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-aminomethylpyridine (PAH) and cidofovir transport was examined along with its effect on the potency of 10 drugs in five different classes (aminosugar, nonsteroidal anti-inflammatory drugs, 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 Burchardt, 2007; Burchardt and Burchardt, 2012; Burchardt, 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; Burchardt and Burchardt, 2011; Burchardt, 2012; Morrissey et al., 2013). In particular, the renal elimination of a number of OAT substrates, including famotidine (Inotsúma 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-aminobenzoic acid (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 higher due to oxidative metabolism and Na-dependent concentration of a plasma level of αKG in CHO cells and basolateral membrane vesicles (BLMVs). Consistent with their hypothesis, an unbound plasma level of αKG influenced the kinetic interaction of ligands with OAT1.

Materials and Methods

Reagents and Chemicals. [3H]PAH (60 Ci/mmol) and [3H]estrone-3-sulfate (50 Ci/mmol) were from American Radiochemicals (St. Louis, MO). [3H]Cidofovir (36.7 Ci/mmol) and [3H]Abc123 (40 Ci/mmol) were from Moravek (Brea, CA). Platinum High Fidelity DNA polymerase, pcDNA5/FRT/V5-His-TOPO mammalian expression plasmid, F12 Kaighn’s modification medium, 10% fetal bovine serum, and 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 biocinchonic 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 (Corvallis, 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 measured by 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:

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

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/V5-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 = 1500 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. After the uptake period, 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 biocinchonic 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:
\[ J = \frac{J_{\text{app}}[S^+]}{IC_{50} + [I]} + D[S^+] \]  

(2)

where \( J \) is the rate of radiolabeled substrate uptake from a concentration of labeled substrate equal to \([S^+]\). \( D \) 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\(_{50}\) is the inhibitor concentration \([I]\) required to reduce substrate uptake by 50%. The apparent maximal transport rate \( (J_{\text{app}}) \) is defined as the \( J_{\text{max}}/(K_m/[I]) \) where \( J_{\text{max}} \) is the maximal rate of substrate transport, \( K_m \) is the Michaelis constant, and \( K_i \) is the inhibitory constant for the test agent. Because substrate concentrations were well below their \( K_m \) value for interaction with OAT1, the IC\(_{50}\) value approximates the \( K_i \) value according to the Cheng–Prusoff relationship:

\[ K_i(1 + [S^+]/K_m). \]  

(3)

\( K_i \) values are reported for \( \alpha \)KG because we provide evidence that it is a competitive inhibitor of OAT1-mediated PAH and cidofovir transport. IC\(_{50}\) 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:

\[ J = \frac{J_{\text{max}}[S]}{K_m + [S]} + D[S] \]  

(4)

where \( J \) is rate of substrate transport from a concentration of labeled and unlabeled substrate equal to \([S]\). \( J_{\text{max}} \) and \( D \) have the same meanings as noted above. The \( K_m \) 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 (Charleston, 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 Sakctor et al. (1981) with modifications. Minced kidneys (10 g) were initially diluted (1:6 wt/vol) in sucrose buffer containing the following (in mM): 250 sucrose and 10 Tris-HCl (pH 7.4 with Tris base). The tissue was homogenized 20 strokes in a glass Teflon homogenizer at 1000–2500 rpm, followed by three 20-second pulses using a Polytron equipped with a PT 20S probe. After a further 1:1 dilution with sucrose buffer, the homogenate was centrifuged at 1500g for 15 minutes. The supernatant was removed and centrifuged at 20,500g for 20 minutes. The outer beige fluffy layer of each pellet was resuspended in sucrose buffer for a combined total of 32 ml and homogenized 18 strokes, as above. Three milliliters of Percoll was added, and the mixture was homogenized three additional strokes. This was then centrifuged at 34,540g for 1 hour, after which the first 6.0 ml of the gradient was discarded. The next 7.0 ml was divided equally between two tubes, diluted to approximately 12.5 ml per tube with sucrose buffer, and centrifuged at 100,000g for 1 hour. The resulting pellet was resuspended in Na-free vesicle buffer (150 mM KCl, 20 mM Tris base, and 50 μM αKG, titrated to pH 7.4 with HEPES) or Na-containing vesicle buffer (150 mM NaCl, 20 mM Tris base, and 50 μM αKG, titrated to pH 7.4 with HEPES) and centrifuged again at 100,000g 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): 150 NaCl, 3 KCl, 20 Tris-HEPES (pH 7.4), and 0.001 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\(_{50}\) 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\(_{50}\) 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 αKG to Human Plasma Proteins.** αKG concentration in human plasma approximates 8–12 μM (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 αKG binds to human plasma proteins. In plasma from three separate donors, binding of αKG 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%. The recovery of both αKG and ABC123 was 100%, indicating that neither compound bound nonspecifically to the equilibrium dialysis apparatus.

