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Identification and Quantitative Assessment of Uremic Solutes as Inhibitors of Renal Organic Anion Transporters, OAT1 and OAT3

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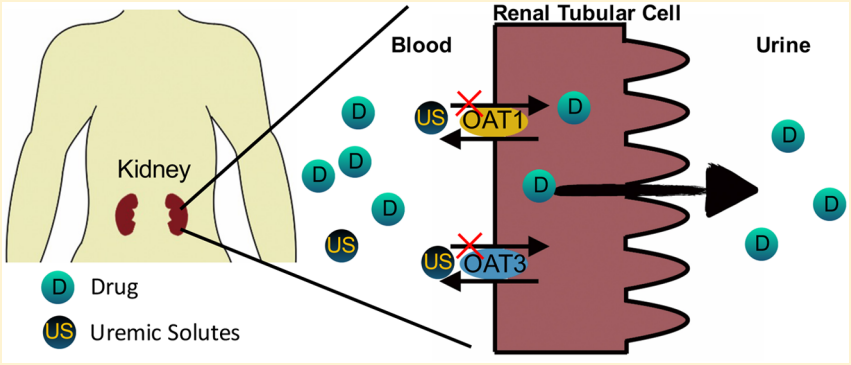
Supporting Information



ABSTRACT: One of the characteristics of chronic kidney disease (CKD) is the accumulation of uremic solutes in the plasma. Less is known about the effects of uremic solutes on transporters that may play critical roles in pharmacokinetics. We evaluated the effect of 72 uremic solutes on organic anion transporter 1 and 3 (OAT1 and OAT3) using a fluorescent probe substrate, 6-carboxyfluorescein. A total of 12 and 13 solutes were identified as inhibitors of OAT1 and OAT3, respectively. Several of them inhibited OAT1 or OAT3 at clinically relevant concentrations and reduced the transport of other OAT1/3 substrates *in vitro*. Review of clinical studies showed that the active secretion of most drugs that are known substrates of OAT1/3 deteriorated faster than the renal filtration in CKD. Collectively, these data suggest that through inhibition of OAT1 and OAT3, uremic solutes contribute to the decline in renal drug clearance in patients with CKD.

KEYWORDS: *organic anion transporter, chronic kidney disease, uremic solute, regulatory science*

1. INTRODUCTION

Chronic kidney disease (CKD) affects around 12% of the U.S. population and is on the rise.[1] Notably, the prevalence of Stages 3, 4, and 5 CKD has increased from 4.74% (1994−1998) to 6.52% (2007−2012).[1] As a result of associated comorbidities, individuals with CKD take many prescription drugs. In fact, the average number of medications prescribed per ambulatory hemodialysis patient is around 12; thus, the potential of drug−drug interactions (DDIs) increased in these patients.[2] Moreover, the pharmacokinetics (PK) of many drugs are altered in patients with CKD; often resulting in higher drug levels and increased toxicities. For example, of the top 200 prescribed drugs in the U.S., more than 30% are primarily eliminated by the kidney and around 90% of those are actively secreted.[3] Whereas reduced renal elimination as a result of reduced glomerular filtration rate (GFR) is expected in CKD, the effect of renal dysfunction on active secretion is poorly understood.

CKD is characterized by the accumulation of uremic solutes, endogenous metabolites that build up in uremia. As renal function deteriorates, many uremic solutes accumulate to very high concentrations in the plasma. Several uremic solutes have been shown to be substrates or inhibitors of organic anion transporter 1 and 3 (OAT1 and OAT3).[4] OAT1 and OAT3 are most abundantly expressed in the kidney and are located on the basolateral surface of renal proximal tubule cells.[3] As the major organic anion uptake transporters in renal cells, OAT1 and OAT3 transport their substrates, which are generally weak acids, from blood into renal cells, contributing to the first step of active renal tubular secretion. OAT1 and OAT3 have a great

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deal of overlapping substrate specificity, but still show some selectivity. In general, OAT3 transports larger and more lipophilic compounds than OAT1.[5] In addition, OAT3 is capable of transporting basic drugs such as cimetidine, famotidine, and ranitidine.[6]

OAT1 and OAT3 have been identified as the targets of potential DDIs.[7−9] Clinical data suggest that uremic solutes may modulate the activity of OAT1 and OAT3, thereby affecting renal clearance.[4] However, so far only a few studies have characterized the interactions between uremic solutes and OAT1 and OAT3. For example, hippuric acid, a canonical uremic solute, has been reported to inhibit the uptake of *p*-aminohippurate and penicillin G by OAT1 and OAT3, respectively.[10] Concentrations of hippuric acid present in the plasma of patients with CKD (∼290 μM) are much higher than the inhibition constants of the two transporters (19 μM for OAT1 and 31 μM for OAT3), suggesting hippuric acid may inhibit the transporters at clinically relevant concentrations.[11] To understand the effect of CKD on renal drug elimination and, in particular, on active secretion mediated by transporters, we tested the hypothesis that uremic solutes, which accumulate in CKD, inhibit the two major anion transporters in the renal tubule, OAT1 and OAT3, thus modulating renal drug clearance in patients with CKD. Specifically, we screened a large set of uremic solutes for inhibition of OAT1 and OAT3 using a medium throughput fluorescent probe assay. Follow-up studies characterizing the potency of inhibition of selected uremic solutes and their effect on OAT1- and OAT3-mediated transport of prescription drugs were performed. Finally, we analyzed clinical data from the literature. The analysis showed that renal secretory clearance (CL_{sec}) of many prescription drug substrates of OAT1 and OAT3 deteriorates more extensively than GFR as kidney function declines. These findings support the hypothesis that inhibitory effect of uremic solutes on renal transporters may manifest clinically.

