A Plasma Concentration of α-Ketoglutarate Influences the Kinetic Interaction of Ligands with Organic Anion Transporter 1

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ABSTRACT

The purpose of the present study was to determine whether a physiologic plasma concentration of α-ketoglutarate (αKG) influences the kinetic interaction of ligands with organic anion transporter 1 (OAT1). The effect of extracellular αKG on the kinetics of para-aminomethylpurpurinate (PAH) and cidofovir transport was examined along with its effect on the potency of 10 drugs in five different classes (uricosuric, nonsteroidal anti-inflammatories, loop diuretics, angiotensin II receptor antagonists, and β-lactam antibiotics) to inhibit OAT1 expressed in Chinese hamster ovary cells. Extracellular αKG competitively inhibited PAH and cidofovir transport with Ki values (~5 μM) approximating its unbound plasma concentration (determined by equilibrium dialysis). When PAH was the substrate, extracellular αKG (5 μM) significantly increased IC50 values for some inhibitors (up to 4-fold), such as probenecid, but not for others (an inhibitor-dependent effect). For some inhibitors, a significant increase in IC50 value was observed when cidofovir was the substrate, but not PAH (a substrate-dependent effect). A significant increase in IC50 value was also observed for inhibition of PAH transport by probenecid in renal basolateral membrane vesicles (5.2-fold). The substrate- and inhibitor-dependent effect of extracellular αKG on ligand interactions with OAT1 highlights the complexity of the OAT1 ligand-binding surface. The effect of extracellular αKG on the potency of OAT1 inhibition should be considered when assessing drug-drug interaction potential at the transporter.

Introduction

Renal proximal tubular secretion is an important pathway for eliminating from the body numerous therapeutic drugs that are negatively charged at physiologic pH, that is, organic anions. For many of these drugs, their rate of tubular secretion influences their systemic concentration and half-life, important determinants of therapeutic potential for efficacy as well as toxicity. Active tubular organic anion secretion involves transporter-mediated uptake across the basolateral membrane of proximal tubule cells, followed by transporter-mediated efflux across the apical membrane. A number of transporters are implicated in mediating apical efflux of organic anions (Pelis and Wright, 2011). The organic anion transporters (OAT) 1 (SLC22A6) and 3 (OAT3; SLC22A8) are important mediators of basolateral organic anion uptake, and possibly, in many cases, the rate-limiting step in their transepithelial secretion (Rizwan and Burchhardt, 2007; Burchhardt and Burchhardt, 2011; Burchhardt, 2012).

OAT1- and OAT3-mediated renal tubular secretion are suspected sites of drug-drug interactions (DDIs), whereby the systemic concentration of a drug substrate (victim drug) is increased in the presence of an inhibitor (perpetrator drug) (Masereeuw and Russel, 2010; Burchhardt and Burchhardt, 2011; Burchhardt, 2012; Morrissey et al., 2013). In particular, the renal elimination of a number of OAT substrates, including famotidine (Inotsume et al., 1990), cidofovir (Cundy et al., 1995), furosemide (Vree et al., 1995), and ciprofloxacin (Jaehde et al., 1995), is slowed in the presence of the well-established OAT inhibitor probenecid. However, due to overlap in substrate selectivity between OAT1, OAT3, and other clearance mechanisms, such as metabolism and other transporters, it is difficult to solely implicate renal OATs in DDIs. Yet, due to their potential involvement in DDIs, the US Food and Drug Administration and the European Medicines Agency, in their recent guidance on drug interaction studies, recommend in vitro testing of the interaction of investigational drugs as inhibitors of OAT1 and OAT3 (European Medicines Agency, 2012; US Food Drug Administration, 2012). In vitro cell systems, such as Chinese hamster ovary (CHO) and human embryonic kidney cell lines that stably express OAT1 and OAT3, are the recommended model systems for these in vitro studies (Giacomini et al., 2010). If the investigational drug is determined to be an in vitro inhibitor, the concentration to produce half-maximal transport is determined (IC50 value). If the maximal therapeutic unbound plasma concentration divided by the in vitro IC50...
value is >0.1, then a clinical DDI study between a probe OAT substrate [para-aminohippurate (PAH) for OAT1 and estrone-3-sulfate for OAT3] and the investigational perpetrator drug is recommended (US Food and Drug Administration, 2012). Consequently, it is important to accurately determine the in vitro inhibitory potency of investigational drugs toward OAT1 and OAT3.

