by rifampin for daprodustat and roxadustat–although by only a less than 2-fold. On the other hand, $t_{1/2}$ increased in case of desidustat and vadadustat. Based on the plasma concentration-time profiles (Figure 5), it is apparent that the terminal $t_{1/2}$, of daprodustat and roxadustat in particular, remained similar or decreased following rifampin treatment. A linear relationship was observed between clearance ratio and VDss ratio (i.e., ratio of parameter in control to rifampin treatment groups) (Figure 6A). This relationship was found to be significant when considering current dustats data alone (n=5, $R^2 = 0.79$, P<0.0001). Moreover, the relationship was significant and consistent when merging the current dataset with several additional OATP1B substrates (n=18) previously evaluated in Cynomolgus monkey (Figure S1). A significant linear relationship ($R^2 = 0.81$, P<0.0001) was also observed between VDss<sub>control</sub> (volume in control group) and VDss ratio, implying that the change in VDss is higher for acids with high VDss<sub>control</sub> (Figure 6B).
DISCUSSION

Hepatic uptake via OATP1B1/1B3 was shown to be the rate-determining process in the clearance and thus clinical PK of structurally diverse drugs (Shitara et al., 2013). This is particularly noted with large MW (>400 Da) acids/zwitterions, irrespective of their lipophilicity and cell membrane permeability, i.e., ECCS classes 1B and 3B (Eng et al., 2021; Varma et al., 2015; Varma, 2022). In this study, we evaluated the significance of OATP1B-mediated uptake in determining the PK of lower MW acids (300-400 Da) using a novel drug class of HIF-PH inhibitors, referred as ‘dustats’. In vitro data from transfected HEK293 cells and primary human and monkey hepatocytes suggest active transport with significant contribution of OATP1B for all acid dustats evaluated. In vivo studies in non-human primates without and with the OATP1B inhibitor, rifampin, substantiated the role of OATP1B in the PK of these dustats. Collectively, these present a clear preclinical basis for the significance of hepatic uptake in the clinical PK of acid dustats. In contrast, PK of neutral compound molidustat is not likely to involve active uptake mechanisms. These findings are of significance in understanding drug-drug interactions (DDIs), pharmacogenomics and disease-state effects on PK of a range of dustats that reached clinical development or are approved for the treatment of anemia in CKD patients. Moreover, OATP1B can be a predominant driver in the PK of not only large MW acids/zwitterions (ECCS class 1B/3B), but also for certain low MW acids/zwitterions (ECCS 1A/3A).

We employed “SLC-phenotyping” approach (Bi et al., 2019) to evaluate uptake transport mechanisms and assess the contribution of specific pathway. In the initial screen against major uptake transporters (OAT2/7, OATP1B1/1B3, NTCP), all dustats except molidustat showed transport by OATP1B1/1B3. Some transport via NTCP was also observed for daprodustat and
desidustat, although the uptake ratios are lower than the arbitrary cut-off of 2. Specific transport via OATP1B1/1B3 was further confirmed in follow up studies using HEK293 cells, wherein, rifamycin SV reduced the cell accumulation in OATP1B1 and 1B3 cells to levels measured in un-transfected cells (i.e., uptake ratio ~1) for the acid dustats (Figure 2). On the basis of transfected cell data, uptake by short-term culture human hepatocytes were measured in the presence of OATP1B inhibitor, rifampin (20µM), and a pan-SLC inhibitor, rifamycin SV (1mM), to estimated $f_{\text{t,OATP1B}}$. For the five acid dustats, transport via OATP1B was found to be the major uptake pathway in the two independent human hepatocyte lots evaluated. There seems to have some differences in the percent inhibition by OATP1B-specific inhibitor versus pan-SLC inhibitor, which was assigned as non-OATP1B based active transport, although in several cases such difference was small and within the experimental variability (10-15% coefficient of variance). Finally, presence of OATP1B-mediated transport was also evident in vitro using monkey hepatocytes with $f_{\text{t,OATP1B}}$ similar to that noted using human hepatocytes, suggesting that the hepatic uptake mechanisms of dustats are similar across the two species.