2. MATERIALS AND METHODS

2.1. Uremic Solutes and Reagents. Uremic solutes described by Duranton et al. were included in this study.[11] All available uremic solutes were obtained for screening except for small proteins or peptides (middle molecules). As middle molecules were less likely to have direct inhibitory effects on transporter-mediated uptake, only 12 of them were chosen randomly. In addition, uremic solutes from the EuTox database (http://www.uremic-toxins.org/) were included in the screening if their highest plasma levels were more than 20-fold higher in CKD Stage 5 than in normal subjects.[12] Those uremic solutes included alpha-*N*-acetylarginine, aribitol, creatine, creatinine, erythritol, gamma-guanidinobutyric acid, mannitol, melatonin, *N*2,*N*2-dimethylguanosine, *N*6-carbamoylthreonyladenosine (t6-Ado), orotic acid, quinolinic acid, *s*-adenosylhomocysteine, and sorbitol. A total of 72 uremic solutes were screened (see Supplementary Table S1).

The inhibitory potencies of uremic solutes were evaluated at concentrations up to 100 times (100×) their plasma levels as described by Duranton et al. and EuTox.[11,12] Unbound concentrations were used for protein bound solutes if available, including acrolein, hippuric acid, indole acetate, indoxyl sulfate, and *p*-cresyl sulfate. Due to their limited solubility, some were tested at the highest soluble concentrations. Those included neopterin (20×), CMPF (8×), homocysteine (50×), pentosidine (20×), uric acid (4×), carboxymethyllysine (25×), dihydroxyphenylalanine (25×), creatine (25×), phenylacetic acid (50×), and orotic acid (4×). 6-Carboxyfluorescein (6-CF) was purchased from Sigma-Aldrich (St. Louis, MO). Tritium labeled adefovir, tenofovir, famotidine, and Ro 64-0802 (oseltamivir carboxylate) were purchased from American Radiolabeled Chemicals (St. Louis, MO). The specific activities of these compounds were 11.9, 10, 10, and 5 Ci/mmole, respectively.

2.2. Cell System and Uptake Assay. Cell culture and uptake assay were as previously described with some modifications.[13] The mRNA expression levels of both OAT1 and OAT3 in stably transfected HEK293 cells were around 0.2 million higher than that of pcDNA5/FRT stably expressing cells (EV cells). Cells were seeded at a density of 800,000 cells per milliliter the day before the experiment. Uptake experiment was conducted by incubating cells for 2 min in HBSS containing the model substrate at 37 °C. The uptake of 6-CF was measured by a fluorescence microplate reader right after the uptake experiment. Afterward, cells were lysed in lysis buffer (0.1% SDS and 0.1 N NaOH) and the intensity of fluorescence was normalized by total protein. Alternatively, cells were lysed in the lysis buffer for the measurement of the uptake of radiolabeled compounds. Aliquots of the cell lysates were mixed with Ecolite (+) scintillation cocktail (VWR, Radnor, PA). The radioactivity was later measured by a scintillation counter and normalized to total cellular protein.

2.3. Inhibitor Screening and IC_{50} Evaluation. An inhibitor was defined as a compound that inhibited more than 50% of the transporter-mediated uptake of the model substrate at the tested concentration mentioned above. Transporter-mediated uptake was calculated by subtracting the uptake of the model substrates in empty vector transfected cells from the uptake in transporter expressing cells. Uremic solutes were added along with the model substrates in the uptake buffer to evaluate the direct inhibition.

Percentages of the model substrate uptake were calculated by normalizing the amount of transporter-mediated uptake of model substrate in the presence of various concentrations of uremic solutes to the control (without any uremic solutes or inhibitors). Uptake percentages were plotted and fitted to the following equation to obtain the IC_{50}:

$$Y = 100/(1 + 10^{C-\log IC_{50}})$$

where Y is the percentage of transporter-mediated uptake and C is the concentration of the uremic solute or inhibitor.

2.4. Literature Review and Meta-Analysis. Prescription drugs that are substrates of OAT1 and OAT3 were identified from the University of Washington Metabolism & Transport Drug Interaction Database (https://www.druginteractioninfo.org/). Only prescribed drugs were included in the analysis. PK studies in CKD patients were searched from Drugs@FDA (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/) and PubMed using the drug name and the keywords, "renal impairment" and "pharmacokinetics". If there was more than one study available, the one that reported the largest change in the area under the curve (AUC) or renal clearance (if AUC was not available) in patients with CKD was selected. The analysis assumes total renal clearance (CL_r) equals the sum of CL_{sec} and CL_{fil}, and CL_{fil} is calculated by multiplying unbound fraction of the drug in plasma (fu) and glomerular filtration rate (GFR) or creatinine clearance (CL_{cr}). Due to limited information available, fu was assumed unchanged in various stages of CKD if data were not available. Only drugs that had a significant secretory component to their renal clearance were

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included in the analysis. The significance is defined as $CL_r/CL_{fil} > 1.5$. If a range of GFR or CL_{cr} was reported, the mean value of the range was used. Information on the unbound fraction of each drug was collected from Micromedex (http://www.micromedexsolutions.com) if not available in the reference papers.

To evaluate if the change in active renal secretion was proportional to the reduction in GFR in CKD, the ratios of the CL_{sec} to GFR (CL_{sec}/GFR) were calculated for subjects with normal renal function and subjects in each CKD stage.

The effect of CKD on CL_{sec} was modeled according to eq 1:

$$CL_{sec\_CKD} = R_{GFR}F_xCL_{sec\_normal} \quad (1)$$

CL_{sec_CKD} is the CL_{sec} in CKD, CL_{sec_normal} is the CL_{sec} in subjects with normal kidney function, and R_{GFR} is the ratio of GFR measured in CKD and in subjects with normal renal function. We assumed a factor F_x for the decrease in CL_{sec} in addition to change related to kidney physiology (measured by R_{GFR}) for each CKD stage.

Stages of CKD were based on the definition by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) as follows: normal (or Stage 1) (GFR ≥ 90 mL/min), Stage 2 (GFR = 60−89 mL/min), Stage 3 (GFR = 30−59 mL/min), Stage 4 (GFR = 15−29 mL/min), and Stage 5 (GFR < 15 mL/min).