OAT1 and OAT3 are exchangers that use the energy in the outwardly directed α-ketoglutarate (αKG; Kreb’s cycle intermediate) gradient to facilitate substrate uptake via an anion exchange mechanism (Pelis and Wright, 2011). Three independent studies have measured αKG concentration in human plasma, with all three finding similar levels, 8.6 μM (Rocchiccioli et al., 1984), 8.9 μM (Wagner et al., 2010), and 10–12 μM (Halamkova et al., 2012). The intracellular concentration of αKG in proximal tubule cells is much higher due to oxidative metabolism and Na-dependent uptake mediated by the Na-dependent dicarboxylate cotransporter 3 (NaDC3) (Pritchard, 1995). Although uptake mediated by the Na-dependent dicarboxylate transporter 3-sulfate (50 Ci/mmol) were from American Radiochemicals (St. Louis, MO). All other chemicals were of the highest purity possible and were reported in the National Center for Biotechnology Information database.

Materials and Methods

Reagents and Chemicals. [3H]PAH (60 Ci/mmole) and [3H]estrone-3-sulfate (50 Ci/mmole) were from American Radiochemicals (St. Louis, MO). [3H]Cidofovir (36.7 Ci/mmole) and [3H]ClO4 (40 mCi/mole) were from Moravek (Brea, CA). Platinum High Fidelity DNA polymerase, pCDNA5/FRT/VS-His-TOPO mammalian expression plasmid, F12 Kaighn’s modification medium, fetal bovine serum (certified, US origin), 1% penicillin-streptomycin solution, zeocin, and hygromycin B were from Life Technologies (Burlington, ON, Canada). Nitrocellulose membrane filters (0.45-μm) were from Millipore (Billerica, MA). The bicinchoninic acid protein assay kit and Rapid Equilibrium Dialysis Device inserts were from Thermo Scientific (Rockford, IL). Oligonucleotide probes for cloning OAT1 and OAT3 were from Integrated DNA Technologies (Coralville, IA). All other chemicals were of the highest purity possible and were obtained from Sigma-Aldrich (St. Louis, MO).

αKG Plasma Protein Binding. Human plasma was from Bioreclamation LLC (Hempstead, NY). Plasma from three male donors was used for protein-binding studies (lot numbers BRH689788, BRH689789, and BRH689790). The age range of the donors was 32–51, and they were verbally screened to be nonsmokers, caffeine-free, and alcohol/drug-free. Plasma protein binding of [14C]αKG and [14C]ABC123 (proprietary control compound) was estimated using the rapid equilibrium dialysis method. The rapid equilibrium apparatus consisted of two chambers (donor and acceptor) separated by a dialysis membrane with a molecular mass cutoff of 12,000 Da. [14C]αKG and [14C]ABC123 each at a nominal concentration of 5 μM were prepared in human plasma, and 0.2 ml of the mixture was added to the donor chamber. The receiver chamber contained 0.35 ml phosphate-buffered saline. The assembled apparatus was incubated at 37°C for 4 hours with shaking at 120 rpm. Aliquots (50 μl) from each chamber were removed and mixed with 5 ml scintillation fluid, and the radioactivity was measured by liquid scintillation counting. Each experiment was performed in triplicate using plasma from the three different donors. The unbound αKG and ABC123 concentrations were calculated as follows:

\[ \text{% unbound} = \frac{[\text{Drug}]\text{receiver chamber}}{[\text{Drug}]\text{donor chamber}} \times 100 \] (1)

Cloning of the Human Orthologs of OAT1 and OAT3. The open reading frames of the human orthologs of OAT1 and OAT3 were amplified from a human kidney cDNA library using Platinum High Fidelity DNA polymerase and sequence-specific oligonucleotide probes. PCR products were purified using the QIAquick gel extraction kit (Qiagen, Toronto, ON, Canada) and subcloned into the pcDNA5/FRT/VS-His-TOPO mammalian expression plasmid. Plasmid DNA was prepared using the MO BIO Ultra Clean plasmid preparation kit (MO BIO Laboratories, Carlsbad, CA), and sequences were confirmed by DNA sequencing (MCLAB, San Francisco, CA). The cDNA sequences of OAT1 (GenBank accession number AF124373) and OAT3 (GenBank accession number AB042505) were the same as reported in the National Center for Biotechnology Information database.

Cell Culture and Stable Expression of OAT1 and OAT3. CHO Flp-In cells (hereafter referred to as CHO cells) were grown in complete media (F12 Kaighn’s modification medium, 10% fetal bovine serum, and 1% penicillin-streptomycin) containing 100 μg/ml Zeocin in a humidified atmosphere of 5% CO2/95% air at 37°C. CHO cells were transfected by electroporation (ECM630, BTX; Harvard Apparatus, Holliston, MA) with 2 μg plasmid DNA. The cuvettes (BTX) had a gap size of 4 mm, and the electroporation settings were as follows: voltage = 260 V, no resistance, and capacitance = 1050 μF. Following electroporation, the cells were placed in a T75 flask until ~50% confluent (~2 days), at which time the medium was switched to complete medium supplemented with 200 μg/ml hygromycin B. Medium was changed every third day until the cells reached near confluence (~2 weeks), at which time they were passaged. Stable expression of OAT1 and OAT3 was confirmed by functional assays ([3H]PAH or [3H]estrone-3-sulfate uptake) in which substrate uptake into the stable cell lines was compared with uptake into the parental CHO cells.