**Effect of Exogenous αKG on PAH and Cidofovir Transport by OAT1 in CHO Cells.** A previous study by Kaufhold et al. (2011) examined the potency (IC\(_{50}\) value) of αKG 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 αKG on transport of two structurally unrelated OAT1 substrates, PAH (a carboxylate) and cidofovir (a phosphonate). The uptakes of [3H]PAH and [3H]cidofovir were reduced 14.5- and 18-fold, respectively, when 100 μM αKG was added to the transport buffer (Fig. 1A). Figure 1B shows the inhibitory kinetic profile of αKG against transport of PAH and cidofovir by OAT1. Despite their structural differences, αKG was an equi potent inhibitor of OAT1-mediated PAH (\(K_i \) value of 5.37 ± 1.1 μM) and cidofovir transport (\(K_i \) value of 5.01 ± 1.6 μM) (Fig. 1B). Inhibitory constants (\(K_i \) values) are reported because we show later that the mechanism of inhibition is competitive. Based on literature values for plasma concentrations of αKG (8–12 μM) and its limited plasma protein binding (data presented in this work), the IC\(_{50}\) value of αKG against OAT1 is near its physiologic unbound plasma concentration. Thus, subsequent experiments to assess the mechanism by which αKG cis inhibits OAT1 and its effects on the potency with which compounds inhibit its activity were conducted using the following (in mM): 30% 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\(_{50}\) 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 K_m values and, accordingly, an approximately 50% reduction in transport efficiency (Table 1). A linear transformation of the kinetic data highlights the pure K_m 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 K_m value (3.20 ± 2.7 versus 2.52 ± 0.82 μM, 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 K_m 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 (ceftriaxone 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.
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 and literature IC₅₀ values for the same inhibitors.

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Jₘₐₓ</th>
<th>Kₘ</th>
<th>Tₘₐₓ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH</td>
<td>Control</td>
<td>480 ± 117</td>
<td>12.7 ± 5.4</td>
<td>41.0 ± 13</td>
</tr>
<tr>
<td></td>
<td>+5 μM αKG</td>
<td>585 ± 159</td>
<td>28.1 ± 6.8*</td>
<td>21.4 ± 7.2*</td>
</tr>
<tr>
<td>Cidofovir</td>
<td>Control</td>
<td>572 ± 384</td>
<td>51.3 ± 13</td>
<td>11.0 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>+5 μM αKG</td>
<td>518 ± 300</td>
<td>125 ± 38**</td>
<td>4.35 ± 2.7*</td>
</tr>
</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 aKG concentration gradient (50 μM in versus 1 μM out) or in the absence of both the Na gradient and aKG 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 aKG 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 aKG 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 aKG completely because the vesicle buffer contained 50 μM aKG 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 aKG 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 aKG. The IC50 value was significantly higher (5.26 ± 2.5-fold) in the presence of 6 μM versus 1 μM extravesicular aKG (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 aKG is available to interact with the OAT ligand-binding surface, we initially set out to determine the unbound concentration of aKG in human plasma. In our analysis, plasma protein binding of aKG was undetectable, whereas plasma protein binding of an investigational drug (ABC123) was high, suggesting that the observed lack of aKG binding was most likely not due to experimental error. Because human plasma contains endogenous aKG, we cannot rule out the possibility that [14C]aKG binding was minimal due to saturation of protein binding by endogenous aKG. Thus, we examined plasma protein binding of aKG 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
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 ($K_i$ values of approximately 5 μM) that approximated its physiologic unbound plasma concentration. The $K_i$ value observed in this study is near-identical to the IC$_{50}$ value (4.7 μM) for αKG inhibition of PAH transport by OAT1 determined elsewhere (Kaufhold et al., 2011). $K_i$ 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 $K_m$ values, without affecting $J_{\text{max}}$ 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 $K_m$ or $J_{\text{max}}$ value associated with estrone-3-sulfate transport by OAT3. This was expected given that αKG cis inhibits OAT3 with an IC$_{50}$ 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 IC$_{50}$ value associated with probenecid inhibition of PAH transport in both CHO-OAT1 cells and BLMVs. Interestingly, the IC$_{50}$ 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 IC$_{50}$ 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 IC$_{50}$ 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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control (μM)</th>
<th>+5 μM αKG (μM)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probencid</td>
<td>10.5 ± 1.9</td>
<td>24.3 ± 8.3*</td>
<td>2.3</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3.79 ± 1.9</td>
<td>9.50 ± 2.6*</td>
<td>2.5</td>
</tr>
<tr>
<td>Naproxen</td>
<td>2.23 ± 1.3</td>
<td>5.22 ± 2.0*</td>
<td>2.3</td>
</tr>
<tr>
<td>Ibufrofen</td>
<td>3.08 ± 1.2</td>
<td>12.2 ± 2.9**</td>
<td>4.0</td>
</tr>
<tr>
<td>Furosemide</td>
<td>17.8 ± 6.1</td>
<td>21.3 ± 9.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>11.9 ± 3.0</td>
<td>23.3 ± 10*</td>
<td>1.9</td>
</tr>
<tr>
<td>Valsartan</td>
<td>8.0 ± 1.7</td>
<td>10.4 ± 1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>0.33 ± 0.10</td>
<td>0.35 ± 0.16</td>
<td>1.0</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>283 ± 30</td>
<td>789 ± 140***</td>
<td>2.8</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>391 ± 134</td>
<td>958 ± 99.7***</td>
<td>2.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control (μM)</th>
<th>+5 μM αKG (μM)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