2.5. Statistical Analysis. Results were summarized by mean ± standard error and analyzed by Prism 5.0 (GraphPad Software, La Jolla, CA). For statistical comparison, ANOVA test was used, and P value less than 0.05 was regarded as statistical significant.

3. RESULTS

3.1. Screening of Uremic Solutes To Identify Inhibitors of OAT1 and OAT3. Overviews of the screening procedure and results are summarized in Figure 1 and Figure 2, respectively. The detailed inhibitory profile of all uremic solutes can be found in Supplementary Table S1. A compound was designated as an inhibitor if it reduced the OAT1 or OAT3 mediated uptake of 6-CF by >50% at the highest concentration tested. We identified 12 inhibitors of OAT1 and 13 for OAT3 (Figure 2b and Table 1). In total, 15 inhibitors were identified. Seven of the compounds belonged to the category of uremic solutes termed free water-soluble solutes and eight belonged to the protein bound solute category.[11] No middle molecule was detected as an inhibitor of OAT1 or OAT3 based on our criteria. Many protein bound solutes (eight out of 19 tested) inhibited OAT1 and OAT3. Among the uremic solutes identified as OAT1 or OAT3 inhibitors, eight were novel, which no inhibitory effects of OAT1 and OAT3 have been reported. Most were identified in the group of free water-soluble solutes (Table 1). In particular, nonenal, decenal, creatinine, nonanal, and phenylacetic acid were novel inhibitors that inhibited both OAT1 and OAT3. Two novel inhibitors were identified in the protein bound solute group: indole-β-D-glucuronide and phenol, and both were specific OAT3 inhibitors.

3.2. Inhibitory Potency of Uremic Solutes for OAT1 and OAT3. The IC_{50} values and the potential clinical relevance of the inhibitors that were identified in the screening assay are summarized in Table 1. Several uremic solutes inhibited OAT1 or OAT3 at clinically relevant concentrations. That is, the free concentrations in plasma that are achieved in patients with CKD are greater than 0.1× the IC_{50} for inhibition of OAT1 or

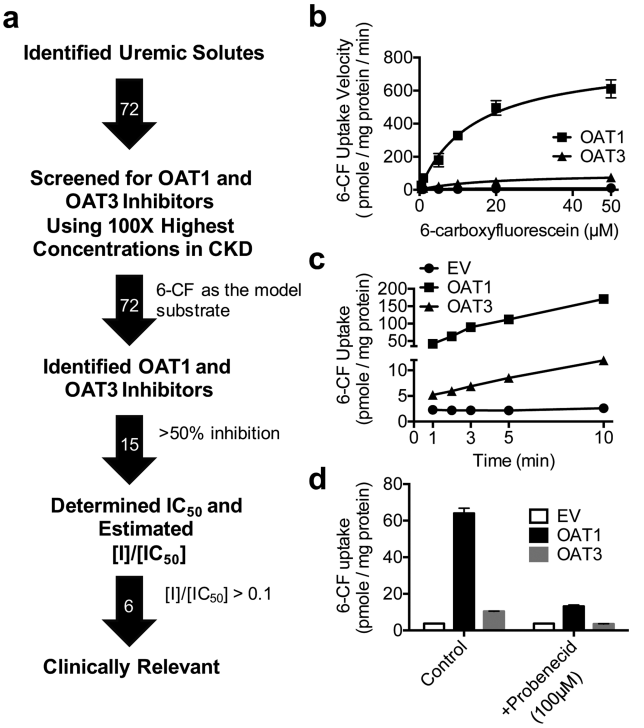

Figure 1. Experimental procedure. (a) Flowchart showing the procedure of uremic solute screening to identify inhibitors of OAT1 and OAT3. The uremic solutes and the corresponding highest concentrations were identified from Eutox database (http://www.uremic-toxins.org/) and relevant publications.[10,11] The number of uremic solutes is shown in the arrows. (b) Michaelis−Menten curves showing the uptake kinetics of 6-carboxyfluorescein (6-CF) by OAT1 and OAT3. The K_m is 14 ± 3 and 19 ± 6 μM for OAT1 and OAT3, respectively. The V_{max} is 790 ± 76 and 88 ± 11 pmol/min/mg protein for OAT1 and OAT3, respectively. 6-CF (1 μM) was used for the inhibitor screening. Data were acquired from two experiments. $N = 3$ for each point. (c) Time-dependent accumulation of 6-CF by OAT1/3 expressing cells and cells stably transfected with empty vector (EV). Two minutes was selected as the uptake time in the inhibitor screening. $N = 3$ replicate determinations for each data point. (d) An example showing the inhibition of 6-CF uptake by OAT1 and OAT3 using 100 μM probenecid ($N = 3$). 6-CF, 6-carboxyfluorescein; [I], highest concentration in CKD patients; IC_{50}, half maximal inhibitory concentration.

OAT3. Thus, the ratios of the unbound concentrations in CKD over IC_{50} ($[I]/IC_{50}$) are greater than 0.1. Overall phenylacetic acid and hippuric acid were most clinically relevant; that is, their IC_{50} values were much lower than plasma concentrations associated with CKD. The elevation of phenylacetic acid and hippuric acid were around 300- and 40-folds in CKD, compared to healthy population.[11] This suggests these two solutes are less likely to inhibit OAT1 or OAT3 in healthy population. CMPF, indoxyl sulfate, p-cresyl sulfate, and uric acid were less significant but conservatively may still be clinically relevant inhibitors. For example, FDA recommends a clinical DDI study should be considered, if clinical unbound concentrations are equal to or greater than 0.1× its IC_{50} in inhibiting OAT1 or OAT3 as they may potentially cause a DDI.[9] Our approach to evaluate clinical relevance is in accordance with the FDA DDI guidance and is more applicable for this study than comparing other PK parameters such as area under a curve (AUC), gastrointestinal concentrations, or dose. The relative inhibition potencies of the uremic solutes, including those that are