Transport Measurement with the Stable Cell Lines. Cells grown to confluence in 24-well flat-bottom plates were used for transport experiments. The transport buffer consisted of Krebs buffer pre-equilibrated (~20-minute pre-equilibration period) to 37°C in a humidified atmosphere of 5% CO2/95% O2. The transport solution contained a tracer amount of radiolabeled substrate (~1 μCi/ml) in the absence or presence of test compounds (αKG, unlabeled substrate, and/or inhibitor drug). The Krebs buffer contained the following (in mM): 117 NaCl, 4.5 KCl, 20 NaHCO3, 6 d-glucose, 1 MgCl2, 1.5 CaCl2, and 10 HEPES (pH 7.25). Transport experiments consisted of aspirating the individual wells, rinsing them quickly with 0.5 ml room temperature Krebs buffer, and then adding transport buffer for the amount of time indicated in the figure legends. The transport buffer was aspirated and the wells were rinsed three times with ice-cold Krebs. Cells were lysed in 0.5 N NaOH containing 1% SDS, and lysates were neutralized with 1 N HCl. Radioactivity content was determined by liquid scintillation counting (LS6500; Beckman Coulter). The bicinchoninic acid method was used to determine protein content in the lysates.

The kinetics of OAT1 inhibition was conducted using [3H]PAH and [3H]Cidofovir as substrates. Their final concentration in the transport solution was in the low nanomolar range (~15 nM). The kinetics of inhibition was determined by nonlinear regression using the following relationship:
Uptake was stopped by adding ice-cold stop solution containing the uptake buffer containing 49 and unlabeled substrate equal to

\[ \text{where } J \text{ is the rate of radiolabeled substrate uptake from a concentration of labeled substrate equal to } [S^*]. D \text{ is defined as the nonsaturable component of uptake that is most likely due to factors such as nonspecific binding, incomplete rinsing of the cell layer, and passive diffusion. IC}_{50} \text{ is the inhibitor concentration } [I] \text{ required to reduce substrate uptake by 50%. The apparent maximal transport rate ( } J_{\text{app}} \text{) is defined as the } J_{\text{max}}/(K_{G}\text{)}—where } J_{\text{max}} \text{ is the maximal rate of substrate transport, } K_{G} \text{ is the Michaelis constant, and } K_{I} \text{ is the inhibitory constant for the test agent. Because substrate concentrations were well below their } K_{m} \text{ value for interaction with OAT1, the IC}_{50} \text{ value approximates the } K_{m} \text{ value according to the Cheng–Prusoff relationship:}

\[ K_{m} (1 + [S^*]/K_{m}). \]

\[ K_{m} \text{ values are reported for } \alpha K G \text{ because we provide evidence that it is a competitive inhibitor of OAT1-mediated PAH and cidofovir transport. IC}_{50} \text{ values are reported for all other test compounds because the mechanism of inhibition was not tested in this study.}

The kinetics of PAH and cidofovir transport was determined by nonlinear regression analysis using the Michaelis–Menten equation:

\[ J = \frac{J_{\text{max}}[S]}{K_{m} + [S]} + D[S^*] \]

where } J \text{ is rate of substrate transport from a concentration of labeled and unlabeled substrate equal to } [S]. J_{\text{max}} \text{ and } D \text{ have the same meanings as noted above. The } K_{m} \text{ value (Michaelis constant) is the substrate concentration resulting in half-maximal transport.}

Transport Measurement with Basolateral Membrane Vesicles. Pig kidneys (sex unknown) were obtained from Stearns Meats (Charlestown, MA) under the University of Connecticut Office of Laboratory Animal Welfare/National Institutes of Health Assurance of Compliance A3124-01, Project E13-013. Pig kidney BLMVs were isolated on a Percoll gradient by the method of Sacktor et al. (1981) with modifications. Minced kidneys (10 g) were initially diluted (1:6 wt/vol) in sucrose buffer, and centrifuged at 100,000 g for 1 hour. The final pellet was resuspended in 250 µl vesicle buffer and frozen in liquid nitrogen. Uptake buffer consisted of the following (in mM): 300 sucrose, 3 Tris base (titrated to pH 7.4 with HEPES), and 0.2 ibuprofen (OAT1 inhibitor). Vesicles were collected and washed on nitrocellulose filters (0.45-µm) by filtration. Transport assays were done in triplicate on four separate BLMV preparations. IC}_{50} \text{ values for inhibition of vesicular PAH uptake by probenecid were determined using eq. 2.}

Statistical Analysis. Data are reported as mean ± S.D. All transport observations were performed in triplicate, and the number of observations based on experiments with a different cell passage or vesicle preparation is reported in the figure legends. Comparison of sample means was done using a two-tailed unpaired Student’s t test. Linear regression analysis was used to compare IC}_{50} \text{ values determined in the present study with those reported in the literature (significance set at } P < 0.05). All graphing, linear regression, nonlinear regression, and statistical analysis were performed with GraphPad Prism (version 5.04; GraphPad Software, La Jolla, CA).