Prototypical OATP1B1/1B3 inhibitor, rifampin, significantly altered the intravenous PK of acid dustats in Cynomolgus monkey. Daprodustat and roxadustat showed larger (~10 fold) total clearance change; while the other acids also showed significant change in total clearance (2-3 fold) and a pronounced decrease in non-renal clearance (4-8 fold). For the acid dustats, monkey VDss ranged from about 0.15 L/kg (desidustat and vadadustat) to about 1.7 L/kg (roxadustat), which was clearly reduced on rifampin treatment. Notably, compounds with high VDss showed relatively higher VDss ratio and clearance ratio (Figure 6). A linear correlation between the clearance ratio and VDss ratio further signifies the role of hepatic uptake in the PK of these compounds (Eng et al., 2021). Inhibition of hepatic metabolism or biliary secretion alone
typically does not alter VDss (Grover and Benet, 2009). Overall, the data suggests that acids with significant OATP1B-mediated active uptake may possess larger than expected VDss with the liver acting as a major distribution compartment for such compounds. The observations with acid dustats are consistent to that noted with high MW lipophilic acids/zwitterions, which showed similar PK behaviour in the absence and presence of OATP1B inhibitor (Figure S1). Considerations to such transporter-mediated hepatic disposition are required to adequately predict or describe plasma-concentration time profiles and dose optimization in early drug development (Zamek-Gliszczynski et al., 2013).

Both the in vitro transport phenotyping based on monkey hepatocytes and the in vivo activity estimated from change in non-renal clearance without/with rifampin treatment in NHP imply OATP1B as the major hepatic uptake pathway and the rate-determining step in the hepatic clearance of the 5 acid dustats. OATP1B contribution estimated in vitro and in vivo are generally aligned (Figure 4), although a trend of relatively lower in vitro $f_{OATP1B}$ was observed compared to that estimated in vivo particularly for roxadustat and vadadustat. This in vitro–in vivo differences may have originated due to down-regulation of OATP1B1/1B3 expression or function in the isolated monkey hepatocytes and general donor variability. Alternatively, other disposition mechanisms and transporter-enzyme interplay may have impacted the in vivo $f_{OATP1B}$ estimation – derived based on simple static calculations (Eq. 6). Further studies including transporter protein quantification and mechanistic modelling of in vivo data may be needed to correct of such differences (Eng et al., 2021; Kumar et al., 2019; Zamek-Gliszczynski et al., 2013). That said, there is a good concordance between the transport characteristics of dustats in human and monkey hepatocytes suggesting the mechanistic relevance of OATP1B in dustats clinical PK. Interestingly, clinical DDI data with OATP1B probe inhibitors such as rifampin and
cyclosporine are not available for the dustats. However, in a clinical DDI study, gemfibrozil treatment produced a marked increase in daprodustat plasma exposure (approximately 18-fold) in healthy subjects, and thus this combination is contraindicated (Johnson et al., 2014). Based on the preclinical data and the mechanistic PBPK modelling and simulations (Bi et al., 2023), it is inferred that the large PK change of daprodustat is due to strong CYP2C8 inhibition and moderate OATP1B inhibition by the gemfibrozil and its acyl glucuronide metabolite. Therefore, considerations to potential OATP1B and/or CYP2C8 modulation by inhibitor drugs or genetic polymorphism and disease state such as hepatic impairment are important in the clinical use of daprodustat (Bi et al., 2023). Similarly, OATP1B modulation, in addition to metabolic or biliary pathways, could be of importance for other acid dustats including desidustat, enarodustat, roxadustat and vadamustat. A recent report by Dong et al., presented a verified PBPK model of roxadustat accounting for OATP1B-CYP2C8/UGT1A9/others interplay in its hepatic disposition (Dong et al., 2023).