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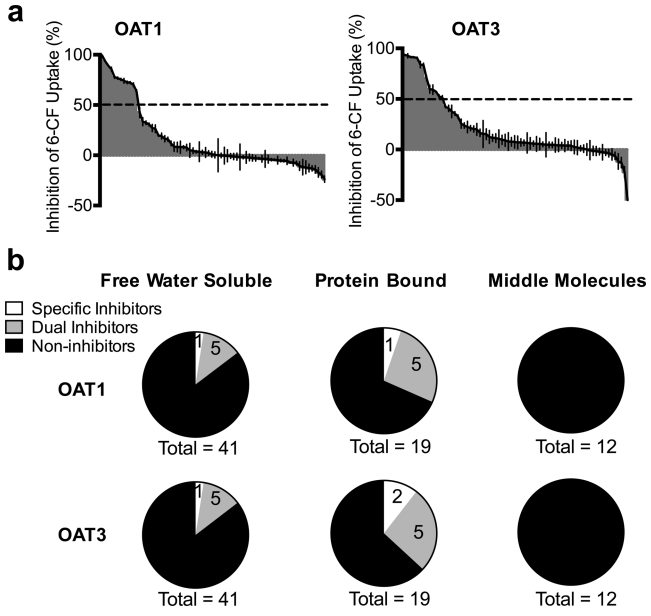

Figure 2. Overview of uremic solutes screening. (a) Summary of the inhibitory effect of uremic solutes. The mean uptake of 6-CF in the presence of each of the 72 uremic solutes is represented as a gray bar; black lines represent standard errors. An inhibitor is designated as a compound that results in greater than 50% inhibition of 6-CF uptake. (b) Distribution of inhibitors versus noninhibitors for each category of uremic solutes. No middle molecules in the study showed inhibitory effects. Most inhibitors identified in the free water-soluble group are novel.

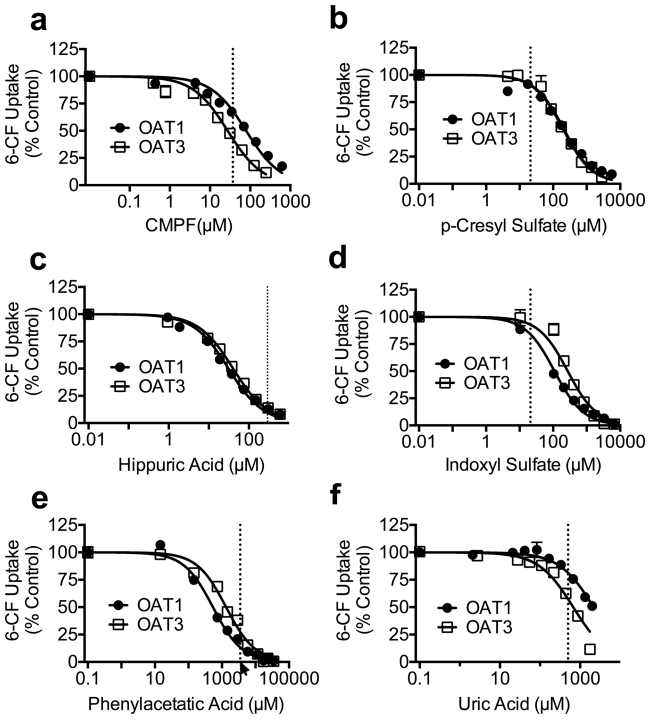

Figure 3. Representative curves of the effects of uremic solutes on the uptake of 6-CF by OAT1 and OAT3. Only clinically relevant inhibitors are shown. The symbols represent the mean of $N = 4$ determinations at each concentration. Dash lines indicate the highest unbound concentrations in CKD patients. Note total concentration was used for CMPF as no unbound concentration was available. CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid.

potentially relevant clinically for OAT1 and OAT3, are shown in Figure 3.

3.3. Effects of Uremic Solutes on OAT-Mediated Transport of Clinically Used Drugs. To further confirm the potential for clinical relevance of CMPF, hippuric acid, and phenylacetic acid in inhibiting OAT1 and OAT3, we evaluated their potencies of inhibition of the OAT-mediated transport of several prescription drugs. Adefovir and tenofovir were chosen as drug substrates of OAT1, whereas famotidine and Ro 64-0802 were selected for OAT3. As shown in Figure 4, the IC_{50} of these uremic solutes were much lower than their plasma levels in CKD, suggesting that the uremic solutes may reduce the renal transport of these drugs in patients with CKD.

Table 1. Inhibitory potencies of selected uremic solutes for OAT1 and OAT3[a]

			OAT1		OAT3	
	Category	[I] (μM)[c]	IC_{50}	[I]/IC_{50}	IC_{50}	[I]/IC_{50}
2-nonenal[b]	W	0.7	19 ± 3	<0.1	60 ± 9	<0.1
4-decenal[b]	W	0.7	38 ± 2	<0.1	53 ± 7	<0.1
CMPF	P	37	79 ± 5	0.5	28 ± 1	1.3
creatinine[b]	W	1200	14000 ± 830	<0.1	40000 ± 2800	<0.1
hippuric acid	P	290	31 ± 1	9.4	41 ± 2	7.1
indole acetate	P	2.5	140 ± 23	<0.1	N/A	
indoxyl sulfate	P	21	110 ± 6	0.2	270 ± 24	<0.1
indoxyl-β-D-glucuronide[b]	P	9.5	N/A		670 ± 57	<0.1
kynurenic acid	P	0.8	34 ± 2	<0.1	23 ± 2	<0.1
*N*2,*N*2-dimethylguanosine[b]	W	1.3	N/A		140 ± 9	<0.1
nonanal[b]	W	0.5	22 ± 1	<0.1	N/A	
p-cresyl sulfate	P	21	210 ± 9	0.1	200 ± 10	0.1
phenol[b]	P	59	N/A		3100 ± 330	<0.1
phenylacetic acid[b]	W	3500	540 ± 30	6.5	1300 ± 100	2.7
uric acid	W	500	2200 ± 240	0.2	670 ± 50	0.8

[a]Data were acquired from 3-4 experiments. All experiments were conducted in quadruplicate. W, free water-soluble solutes; P, protein bound solutes; IC_{50}, half maximal inhibitory concentration; [I]/IC_{50}, ratio of highest concentration in CKD over IC_{50}; CMPF, 3-carboxy-4-methyl-5-propyl-2- furanpropanoic acid; NA, not available. [b]Novel inhibitor. [c]Highest unbound concentrations in CKD; total concentrations are shown for CMPF, indoxyl-β-D-glucuronide, kynurenic acid, and phenol as unbound concentrations are not available.