Results

Binding of }\alpha K G \text{ to Human Plasma Proteins. } \alpha K G \text{ concentration in human plasma approximates } 8–12 \mu M \text{ (Rocchiccioli et al., 1984; Wagner et al., 2010; Halamkova et al., 2012), but, to our knowledge, the unbound concentration that is free to interact with the renal uptake transporters OAT1 and OAT3 is unknown. Rapid equilibrium dialysis was used to determine the extent to which } \alpha K G \text{ binds to human plasma proteins. In plasma from three separate donors, binding of } \alpha K G \text{ to human plasma protein was negligible (∼100% was unbound; data not shown). As a control, we examined the extent of plasma protein binding of a proprietary drug (ABC123). The fraction of ABC123 bound to human plasma protein was } ∼99.5\%. \text{ The recovery of both } \alpha K G \text{ and ABC123 was } 100\%, \text{ indicating that neither compound bound nonspecifically to the equilibrium dialysis apparatus.}

Effect of Exogenous }\alpha K G \text{ on PAH and Cidofovir Transport by OAT1 in CHO Cells. A previous study by Kaufhold et al. (2011) examined the potency (IC}_{50} \text{ value) of } \alpha K G \text{ to inhibit OAT1 using PAH as a substrate. Given the potential for ligand interactions to be substrate-dependent, as shown for other members of the solute carrier family, including OATP1B3 (Roth et al., 2011), organic cation transporter 2 (Belzer et al., 2013), and multidrug and toxin extrusion 1 (Martinez-Guerrero and Wright, 2013), we examined the effect of } \alpha K G \text{ on transport of two structurally unrelated OAT1 substrates, PAH (a carboxylate) and cidofovir (a phosphonate). The uptakes of } ^{3}H\text{PAH and } ^{3}H\text{cidofovir were reduced 14.5- and 18-fold, respectively, when } 100 \mu M \alpha K G \text{ was added to the transport buffer (Fig. 1A). Figure 1B shows the inhibitory kinetic profile of } \alpha K G \text{ against transport of PAH and cidofovir by OAT1. Despite their structural differences, } \alpha K G \text{ was an equipotent inhibitor of OAT1-mediated PAH ( } K_{I} \text{ value of } 5.37 \pm 1.1 \mu M \text{) and cidofovir transport ( } K_{I} \text{ value of } 5.01 \pm 1.6 \mu M \text{) (Fig. 1B). Inhibitory constants ( } K_{I} \text{ values) are reported because we show later that the mechanism of inhibition is competitive. Based on literature values for plasma concentrations of } \alpha K G \text{ (8–12 } \mu M \text{) and its limited plasma protein binding (data presented in this work), the IC}_{50} \text{ value of } \alpha K G \text{ against OAT1 is near its physiologic unbound plasma concentration. Thus, subsequent experiments to assess the mechanism by which } \alpha K G \text{ cis inhibits OAT1 and its effects on the potency with which compounds inhibit its activity were conducted using the following (in mM): 300 sucrose, 3 Tris base (titrated to pH 7.4 with HEPES), and 0.2 ibuprofen (OAT1 inhibitor). Vesicles were collected and washed on nitrocellulose filters (0.45-µm) by filtration. Transport assays were done in triplicate on four separate BLMV preparations. IC}_{50} \text{ values for inhibition of vesicular PAH uptake by probenecid were determined using eq. 2.}
a near-physiologic concentration of αKG added to the transport buffer (in most cases, 5 μM).