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

**Fig. 7.** Correlation between IC$_{50}$ 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 ceftriaxone were omitted from the linear regression analysis because their large IC$_{50}$ values skewed the regression line.
influenced by changes or differences in plasma concentrations. In contrast, the potency of inhibition can be affected by extracellular 

For perpetrator drugs in which inhibition potency is reduced through an increase in the transport activity in our cultured system was a decrease in activity, so the dominant effect of exogenous binding surface. CHO cells presumably do not express the potency with which competitive ligands interact with the substrate and inhibitor dependency of extracellular ligands at the binding surface are competitive. The apparent occurs in cases in which the interactions between ligand and 

Fig. 8. Uptake of PAH into porcine renal cortical BLMVs. (A) Time course of PAH uptake in the presence of a 50:1 (in/out) outwardly directed αKG and inwardly directed Na gradient (Gradient) or in the absence of a Na gradient and αKG gradient (No Gradient) (details in Materials and Methods). (B) Four replicate experiments showing the inhibitory effect of probenecid on PAH uptake (1-minute uptakes) done either with a 50:1 (control) or 50:6 (in/out) outwardly directed αKG gradient. These experiments were done in the presence of an inwardly directed Na gradient. Nonlinear regression analysis was performed using eq. 2. We did not show a graph depicting mean ± S.D. because we used different probenecid concentrations in each of the experiments. The data are expressed as a percentage of the control uptake done in the absence of inhibitor drug.

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 Na gradient, which stimulates 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 appreciable levels of Na-dependent dicarboxylate cotransport activity, so the dominant effect of exogenous αKG on absolute transport activity in our cultured system was a decrease through an increase in the Km value. Thus, the magnitude of OAT1-mediated tubular secretion of organic anions is most likely well-preserved over a wide range of plasma αKG concentrations. In contrast, the potency of inhibition can be influenced by changes or differences in plasma αKG levels. 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. Variability in plasma αKG levels could contribute to interindividual 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 Kᵢ and Kₘ values will change in the presence of αKG. Approximately half of the binding region will be occupied by αKG under physiologic conditions (plasma levels of approximately 5 μM), leaving the other half available for competitive ligands. Thus, for a competitive interaction between ligand and αKG, Kᵢ and Kₘ values should increase 2-fold, and this fold change should increase further under conditions in which plasma αKG levels are elevated. Accordingly, the Kₘ values for both PAH and cidofovir increased approximately 2-fold in the presence of a αKG concentration, approximating its Kᵢ 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 substrate and 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.
Conducted experiments: Ingraham, Li, Parker, Vapurcuyan.
Performed data analysis: Ingraham, Li, Parker, Vapurcuyan.
Wrote or contributed to the writing of the manuscript: Pelis, Hanna, Renfro.

References


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Title: A plasma concentration of αKG influences the kinetic interaction of ligands with OAT1

Authors: Leslie Ingraham, Mansong Li, J Larry Renfro, Sonda Parker, Arpine Vaparcuyan, Imad Hanna and Ryan M. Pelis

Journal: Molecular Pharmacology

Supplemental Table 1. Performance of a QSAR model for predicting the fraction of drug unbound in human plasma.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Predicted f_{up}</th>
<th>Observed f_{up}</th>
</tr>
</thead>
<tbody>
<tr>
<td>probenecid</td>
<td>0.053</td>
<td>0.05 – 0.25</td>
</tr>
<tr>
<td>indomethacin</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>naproxen</td>
<td>0.026</td>
<td>0.01</td>
</tr>
<tr>
<td>ibuprofen</td>
<td>0.01</td>
<td>0.01 – 0.1</td>
</tr>
<tr>
<td>cidofovir</td>
<td>0.91</td>
<td>0.94</td>
</tr>
<tr>
<td>furosemide</td>
<td>0.065</td>
<td>0.05</td>
</tr>
<tr>
<td>bumetanide</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>valsartan</td>
<td>0.003</td>
<td>0.03</td>
</tr>
<tr>
<td>telmisartan</td>
<td>0.0005</td>
<td>0.005</td>
</tr>
<tr>
<td>cephalothin</td>
<td>0.28</td>
<td>0.2 – 0.35</td>
</tr>
<tr>
<td>methotrexate</td>
<td>0.64</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The QSAR model used was contained in the Simcyp Simulator version 12 (Simcyp Ltd, Sheffield, UK). The physicochemical properties of the organic anion drugs listed, which was input into Simcyp for the predictions were obtained from the DrugBank database (http://www.drugbank.ca). The observed values for protein binding were also obtained from the DrugBank database. Organic anions for which there is a range reported for observed f_{up} values exhibit non-linear plasma protein binding. f_{up} refers to the fraction of drug unbound in plasma.