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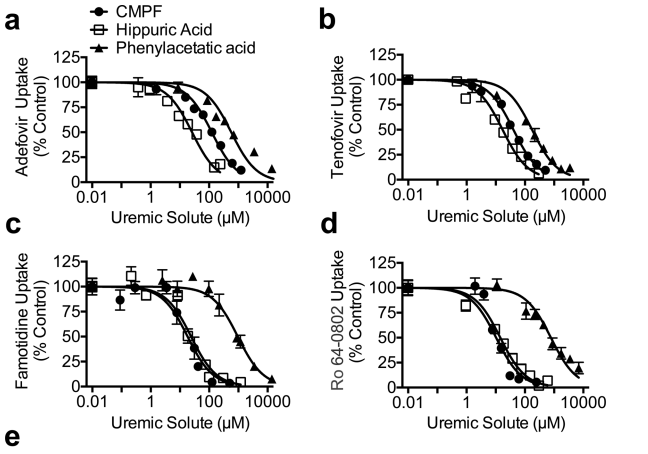

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		CMPF		Hippuric Acid		Phenylacetic Acid	
	Transporter	IC_{50} (μM)	[I]/IC_{50}	IC_{50} (μM)	[I]/IC_{50}	IC_{50} (μM)	[I]/IC_{50}
Adefovir	OAT1	120 ± 10	0.3	26 ± 2	11	570 ± 53	6
Tenofovir	OAT1	40 ± 3	0.9	17 ± 1	17	175 ± 15	20
6-CF	OAT1	79 ± 5	0.5	31 ± 1	9.4	540 ± 30	6.5
Famotidine	OAT3	18 ± 3	2	24 ± 5	12	950 ± 170	4
Ro 64-0802	OAT3	10 ± 1	4	14 ± 2	20	780 ± 100	5
6-CF	OAT3	28 ± 1	1.3	41 ± 2	7.1	1300 ± 100	2.4

Figure 4. Effect of uremic solutes on the uptake of adefovir (a) and tenofovir (b) by OAT1, and famotidine (c) and Ro 64-0802 (d) by OAT3. $N = 4$ determinations for each point. (e) Table summarizes the potencies of uremic solutes in inhibiting the OAT-mediated transport of the four evaluated drugs. Data were acquired from 2 to 3 independent experiments. [I], highest unbound concentration in CKD patients except for CMPF, which no unbound concentration was available and total concentration was used; IC_{50}, half maximal inhibitory concentration; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid.

3.4. Effect of CKD on Active Secretion: Review of Clinical PK of 18 Drugs in CKD Patients.

To evaluate the effect of CKD on secretory clearance of drugs, we compared the ratio of CL_{sec} to GFR in patients with normal renal function and those with CKD. Data from PK studies in patients with CKD were analyzed. We identified 68 clinically used drugs as substrates of OAT1 or OAT3 (Figure 5a). Among these, 41 were mainly eliminated by the kidney (Fe > 0.3). PK studies for 23 of the 41 drugs have been performed in patients with CKD. The total number of publications reviewed included 173 papers and 54 New Drug Applications (NDAs). We excluded ranitidine from the analysis because it is a dual substrate of organic anion and organic cation transporters such as OCT2. Although famotidine is also a substrate of OCT2 *in vitro*, addition of an OCT2 inhibitor, cimetidine, produced little effect on its renal clearance suggesting that OCT2 plays little or no role in its transport *in vivo*.[14] In the end, we analyzed 18 drugs from 13 published papers[15−27] and five NDAs.[28−32] Our analysis showed that for many drugs, CL_{sec} normalized to GFR was reduced in patients with CKD Stage 4 (GFR = 15−29 mL/min) compared with patients with normal renal function (Figure 5b, $p < 0.001$). The disproportionate deterioration of CL_{sec} in CKD was modeled using eq 1, which introduces a factor F_x causing decreased CL_{sec} in addition to decreased filtration as demonstrated by the ratio of CL_{sec} to GFR between subjects with CKD and subjects with normal renal function. The detailed calculation of kinetic changes of all drugs can be found in Supplementary Table S2 and Figure S3. The median F_x values appear to decrease as renal function declines between CKD Stage 2 and CKD Stage 4 (Supplemental Figure S3). The median values of calculated F_x were 0.73 (0.31−1.33), 0.41 (0.02−1.00), and 0.61 (0.36−0.98) for CKD Stages 3, 4, and 5, respectively (Figure 6).