**Mechanism by which Exogenous αKG Cis Inhibits OAT1.** The inhibition kinetics presented above and by others (Kaufhold et al., 2011) offer no insight into the mechanism by which αKG cis inhibits OAT1. To determine the mechanism, we examined the kinetics of PAH and cidofovir transport in the absence versus presence of exogenous αKG. Initial experiments were done to determine the time course of [3H]PAH and [3H]cidofovir uptakes into CHO-OAT1 cells and the effect of exogenous αKG (Fig. 2). Uptake of PAH was linear for approximately 1 minute in both the absence and presence of αKG (5 μM) and approached steady state at approximately 2 minutes in both cases (Fig. 2A). In comparison, the uptake of [3H]cidofovir was linear for a much longer period (Fig. 2B). In both cases, exogenous αKG reduced PAH and cidofovir uptake by approximately half at each time point examined, which is consistent with the IC50 value of αKG against OAT1-mediated PAH and cidofovir transport. Figure 3 shows the kinetic profile of PAH and cidofovir uptake (at initial rate) into CHO-OAT1 cells. The uptakes of both PAH and cidofovir were saturable in the absence and presence of exogenous αKG (Fig. 3, A and B). Exogenous αKG had no effect on the Jmax associated with PAH or cidofovir transport, but caused an approximately 2-fold increase in the Km values and, accordingly, an approximately 50% reduction in transport efficiency (Table 1). A linear transformation of the kinetic data highlights the pure Km effect of exogenous αKG on OAT1-mediated transport of PAH and cidofovir (Fig. 3, C and D, respectively). In contrast, exogenous αKG had no effect on the Km value (3.20 ± 2.7 versus 2.52 ± 0.82 μM, n = 3) or Jmax value (28.5 ± 23 versus 21.6 ± 8.9 pmol × mg protein⁻¹ × min⁻¹, n = 3) associated with estrone-3-sulfate transport by OAT3 stably expressed in CHO cells.

**Effect of αKG on Potency of OAT1 Inhibition in CHO Cells.** Because an increase in Km value for substrate transport was observed in the presence of a near-physiologic concentration of αKG, we hypothesized that it would also reduce the potency of OAT1 inhibition. To assess this possibility, we examined the effect of exogenous αKG on the potency (IC50 values) with which drugs in structurally diverse classes inhibit PAH and cidofovir transport by OAT1 expressed in CHO cells. The drug classes examined were a uricosuric (probenecid), nonsteroidal anti-inflammatory drugs (indomethacin, naproxen, and ibuprofen), loop diuretics (furosemide and bumetanide), angiotensin II receptor antagonists (valsartan and telmisartan), and β-lactam antibiotics (ceftaxone and cephalothin). These drugs were chosen because of the following: 1) they inhibit OAT1; 2) IC50 values are available from the literature; 3) they are clinically (or were) relevant; and 4) they are structurally diverse, as shown in Fig. 4. Regardless of whether exogenous αKG was present or not, all of the drugs at the highest concentration tested reduced PAH and cidofovir uptake to ≤20% of the control value (data for selected drugs in each of the classes are shown in Figs. 5 and 6). In the absence of inhibitor, exogenous αKG reduced the rate of PAH and cidofovir transport to approximately 50% of the control value—control level of transport being that done in the absence of inhibitor and exogenous αKG (data not shown). Figure 5 shows the inhibitory kinetic profiles against PAH transport for selected drugs within each of the therapeutic classes, and Table 2 summarizes the IC50 values determined for all of the drugs tested. Exogenous

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**Fig. 1.** (A) One-minute uptake of [3H]PAH and [3H]cidofovir by CHO-OAT1 cells done either in the absence (control) or presence of αKG (100 μM) added to the transport buffer. Significant differences from control were determined by unpaired Student’s t test, *P < 0.05; **P < 0.001. (B) One-minute uptake of [3H]PAH and [3H]cidofovir by CHO-OAT1 cells done in the presence of increasing concentrations of αKG (0–100 μM) added to the transport solution. Data are expressed as a percentage of the control uptake done in the absence of exogenous αKG. Km values were determined by nonlinear regression analysis (eq. 2).

**Fig. 2.** Time course of [3H]PAH (A) and [3H]cidofovir (B) uptake by CHO-OAT1 cells done in the absence or presence of αKG (5 μM) added to the transport solution. Data are from one representative experiment.
αKG (5 μM) caused a significant rightward shift in IC₅₀ values (reduced potency) for some drugs, but not others (Fig. 5; Table 2). The greatest fold increase in IC₅₀ value was for ibuprofen (4-fold) and the lowest for telmisartan (1-fold) (Table 2). In a separate set of experiments, with probenecid as the inhibitor and PAH as the substrate, we used a higher concentration of exogenous αKG (12 μM), as this concentration represents the higher end of the range for measured plasma αKG in healthy humans (Halamkova et al., 2012). In four separate experiments including 12 μM αKG in the transport, solution increased the probenecid IC₅₀ value 3.67 ± 1.7-fold (P < 0.01, two-tailed unpaired Student’s t test). Figure 6 shows the inhibitory kinetic profiles against cidofovir transport for selected drugs within each of the therapeutic classes, and Table 2 summarizes the IC₅₀ values determined for each of the drugs tested. Inclusion of αKG (5 μM) in the transport buffer caused a ~2-fold significant increase in IC₅₀ value for all inhibitors tested (Fig. 6; Table 2). A comparison between the control IC₅₀ data in which PAH was the substrate and literature IC₅₀ values for the same inhibitors revealed a significant correlation that was near unity (Fig. 7A). However, adding αKG (5 μM) to the transport buffer caused most of the IC₅₀ values to shift above the line of unity, and the correlation between literature IC₅₀ values and IC₅₀ values determined in the presence of exogenous αKG was not significant (Fig. 7B).