Dustats that reached clinical development, with the exception of molidustat, carry a common structural group of formyl glycine (carbonyl amino acetic acid) (Figure 1). Nonetheless, preclinical structure activity studies also identified several other chemical series including aromatic carboxylic acids to be potent HIF-PH inhibitors (Rabinowitz, 2013). In a previous study, evaluating a set of 25 low MW acids/zwitterions (ECCS 1A), we identified only four compounds to be substrates to OATP1B1 using transfected cells (Kimoto et al., 2018). These four compounds` MW ranged from 305-335 Da and possess diverse acidic groups including phenyl propanoic acid (nateglinide), benzoic acid (fluorescein), phenyl acetic acid (bromfenac) and nitro phenyl (entacapone). Additionally, carboxylic acid and related bioisosteres (pKa <6.0) such as acyl/hetaryl sulfonamide and tetrazole are common structural elements of known high
MW OATP1B substrates (ECCS 1B/3B) (Eng et al., 2021). It remains to be seen if certain modifications in formyl glycine group of dustats impact the substrate recognition by OATP1B. However, considering the variety in the acidic groups of known OATP1B substrates, adequate testing should be done in the preclinical and clinical development for lower MW acids.

CONCLUSIONS

In conclusion, our preclinical in vitro and in vivo studies suggest that active hepatic uptake mediated by OATP1B1/1B3 is a major determinant of PK for many dustats and thus act as a target for potential DDIs. Moreover, OATP1B substrate activity for low MW (300-400 Da) acids (ECCS class 1A and 3A) seem to be more prevalent than previously thought.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Bi Y, West M, Jordan S, King-Ahmad A, Varma M.

 Conducted experiments: Bi Y, West M, Jordan S, King-Ahmad A, Yamaguchi E, Ryu S, Mathialagan S.

Performed data analysis: Bi Y, Jordan S, King-Ahmad A, Tess D, Varma M

Wrote or contributed to the writing of the manuscript: Bi Y, West M, Jordan S, King-Ahmad A, Yamaguchi E, Ryu S, Mathialagan S, Tess D, Varma M
References


Footnotes:

FUNDING

This work received no external funding.

DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are contained within the paper.

CONFLICT OF INTEREST

All authors are full-time employees of Pfizer Inc, at the time of this study. Authors declare no conflicts of interest that are directly relevant to this study.
Figure Legends:

Figure 1. Structures of hypoxia-inducible factor prolyl hydroxylase inhibitors (Dustats). Formyl glycine group, MW and acid pKa are highlighted.

Figure 2. Dustats transport by OATP1B1 and OATP1B3 in transfected HEK293 cells. Ratio of cell accumulation in transfected to un-transfected cells was measured at 1 and 3 minutes in the absence and presence of rifamycin SV for (A) daprodustat, (B) desidustat, (C) enarodustat, (D) molidustat, (E) roxadustat and (F) vadadustat. *P<0.05, unpaired t-test, significant difference compared to control (n=3, Mean ± s.d.).

Figure 3. Mean percent contribution of OATP1B to the overall uptake rate in human hepatocytes. Uptake was measured in the presence of rifampin and rifamycin SV to estimate passive, OATP1B and other transporters contribution to hepatic uptake in 2 independent human hepatocyte lots, (A) Hu8246 and (B) HH1062.

Figure 4. In vitro and in vivo percent contribution of OATP1B to the uptake or overall hepatic clearance of dustats in non-human primates. A, uptake was measured in the presence of rifampin and rifamycin SV to estimate passive, OATP1B and other transporters contribution to hepatic uptake in plated monkey hepatocytes. B, in vivo OATP1B contribution to overall hepatic clearance was estimated (Eq. 6) based on non-renal clearance observed in the control and rifampin-treated Cynomolgus monkeys.

Figure 5. Intravenous pharmacokinetics of dustats in the absence and presence of rifampin, an OATP1B inhibitor, in Cynomolgus monkeys. Plasma concentration-time profiles of (A) daprodustat, (B) desidustat, (C) enarodustat, (D) molidustat, (E) roxadustat and (F) vadadustat are presented. Closed points and open points are individual subject data, and solid curves and
dashed curves represent mean profiles, in the control and rifampin treatment groups (n=3), respectively.