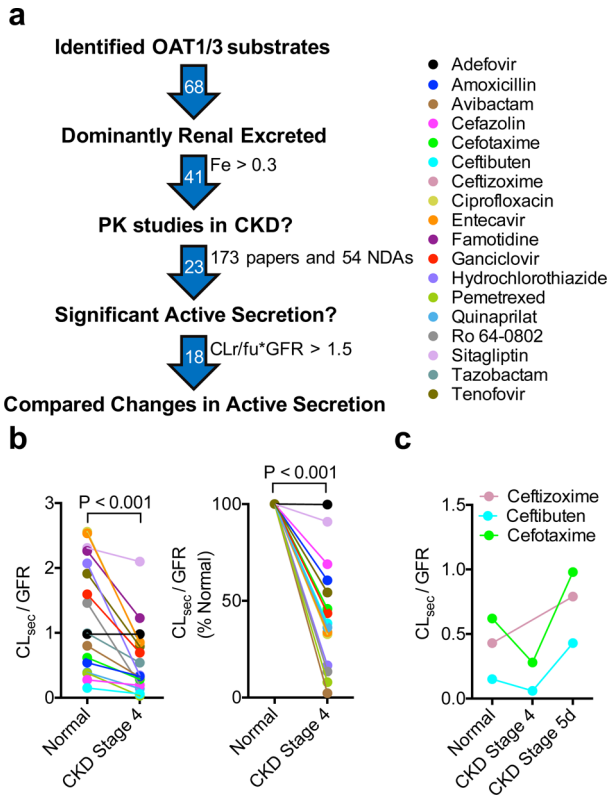

Figure 5. Clinical PK changes of OAT1/3 substrates in response to deteriorating renal function. (a) Flowchart of the analytical procedures. Number of prescribed drugs identified as OAT1/3 substrates are shown in the arrows. All drugs in the analysis have significant active secretion as their renal clearances are at least 1.5-fold of the filtration clearance (CL_r/fu·GFR > 1.5). (b) Ratio of secretory clearance (CL_{sec}) over GFR in normal and in CKD Stage 4 patients. More extensive deterioration of active secretion was seen in CKD Stage 4 patients ($p < 0.001$ by ANOVA). CL_{sec} was calculated by subtracting CL_{fil} from renal clearance: $CL_{sec} = CL_r$/fu·GFR. Note there was only patient in CKD Stage 4 for pemetrexed and hydrochlorothiazide. Normalized data of the relative changes in CKD Stage 4 patients are shown in the right panel. (c) Influence of dialysis on active secretion changes in CKD patients. Cefotaxime and Ceftibuten both display greater reduction of CL_{sec} in CKD Stage 4 patients when compared to GFR. Dialysis ameliorates or even improves the deteriorated CL_{sec} in CKD Stage 5d patients (Stage 5 CKD patients on dialysis). Note the clearances were measured in the off dialysis period. The calculated CL_{sec} of ceftizoxime in CKD Stage 4 patients was negative and is not shown here. CLr, renal clearance; fu, unbound fraction; GFR, glomerular filtration rate.

4. DISCUSSION

There are about 26 million adults in the U.S. with different degrees of CKD, while millions of others are at increased risk.[1] Because active tubular secretion contributes to the removal of most renally eliminated drugs and because multiple medications are commonly prescribed to patients with CKD, it is critical to understand the influence of CKD on renal active secretion. In patients with CKD, many endogenous substances termed uremic solutes accumulate in the body directly or indirectly as a result of reduced renal function.[4,11,33] In addition, altered dietary intake, gut flora, and the inflammatory microenvironment in uremic patients may potentially result

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$$F_x = \frac{\frac{CL_{sec\_CKD}}{CL_{sec\_normal}}}{\frac{GFR_{CKD}}{GFR_{normal}}}$$

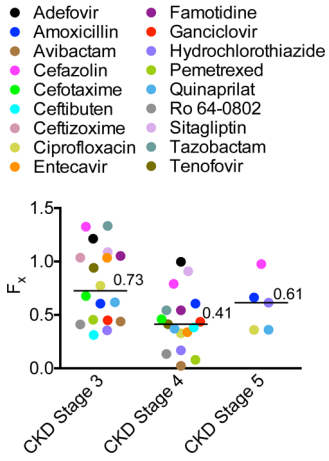

Figure 6. Distribution of the F_x for individual drugs in CKD Stage 3, 4, and 5 patients. The median values are shown in the figure. F_x was calculated based on the following equation: $CL_{sec\_CKD} = GFR_{CKD}/GFR_{normal} \times CL_{sec\_normal} \times F_x$ Note there was only one patient for pemetrexed in CKD Stage 4 and hydrochlorothiazide in CKD Stages 4 and 5.

in increased levels of uremic solutes in CKD.[33] Satoh et al. reported that the administration of hippuric acid or indole acetate promoted renal injury and reduced inulin clearance in rats.[34] However, no single uremic solute so far has been identified to be responsible for all the clinical manifestations in CKD. Active renal secretion appears to play an important role in the elimination of several uremic solutes, particularly for protein bound solutes.[4] Recent data suggest free water-soluble solutes could also interact with OAT1/3.[35,36]

4.1. Uremic Solutes Screening. Here we conducted the first comprehensive screening to evaluate the inhibitory effects of uremic solutes on two major renal transporters, OAT1 and OAT3. Among the 72 uremic solutes, 12 inhibitors of OAT1 and 13 of OAT3 were identified based the criteria that the highest concentration results in >50% inhibition (Figure 2). Most previous studies of uremic solutes have focused on solutes with a high degree of plasma protein binding, as these molecules are poorly dialyzable. Indeed, about half of the inhibitors identified in our screen (6 for OAT1 and 7 of OAT3) were protein bound solutes. Of these, two were novel (indoxyl-β-D-glucuronide and phenol). In contrast, few studies have focused on free water-soluble solutes because they are dialyzable. Our data suggest that this group of uremic solutes is also important for inhibiting drug transporters.

For example, 7 out of 41 water-soluble uremic solutes screened in our study were inhibitors of OAT1 or OAT3. Importantly, most of these were novel inhibitors except for uric acid, which has long been recognized as a uremic solute. OAT3 was reported to contribute to the elimination of creatinine in mice.[37,38] There are also two publications showing that human OAT3 takes up creatinine.[39,40] In contrast to the previous studies, Lepist et al. reported that creatinine was not transported by human OAT1 or OAT3.[41] Here we showed that creatinine inhibited the uptake of 6-CF mediated by OAT1 and OAT3 at a very high concentration. Phenylacetic acid has the potential to be highly clinically relevant and is a novel inhibitor for OAT1 and OAT3. Of note, different plasma concentrations of phenylacetic acid in CKD are reported, ranging from about 70 μM to as high as 3500 μM.[42,43] Besides, the unbound concentrations of several uremic solutes are not available, further studies are needed to understand if those uremic solutes indeed play a role in modulating OAT1 and OAT3 activities in patients with CKD. Because of their large size, we did not expect any direct interactions of OAT1 and OAT3 with middle molecules, proteins, and peptides with molecular weight <60 kDa,[44] and the results confirmed our expectations (Supplementary Table S1).