**Effect of αKG on OAT Transport Activity in BLMV.** Renal cortical BLMVs have proven an excellent model for studying energetic and kinetic mechanisms of OAT-mediated transport. For these reasons, it is useful to compare effects of exogenous αKG seen with OAT1 overexpressed in cultured cells with that seen in BLMVs that presumably have the full complement of machinery that energizes OAT1-mediated transport in the proximal tubule, such as NaDC3. The vesicles were predominately enriched with Na,K-ATPase (31.6 ± 6.3-fold), but less so with the apical marker alkaline phosphatase (4.87 ± 1.1-fold) or with oligomycin-sensitive ATPase activity (2.55 ± 0.34-fold), a marker of mitochondrial membranes. An initial experiment examined the time course of PAH uptake in BLMVs (Fig. 3). Kinetics of PAH (A and C) and cidofovir (B and D) uptake by CHO-OAT1 cells done in the absence or presence of αKG (5 μM) added to the transport solution. Uptakes for both substrates were conducted for 1 minute. Data are the mean of four observations. S.D. are not included in A and B, but they are shown in C and D. C and D are linear transformations of the data in A and B, respectively. The kinetic values are summarized in Table 1, and the analyses were performed using eq. 4.

### Table 1

<table>
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<th>Substrate</th>
<th>Treatment</th>
<th>J₅₀</th>
<th>Kₐₐ₅₀</th>
<th>Tₑff</th>
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<tr>
<td>PAH</td>
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<td>12.7 ± 5.4</td>
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<td></td>
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<td>585 ± 159</td>
<td>28.1 ± 6.8*</td>
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<td>Cidofovir</td>
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<td>518 ± 300</td>
<td>125 ± 38**</td>
<td>4.35 ± 2.7*</td>
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</tbody>
</table>

*P < 0.05; **P < 0.01.
into porcine renal cortical BLMVs either in the presence of an inwardly directed Na gradient and a 50-fold outwardly directed αKG concentration gradient (50 μM in versus 1 μM out) or in the absence of both the Na gradient and αKG gradient (50 μM in versus 50 μM out) (Fig. 8A). Uptake of PAH was linear for approximately 1 minute, and an overshoot was observed between 1 and 90 minutes, indicating active PAH accumulation in the presence of the outwardly directed αKG gradient. At the 1-minute time point, the uptake of PAH was 2.65-fold higher in the presence of the inwardly directed Na gradient and outwardly directed αKG gradient than in their absence. The intention of the vesicle experiments was to mimic as close to possible the extracellular conditions in the in vitro experiments with CHO-OAT1 cells. We could not eliminate extravesicular αKG completely because the vesicle buffer contained 50 μM αKG and the vesicles were diluted 500-fold with uptake buffer, leaving a minimum final extravesicular concentration of 1 μM. However, we established conditions in which extravesicular αKG concentration was different by 5 μM, similar to the in vitro experiments with CHO-OAT1 cells. Figure 8B shows the effect (in four separate experiments) of increasing concentrations of probenecid on the uptake of PAH in the presence of either 1 or 6 μM extravesicular αKG. The IC₅₀ value was significantly higher (5.26 ± 2.5-fold) in the presence of 6 μM versus 1 μM extravesicular αKG (7.13 ± 4.06 μM versus 1.37 ± 0.82 μM, respectively; P < 0.05, two-tailed unpaired Student’s t test).

**Discussion**

Because only the unbound fraction of αKG is available to interact with the OAT ligand-binding surface, we initially set out to determine the unbound concentration of αKG in human plasma. In our analysis, plasma protein binding of αKG was undetectable, whereas plasma protein binding of an investigational drug (ABC123) was high, suggesting that the observed lack of αKG binding was most likely not due to experimental error. Because human plasma contains endogenous αKG, we cannot rule out the possibility that [¹⁴C]αKG binding was minimal due to saturation of protein binding by endogenous αKG. Thus, we examined plasma protein binding of αKG using a protein-binding predictor in the Simcyp Simulator version 12 (Simcyp Limited, Sheffield, UK). The quantitative structure activity relationship model in Simcyp predicted an unbound
fraction of 56% for αKG. An independent investigation of the quantitative structure-activity relationship model performance for a variety of organic anions suggests that the model is predictive (Supplemental Table 1). Based on reported total plasma αKG concentrations (8–12 μM), and our experimental and predicted results for plasma protein binding, the unbound

Fig. 5. Inhibitory effect of selected drugs on OAT1-mediated [3H]PAH uptake into CHO-OAT1 cells done either in the absence or presence of αKG (5 μM). Uptake of [3H]PAH was conducted for 1 minute. Data are the mean ± S.D. of four observations and are expressed as a percentage of the control uptake done in the absence of inhibitor drug. In the absence of inhibitor drug, αKG (5 μM) reduced uptake by ~50% (data not shown). IC50 values are summarized in Table 2, and the analyses were performed using eq. 2. Significant difference in IC50 values (between control and +5 μM αKG) was determined by unpaired Student’s t test, *P < 0.05; ***P < 0.001.