Figure 6. Inter-relationship between Cynomolgus monkey pharmacokinetic parameters of acid dustats. A, correlation between change in clearance and VDSS of dustats on single dose rifampin treatment. B, correlation between VDss in the control group and change in VDss on rifampin treatment (VDss ratio). Datapoints depict mean ± s.d (n=3). Curves represent linear regression fit and 95% confidence interval. Neutral compound molidustat was not included in regression analysis. Statistical significance of the linear relationship was tested at P-value 0.001.
Table 1. Summary of transcellular permeability and hepatic transporter substrate data of dustats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>P_{app} (x10^{-6} cm/sec)</th>
<th>OATP1B1 uptake ratio</th>
<th>OATP1B3 uptake ratio</th>
<th>NTCP uptake ratio</th>
<th>OAT2 uptake ratio</th>
<th>OAT7 uptake ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daprodustat</td>
<td>3.4 ± 0.3</td>
<td>2.46 ± 0.25</td>
<td>2.27 ± 0.08</td>
<td>1.60 ± 0.04</td>
<td>1.06 ± 0.14</td>
<td>1.17 ± 0.14</td>
</tr>
<tr>
<td>Desidustat</td>
<td>2.0 ± 0.3</td>
<td>6.77 ± 0.18</td>
<td>3.49 ± 0.19</td>
<td>1.59 ± 0.12</td>
<td>1.01 ± 0.06</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>Enarodustat</td>
<td>2.0 ± 0.2</td>
<td>4.01 ± 0.12</td>
<td>2.85 ± 0.06</td>
<td>1.12 ± 0.11</td>
<td>1.32 ± 0.04</td>
<td>0.91 ± 0.08</td>
</tr>
<tr>
<td>Molidustat</td>
<td>8.9 ± 0.2</td>
<td>1.09 ± 0.06</td>
<td>0.67 ± 0.06</td>
<td>1.15 ± 0.22</td>
<td>0.94 ± 0.04</td>
<td>0.91 ± 0.13</td>
</tr>
<tr>
<td>Roxadustat</td>
<td>22.8 ± 1.3</td>
<td>1.72 ± 0.07</td>
<td>1.60 ± 0.02</td>
<td>1.30 ± 0.13</td>
<td>0.84 ± 0.03</td>
<td>1.03 ± 0.05</td>
</tr>
<tr>
<td>Vadadustat</td>
<td>8.3 ± 0.6</td>
<td>2.99 ± 0.13</td>
<td>2.49 ± 0.64</td>
<td>1.28 ± 0.13</td>
<td>0.85 ± 0.02</td>
<td>1.06 ± 0.07</td>
</tr>
</tbody>
</table>

Papp, apparent permeability. Data represent mean ± s.d. (n=3).
Table 2. In vitro uptake transport and biliary clearance of dustats in human and monkey hepatocytes.

<table>
<thead>
<tr>
<th>Hepatocytes</th>
<th>PS_{inf,control} (µL/min/mg protein)</th>
<th>Uptake (% of control)</th>
<th>Biliary Excretion Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ 20 uM rifampin</td>
<td>+ 1 mM rifamycin SV</td>
</tr>
<tr>
<td>Daprodustat</td>
<td>Human, lot 1</td>
<td>21.2±1.9</td>
<td>15±13</td>
</tr>
<tr>
<td></td>
<td>Human, lot 2</td>
<td>18.3±1.2</td>
<td>39.5±12</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>14.4±4.2</td>
<td>18±9</td>
</tr>
<tr>
<td>Desidustat</td>
<td>Human, lot 1</td>
<td>2.0±0.3</td>
<td>33±5</td>
</tr>
<tr>
<td></td>
<td>Human, lot 2</td>
<td>2.4±0.2</td>
<td>40±3</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>0.7±0.3</td>
<td>51±11</td>
</tr>
<tr>
<td>Enarodustat</td>
<td>Human, lot 1</td>
<td>1.1±0.1</td>
<td>57±3</td>
</tr>
<tr>
<td></td>
<td>Human, lot 2</td>
<td>1.0±0.2</td>
<td>58±5</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>2.5±0.7</td>
<td>33±8</td>
</tr>
<tr>
<td>Molidustat</td>
<td>Human, lot 1</td>
<td>1.3±0.5</td>
<td>100±10</td>
</tr>
<tr>
<td></td>
<td>Human, lot 2</td>
<td>1.0±0.2</td>
<td>85±8</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>0.5±0.1</td>
<td>96±9</td>
</tr>
<tr>
<td>Roxadustat</td>
<td>Human, lot 1</td>
<td>15.8±2.7</td>
<td>53±10</td>
</tr>
<tr>
<td></td>
<td>Human, lot 2</td>
<td>15.1±1.3</td>
<td>58±7</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>10.6±2.4</td>
<td>37±11</td>
</tr>
<tr>
<td>Vadadustat</td>
<td>Human, lot 1</td>
<td>10.3±1.9</td>
<td>45±4</td>
</tr>
<tr>
<td></td>
<td>Human, lot 2</td>
<td>5.8±2.3</td>
<td>38±4</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>3.2±1.7</td>
<td>60±4</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Human, lot 1</td>
<td>8.4±0.4</td>
<td>27±2</td>
</tr>
<tr>
<td></td>
<td>Human, lot 2</td>
<td>8.7±0.6</td>
<td>48±5</td>
</tr>
<tr>
<td></td>
<td>Monkey</td>
<td>11.4±2.2</td>
<td>32±5</td>
</tr>
</tbody>
</table>