OAT1 and OAT3 play an important role in elimination of several uremic solutes. CMPF, indoxyl sulfate, kynurenic acid, *p*-cresyl sulfate, and uric acid have been reported to be transported by both OAT1 and OAT3, while indole acetate and hippuric acid are transported by OAT1 (Supplementary Table S4).[10,35,36,45,46] Metabolomics studies in OAT1 or OAT3 knockout mice revealed more uremic solutes as potential substrates of OAT1 and OAT3 and confirmed their biological relevance (Supplementary Table S4).[38,45−49] Interestingly, several of them found in metabolomics studies are free water-soluble solutes. This again suggests the importance of this group of solutes in modulating OAT1 and OAT3 in CKD.

Inhibitors of transporters have been shown to have different potencies, depending on the substrates used in the *in vitro* assays.[50] Therefore, to determine the relevance of OAT1/3 inhibition identified in our screening study to clinically used drugs, we tested CMPF, hippuric acid, and phenylacetic acid as inhibitors of OAT1- and OAT3-mediated uptake of prescription drug substrates (Figure 4). The results showed that these uremic solutes may inhibit drug transport by the two OATs and the values of IC_{50} differed slightly when different substrates were used. Overall, in agreement with the results using 6-CF as the substrate, the IC_{50} of CMPF, hippuric acid, and phenylacetic acid as inhibitors of adefovir, tenofovir, famotidine, and Ro 64-0802 uptake were significantly lower than their concentrations in CKD patients suggesting that transporter inhibition may occur *in vivo* and thus lead to reduced drug clearance.

4.2. Clinical Evidence Supporting the Role of Uremic Solutes in Modulating OAT Activity in CKD Patients. Deteriorating renal active tubular secretion is expected in patients with CKD.[51−53] In fact, reduced expression levels of OAT1 and OAT3 and impaired active secretion have been reported in many rat models of renal impairment.[54−62] Pharmacokinetic studies in rats with acute kidney injury showed greater reduction in active secretion clearance when compared to the reduction of GFR.[63,64] However, little is known about the degree of deterioration of active secretion in patients with CKD in comparison to the reduction in glomerular filtration rates, a measure generally used to reflect kidney function. To our knowledge, our review of published clinical PK data for renally eliminated drugs is the first to examine changes in CL_{sec} versus GFR in patients with CKD. Our analysis suggests that for drugs that are cleared substantially by tubular secretion (i.e., $CL_r/fu \cdot GFR > 1.5$), CL_{sec} (likely mediated by OAT1 and OAT3) deteriorates more extensively than GFR in patients with CKD (Figure 5b).

We used eq 1 to quantify factor (F_x) responsible for additional deterioration in CL_{sec}, which cannot be fully accounted for by change in GFR. The median F_x values appear to decrease as renal function declines between CKD Stage 2

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and CKD Stage 5 (Supplemental Figure S3). For CKD Stage 4, the calculated F_x ranges from 0.02 to 1.00, with a median of 0.41. The wide ranges of calculated F_x values were observed for all CKD stages. Together with *in vitro* inhibition data reported in this study, we hypothesize that transporter inhibition by uremic solutes accumulated in CKD patients may at least partially explain the observed additional percent decrease in CL_{sec} in CKD patients. That is, the calculated F_x value might represent an inhibition mechanism. Assuming reversible inhibition of OATs by all responsible uremic solutes, F_x can be mechanistically related as shown in eq 2:[65]

$$\frac{CL_{int,Inh}}{CL_{int}} = 1/(1 + \sum_{k=1}^{n} [I]_k / K_{ik}) \quad (2)$$

$CL_{int,Inh}$ and CL_{int} are intrinsic clearance of transporter in the presence and absence of inhibitor(s), $[I]_k$ and K_{ik} are the concentration and reversible inhibition constant for *k*th inhibitory uremic solute.

The observed range of F_x within each CKD stage is wide. Under the assumption that F_x is related to fold difference in CL_{int} in eq 2, such high variability can be explained by several plausible reasons. First, the compositions and the concentrations of uremic solutes could be different in various clinical studies that we analyzed. These differences may lead to different CL_{int} ratios in patients of the same CKD stage. Second, small numbers of patients were sometimes seen in some studies and thus the corresponding F_x might not be representative. For example, the numbers of patients in CKD Stage 5 were 3, 1, and 2 for cefozolin, hydrochlorothiazide, and pemetrexed, respectively (Supplementary Table S2). This might potentially explain the slightly higher median value in CKD Stage 5 compared to CKD Stage 4. Third, in spite that all drugs were net secreted (CL_{sec} > fu·GFR), reabsorption could still occur. CL_{sec} was calculated based on the assumption of no reabsorption, which might lead to underestimation of CL_{sec}. Each drug could have a different degree of reabsorption and thus different underestimation of CL_{sec}. Furthermore, the contribution of OAT1 and OAT3 to CL_{sec} could vary by drugs. Other transporters might be involved. Different efflux transporters and/or reabsorption transporters among those drugs might also be expected. This complexity would increase the variability of F_x. Forth, due to limited information available, we assumed that there were no changes in fu for drugs in CKD if fu in various stages of CKD were not available. Several drugs in our analysis have fu > 0.8 and therefore changes in fu in CKD might not be important. The effects of CKD on fu have been reported for some drugs with medium to high protein binding capacities such as cefazolin, pemetrexed, and sitagliptin.[20,25,66] There were no differences between the fu values measured in healthy volunteers and patients with CKD for pemetrexed (fu = 0.23) and sitagliptin (fu = 0.64).[20,66] The values of fu of cefazolin increased from 0.16 to 0.28 in patients with CKD Stage 4.[25] An increased fu is often observed in CKD for highly protein-bound anionic drugs as a result of reduced serum albumin levels, and thus, the "true" CL_{sec} in CKD as well as F_x would be even smaller than those we estimated in our analysis, particularly in later stages of CKD; the impact of fu change in CKD Stage 5 would be greater than CKD Stage 4. Collectively, our data suggests CL_{sec} deteriorate more extensively than GFR. Renal clearance by secretion measured in off-dialysis period recovered in patients with CKD Stage 5d (Figure 5c), consistent with the removal of dialyzable uremic solutes (unbound protein-bound solutes and free water-soluble solutes) that inhibit renal transporters. These results demonstrate that several uremic solutes inhibit OAT1 and OAT3 and are consistent with the contribution of uremic solutes to F_x.