Fig. 6. Inhibitory effect of selected drugs on OAT1-mediated [3H]cidofovir uptake into CHO-OAT1 cells done either in the absence or presence of αKG (5 μM). Uptake of [3H]cidofovir was conducted for 1 minute. Data are the mean ± S.D. of four observations and are expressed as a percentage of the control uptake done in the absence of inhibitor drug. In the absence of inhibitor drug, αKG (5 μM) reduced uptake by ~50% (data not shown). IC50 values are summarized in Table 2, and the analyses were performed using eq. 2. Significant differences in IC50 values (between control and +5 μM αKG) were determined by unpaired Student’s t test, *P < 0.05.
plasma αKG concentration is most likely in the range of 5–12 μM.

Exogenous αKG inhibited OAT1-mediated PAH and cidofovir transport with near-identical potencies (Ki values of approximately 5 μM) that approximated its physiologic unbound plasma concentration. The Ki value observed in this study is near-identical to the IC50 value (4.7 μM) for αKG inhibition of PAH transport by OAT1 determined elsewhere (Kaufhold et al., 2011). Ki values are reported because αKG behaved as a true competitive inhibitor of both PAH and cidofovir transport. That is, addition of αKG to the transport solution caused an increase in Km values, without affecting Jmax values. Despite their apparent structural differences, these data indicate that αKG (dicarboxylate) and PAH (monocarboxylate), and αKG and cidofovir (phosphonate), share a common binding region in OAT1. We can infer from these results that PAH and cidofovir also share a common binding region, although this needs to be confirmed experimentally. Unlike OAT1, exogenous αKG had no significant effect on the Km or Jmax value associated with estrone-3-sulfate transport by OAT3. This was expected given that αKG cis inhibits OAT3 with an IC50 value approximately 20-fold higher (92.8 μM) than the concentration used in our transport solution (Kaufhold et al., 2011).

Because exogenous αKG influenced the interaction of substrates with the ligand-binding region of OAT1, we speculated that it would have a similar effect on the kinetic interaction of inhibitors with OAT1. Indeed, exogenous αKG increased the IC50 value associated with probenecid inhibition of PAH transport in both CHO-OAT1 cells and BLMVs. Interestingly, the IC50 value was approximately 10-fold lower in BLMVs than CHO-OAT1 cells, potentially a result of species differences (porcine versus human) in the OAT1 binding region. Although exogenous αKG increased the IC50 values against PAH transport by CHO-OAT1 cells for some inhibitors, a significant shift was not seen for furosemide, valsartan, or telmisartan, indicating that the effect of αKG on inhibitor potency is inhibitor-dependent. The effect of αKG on inhibition potency was also substrate-dependent, as significant increases in IC50 values for furosemide and valsartan were observed when cidofovir was the substrate, but not PAH. Taken together, these findings highlight the complexity of the ligand-binding surface of OAT1.

There are reports of interindividual variability in plasma αKG levels (Rocchiccioli et al., 1984), and, at least in the case of individuals with hyperammonemia, plasma concentrations increase nearly 4-fold (Batshaw et al., 1980). Yet, even these concentrations are not expected to inhibit OAT1 uptake activity into proximal tubule, as concentrations up to 100 μM, when applied to the bath surrounding perfused and freshly