Human lot 1, Hu8246; Human lot 2, HH1062. Data represent mean ± s.d. (n=3).
### Table 3. Summary of intravenous PK parameters of dustats dosed without and with single-dose oral rifampin in Cynomolgus monkey.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treatment (+ Rifampin 30 mg/kg)</th>
<th>Control-to-treatment ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Daprodustat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;total&lt;/sub&gt; (mL/min/kg)</td>
<td>4.51 ± 1.21</td>
<td>0.45 ± 0.09*</td>
<td>9.9 ± 1.2</td>
</tr>
<tr>
<td>Vdss (L/kg)</td>
<td>0.59 ± 0.22</td>
<td>0.12 ± 0.02*</td>
<td>5.0 ± 1.1</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>5.93 ± 0.70</td>
<td>4.05 ± 0.14*</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>% Excreted in Urine</td>
<td>0.40 ± 0.46</td>
<td>11.5 ± 12.3</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>CL&lt;sub&gt;renat&lt;/sub&gt; (mL/min/kg)</td>
<td>0.019 ± 0.022</td>
<td>0.057 ± 0.064</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>CL&lt;sub&gt;non-renal&lt;/sub&gt; (mL/min/kg)</td>
<td>4.49 ± 1.20</td>
<td>0.39 ± 0.06*</td>
<td>11.3 ± 1.7</td>
</tr>
<tr>
<td><strong>Desidustat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;total&lt;/sub&gt; (mL/min/kg)</td>
<td>0.60 ± 0.11</td>
<td>0.30 ± 0.03*</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Vdss (L/kg)</td>
<td>0.14 ± 0.03</td>
<td>0.16 ± 0.06</td>
<td>0.9 ± 0.14</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>5.7 ± 1.0</td>
<td>10.0 ± 2.6*</td>
<td>0.6 ± 0.06</td>
</tr>
<tr>
<td>% Excreted in Urine</td>
<td>8 ± 3</td>
<td>51 ± 34</td>
<td>0.22 ± 0.13</td>
</tr>
<tr>
<td>CL&lt;sub&gt;renat&lt;/sub&gt; (mL/min/kg)</td>
<td>0.048 ± 0.020</td>
<td>0.15 ± 0.10</td>
<td>0.44 ± 0.27</td>
</tr>
<tr>
<td>CL&lt;sub&gt;non-renal&lt;/sub&gt; (mL/min/kg)</td>
<td>0.56 ± 0.11</td>
<td>0.14 ± 0.09*</td>
<td>4.9 ± 2.3</td>
</tr>
<tr>
<td><strong>Enarodustat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;total&lt;/sub&gt; (mL/min/kg)</td>
<td>1.53 ± 0.44</td>
<td>0.61 ± 0.20</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>Vdss (L/kg)</td>
<td>0.30 ± 0.11</td>
<td>0.20 ± 0.10</td>
<td>1.6 ± 0.38</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>7.2 ± 1.2</td>
<td>8.3 ± 2.2</td>
<td>0.88 ± 0.12</td>
</tr>
<tr>
<td>% Excreted in Urine</td>
<td>5.7 ± 3.5</td>
<td>51.5 ± 38.0</td>
<td>0.14 ± 0.08</td>
</tr>
<tr>
<td>CL&lt;sub&gt;renat&lt;/sub&gt; (mL/min/kg)</td>
<td>0.09 ± 0.06</td>
<td>0.36 ± 0.35</td>
<td>0.36 ± 0.17</td>
</tr>
<tr>
<td>CL&lt;sub&gt;non-renal&lt;/sub&gt; (mL/min/kg)</td>
<td>1.44 ± 0.41</td>
<td>0.25 ± 0.15*</td>
<td>7.7 ± 4.8</td>
</tr>
<tr>
<td><strong>Molidustat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;total&lt;/sub&gt; (mL/min/kg)</td>
<td>14.26 ± 1.10</td>
<td>11.20 ± 0.92*</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Roxadustat</td>
<td>Vadadustat</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------------------------</td>
<td>------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Vdss (L/kg)</strong></td>
<td>1.68 ± 0.83</td>
<td>0.15 ± 0.04</td>
<td></td>
</tr>
<tr>
<td><strong>t\textsubscript{1/2} (h)</strong></td>
<td>7.3 ± 1.0</td>
<td>4.14 ± 0.94</td>
<td></td>
</tr>
<tr>
<td><strong>% Excreted in Urine</strong></td>
<td>0.44 ± 0.32</td>
<td>2.7 ± 1.9</td>
<td></td>
</tr>
<tr>
<td><strong>CL\textsubscript{renal} (mL/min/kg)</strong></td>
<td>0.04 ± 0.03</td>
<td>0.02 ± 0.02</td>
<td></td>
</tr>
<tr>
<td><strong>CL\textsubscript{non-renal} (mL/min/kg)</strong></td>
<td>8.16 ± 2.17</td>
<td>0.80 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