4.3. Utility of *In Vitro* OAT Inhibition Data in Quantitatively Predicting Pharmacokinetics of Renally Cleared Drugs in CKD Patients. If the connection between F_x and eq 2 can be confirmed, and if the *in vitro*−*in vivo* extrapolation for OAT inhibition by uremic solutes can be established, one can integrate *in vitro* inhibition data generated in this work and exposure information on uremic solutes in CKD patients into eq 1 to quantitatively predict $CL_{sec,CKD}$. Such prediction may have the potential to support dosing recommendations for renally cleared drugs in CKD patients in drug development, especially for drugs that are significantly secreted (i.e., CL_r/fu·GFR > 1.5).

4.4. Other Circumstances When Serum Uremic Solutes Elevate. The results of our studies have important implications beyond patients with CKD. It is of note that many uremic solutes are endogenous metabolites that are continuously synthesized in the human body. Various factors could lead to elevated concentrations of these uremic solutes, including disease, genetics, dietary factors, and medications. For example, patients with gout, impaired glucose tolerance, and diabetes mellitus have been reported to have elevated plasma uric acid concentrations comparable to those in CKD patients.[67] Canavan disease is an autosomal recessive disease characterized by progressive loss of myelin in brain neuron cells. Patients with Canavan have plasma uric acid concentration as high as ∼1700 μM, which is 3-fold higher than the concentrations in CKD patients.[68] Hippuric acid is a clinically relevant uremic solute that inhibited OAT1 and OAT3 in our study. It can be acquired from consumption of food enriched in phenolic compounds such as wine, grape juice, tea, etc.[69,70] It was reported that the fasting plasma hippuric acid and CMPF levels increased 3.3- and 2.6-fold in subjects on a Nordic diet.[71] Thus, levels of hippuric acid in subjects on a Nordic diet might be sufficiently high to inhibit OAT1 and OAT3. Interestingly, phenylacetic acid and benzoic acid are used to treat hyperammonemia in patients with urea cycle disorders under the brand name, Ammonul.[72] Following administration of Ammonul[72] the maximum plasma concentrations of phenylacetic acid and hippuric acid (a metabolite of benzoic acid) are around 2200 and 350 μM, respectively. These concentrations are much higher than their *in vitro* IC_{50} values for OAT1 and OAT3, suggesting that Ammunol coadministered with drugs that are eliminated by OAT1 or OAT3 may result in clinical DDIs. In addition to hyperammonemia treatment, phenylacetic acid might be applied to other diseases such as cancer and Alzheimer's disease, and again may have the potential to cause clinical DDIs.[73,74]

4.5. Limitation in This Study. OATP4C1, another renal organic anion transporter, was not studied but is expressed at low levels in our HEK293 cells. Our studies focus largely on the effects of uremic toxins on influx transporters in the kidney; however, studies are clearly needed to understand the effects of uremic solutes on renal efflux transporters as well. Although there are studies showing interaction between uremic solutes and efflux transporters,[75−77] intracellular concentrations of those solutes are not well studied. It is difficult to evaluate the clinical relevance.

Though our data suggest that uremic toxins directly inhibit OATs, it is also possible that uremic toxins indirectly modulate

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expression levels of OATs. In fact, several animal studies suggest altered renal transporters expression by CKD. OAT1, OAT3, and OCT2 were down-regulated.[54,56−58,61,62,78] MRP2 was up-regulated,[61,78,79] while renal P-gp showed no change.[79] Effect of uremic solutes on regulating renal transporter expression is still unclear. An oral adsorbent, AST-120, which could remove uremic solutes such as indoxyl sulfate has been shown to increase the expression of OAT1 in uremic rats.[55] To our knowledge, there is no data showing the effect of CKD or uremic solutes in human. The expression of OAT1 and OAT3 mRNA reduced by 50% in the diabetic kidney.[80] Treating a human proximal tubule cell line, HK-2 with sera from CKD rats resulted in reduced NPT1, OAT3, OATP1, and P-gp protein expression.[61] It might be argued if the findings in animal models, diabetic kidneys, and in vitro data could be extrapolated well into the clinical manifestation in CKD. Further studies will be needed to clarify this issue. The F_x values in this studies should be regarded as the products of direct inhibition of uremic solutes on transporters, potential changes of transporter expression, and other factors that alter the pharmacokinetics in CKD.

4.6. Conclusion. In this study, we identified uremic solutes, including several novel ones, that inhibit two clinically important renal anion transporters, OAT1 and OAT3. Our analysis of clinical reports suggests that active secretory clearance of drugs that are highly secreted deteriorates more than GFR in patients with CKD. Though further studies are needed, uremic solutes that inhibit OAT1 and OAT3 and accumulate in CKD may explain these data. Our suggested prediction factor, F_x, may be useful in quantitatively predicting secretory clearance of OAT1/OAT3 substrate drugs in CKD patients and supporting dosing recommendations for these renally cleared drugs in CKD.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00332.

(PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

6-CF, 6-carboxyfluorescein; CL_{cr}, creatinine clearance; CL_{sec}, secretory clearance; CKD, chronic kidney disease; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; DDI, drug−drug interaction; GFR, glomerular filtration rate; IC_{50}, half maximal inhibitory concentration; OAT1, organic anion transporter 1; OAT3, organic anion transporter 3; OCT2, organic cation transporter 2; PK, pharmacokinetic(s)

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