**TABLE 2**

The effect of extracellular αKG (5 μM) on potency of OAT1 inhibition

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control</th>
<th>+5 μM αKG</th>
<th>Fold Change</th>
<th>Control</th>
<th>+5 μM αKG</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probencid</td>
<td>10.5 ± 1.9</td>
<td>24.3 ± 8.3*</td>
<td>2.3</td>
<td>7.63 ± 3.5</td>
<td>14.8 ± 3.9*</td>
<td>1.9</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3.79 ± 1.9</td>
<td>9.50 ± 2.6*</td>
<td>2.5</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Naproxen</td>
<td>2.23 ± 1.3</td>
<td>5.22 ± 2.0*</td>
<td>2.3</td>
<td>2.05 ± 0.6</td>
<td>5.22 ± 2.4*</td>
<td>2.5</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>3.08 ± 1.2</td>
<td>12.2 ± 2.9**</td>
<td>4.0</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Furosemide</td>
<td>17.8 ± 6.1</td>
<td>21.3 ± 9.2</td>
<td>1.2</td>
<td>9.67 ± 4.7</td>
<td>24.1 ± 13*</td>
<td>2.5</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>11.9 ± 3.0</td>
<td>23.3 ± 10*</td>
<td>1.9</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Valsartan</td>
<td>8.0 ± 1.7</td>
<td>10.4 ± 1.6</td>
<td>1.3</td>
<td>4.93 ± 1.2</td>
<td>9.21 ± 3.60*</td>
<td>1.9</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>0.33 ± 0.10</td>
<td>0.35 ± 0.16</td>
<td>1.0</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>283 ± 30</td>
<td>789 ± 140***</td>
<td>2.8</td>
<td>397 ± 88</td>
<td>972 ± 393*</td>
<td>2.4</td>
</tr>
<tr>
<td>Celtrixone</td>
<td>391 ± 134</td>
<td>958 ± 99.7**</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

*P < 0.05; **P < 0.01; ***P < 0.001.

**Fig. 7.** Correlation between IC50 values reported in the literature and those determined in this study (data from Table 2) for inhibition of OAT1-mediated PAH transport done in the absence (A) or presence (B) of αKG (5 μM) added to the transport solution. The literature data were taken from a review by Burechardt (2012). The dashed line is the line of unity (slope = 1). The inset figures show the linear regression analysis of the data. Data for cephalothin and celtrixone were omitted from the linear regression analysis because their large IC50 values skewed the regression line.
isolated proximal tubules in suspension, do not inhibit activity; concentrations in excess of 200 μM are required (Welborn et al., 1998; Shuprisha et al., 1999). This is in large part due to the ability of NaDC3 to maintain a steep KG gradient, thus stimulating trans-transporter turnover and maximal transport rate, which masks the inhibitory effect of αKG acting in a cis configuration to reduce the potency with which competitive ligands interact with the binding surface. CHO cells presumably do not express the potency with which competitive ligands interact with the OAT1-binding surface. The effect of extracellular ligand interactions highlights the complexity of the OAT1 substrate and inhibitor dependency of extracellular ligands at the binding surface are competitive. The apparent Km value. Thus, the magnitude of ligand-binding surface. For perpetrator drugs in which inhibition potency is reduced by extracellular αKG, heightened plasma αKG levels may protect against DDIs at OAT1 while maintaining a high level of victim drug clearance due to NaDC3 involvement. Vari-ability in plasma αKG levels could contribute to interindivid-ual variability in DDI magnitude at OAT1.

There are several methods outlined by the US Food and Drug Administration for predicting transporter-mediated DDIs from in vitro studies, including static and mechanistic models. Regardless of whether the model is static or mechanistic, accurate predictions of DDIs at OAT1 require that the kinetic interaction of ligands with the OAT1-binding surface measured in vitro reflects those occurring in vivo. Based on our results, we can quantitatively predict, at least for simple competitive interactions, how Km and Ki values will change in plasma levels of approximately 5 μM, leaving the other half available for competitive ligands. Thus, for a competitive interaction between plasma αKG levels are elevated. Accordingly, the Km values for both PAH and cidofovir increased approximately 2-fold in the presence of αKG, approximating its Km value for inhibition of OAT1 transport of PAH and cidofovir. We contend that for drugs in which IC50 values shifted approximately 2-fold, they share a common binding region with αKG and substrate, although it is possible for mixed-type inhibitors to cause a similar shift. In fact, cephalothin, ceftriaxone, and probenecid, all of which had their IC50 values against PAH transport increase approximately 2-fold in the presence of exogenous αKG, were previously characterized as competitive inhibitors of human OAT1-mediated PAH transport (Takeda et al., 2001, 2002). For drugs in which IC50 values did not shift, such as telmisartan, they are most likely non-competitive inhibitors.

In conclusion, the potency with which some ligands interact with the OAT1 ligand-binding surface is reduced by a physiologic unbound plasma concentration of αKG. This most likely occurs in cases in which the interactions between αKG and ligands at the binding surface are competitive. The apparent ligand and substrate inhibitor dependency of extracellular αKG on ligand interactions highlights the complexity of the OAT1 ligand-binding surface. The effect of extracellular αKG on the potency of OAT1 inhibition should be considered when assessing drug-drug interaction potential at the transporter.

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**Authorship Contributions**

**Participated in research design:** Pelis, Hanna, Renfro.

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**Performed data analysis:** Ingraham, Li, Parker, Vapurcuyan.

**Wrote or contributed to the writing of the manuscript:** Pelis, Hanna, Renfro.

**References**