**Roxadustat**

|                          | 8.20 ± 2.20                                    | 0.82 ± 0.13                                    |
| **CL\textsubscript{total} (mL/min/kg)** | 0.74 ± 0.10*                                  | 0.34 ± 0.12*                                  |

**Vadadustat**

|                          | 1.16 ± 1.33                                    | 0.13 ± 0.14                                    |
| **CL\textsubscript{renal} (mL/min/kg)** | 1.16 ± 1.33                                    | 0.13 ± 0.14                                    |
| **CL\textsubscript{non-renal} (mL/min/kg)** | 14.15 ± 1.08                                   | 0.80 ± 0.13                                    |

* P<0.05, significantly different from control. Paired t-test. Each subject served as own control. All statistical analyses are carried in GraphPad Prism 9.5.1 (GraphPad Software LLC., CA).
**Figure 1**

- **Daprodustat**
  - MW: 393.4
  - Acid pKa: 2.8

- **Desidustat**
  - MW: 332.3
  - Acid pKa: 5.1

- **Enarodustat**
  - MW: 340.3
  - Acid pKa: 5.8

- **Molidustat**
  - MW: 314.3
  - Acid pKa: > 9

- **Roxadustat**
  - MW: 352.3
  - Acid pKa: 3.5

- **Vadadustat**
  - MW: 306.7
  - Acid pKa: 2.9
Figure 2
Figure 3
**Figure 4**

(A) In vitro and (B) in vivo contributions of OATP1B, other, and passive transport for different drugs. The graphs show the percentage contributions of each transport mechanism for Daprodustat, Desidustat, Enarodustat, Moldustat, Roxadustat, Vadadustat, and Rosuvastatin.
Figure 5
Figure 6

**Goodness of Fit**
- **R squared**: 0.7869
- **Sy.x**: 1.550

- **Is slope significantly non-zero?**
  - **F**: 48.00
  - **DFn, DFd**: 1, 13
  - **P value**: <0.0001
  - **Deviation from zero?**: Significant

**Goodness of Fit**
- **R squared**: 0.8144
- **Sy.x**: 1.446

- **Is slope significantly non-zero?**
  - **F**: 57.06
  - **DFn, DFd**: 1, 13
  - **P value**: <0.0001
  - **Deviation from zero?**: Significant