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 (aKG) influences the kinetic interaction of ligands with organic anion transporter 1 (OAT1). The effect of extracellular aKG on the kinetics of para-aminohippurate (PAH) and cidofovir transport was examined along with its effect on the potency of 10 drugs in five different classes (cisurotic, nonsteroidal anti-inflammatories, loop diuretics, angiotensin II receptor antagonists, and β-lactam antibiotics) to inhibit OAT1 expressed in Chinese hamster ovary cells. Extracellular aKG competitively inhibited PAH and cidofovir transport with IC50 values (∼5 μM) approximating its unbound plasma concentration (determined by equilibrium dialysis). When PAH was the substrate, extracellular aKG (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 aKG on ligand interactions with OAT1 highlights the complexity of the OAT1 ligand-binding surface. The effect of extracellular aKG 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, 2011; 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 (Inotsu 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

ABBREVIATIONS: aKG, α-ketoglutarate; BLMV, basolateral membrane vesicle; CHO, Chinese hamster ovary; DDI, drug-drug interaction; Jmax, maximal transport rate; NaDC3, Na-dependent dicarboxylate cotransporter 3; OAT, organic anion transporter; PAH, para-aminohippurate.
value is >0.1, then a clinical DDI study between a probe OAT
substrate [para-aminomhippurate (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 inter-
mediate) 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
transporter 3 (NaDC3) (Pritchard, 1995). Although
intracellular αKG is an important trans-substrate driving
OAT activity, extracellular αKG is a substrate and cis
inhibits OAT1 and OAT3 with IC50 values of 4.7 and 92.8 μM,
respectively (Kaufhold et al., 2011). Given that the IC50 value
of αKG against OAT1 is near its plasma concentration, we
hypothesized that a physiologic plasma concentration of αKG
would alter the kinetic interaction of ligands with the trans-
porter. To test this hypothesis, we examined the effect of a
near-physiologic unbound plasma concentration of αKG
on the kinetic interaction of substrates and inhibitors with
OAT1 in CHO cells and basolateral membrane vesicles
(BLMVs). Consistent with our 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]ClO4 (40 mCi/mmol)
were from Moravek (Brea, CA). Platinum High Fidelity DNA
polymerase, pCDNA5/FRT/V5-His-TOPO mammalian expression
plasmid, P12 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 bichinoninic 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 (Corvalis, OR).
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 by the
rapid equilibrium dialysis method. The rapid equilibrium
 apparatur consisted of two chambers (donor and acceptor) sepa-
rated by a dialysis membrane with a molecular mass cutoff of
12,000 Da. [14C]αKG and [14C]ABC123 each at a nominal concen-
tration 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 appa-
rratus 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:

% unbound = \frac{|\text{Drug}|_{\text{receiver chamber}}}{|\text{Drug}|_{\text{donor chamber}}} \times 100 \tag{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/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
Fip-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 Appar-
atus, 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. 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 de-
termined by liquid scintillation counting. Each experiment was
performed in triplicate using plasma from the three different donors.

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 were 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 \( J_{\text{max}}/(K_m/K_i) \) —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:

\[ J = \frac{J_{\text{max}}[S]}{K_m + [S]} + DS \]  
(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 Basalateral Membrane Vesicles. Pig kidneys (sex unknown) were obtained from Stearns Meats (Charleton, 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 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 \( \alpha KG \), titrated to pH 7.4 with HEPES) or Na-containing vesicle buffer (150 mM NaCl, 20 mM Tris base, and 50 μM \( \alpha KG \), 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 PAH, and contained no \( \alpha KG \), 5 μM \( \alpha KG \), or 49 μM \( \alpha KG \). \(^3\)H)PAH (2 μCi/ml) uptake was determined by adding 480 μl uptake buffer to 10 μl vesicles at room temperature. Given the 50-fold dilution of vesicles with uptake buffer, the outwardly directed \( \alpha KG \) gradient was 50-fold (50 μM in versus 1 μM out) in vesicles incubated in uptake buffer lacking \( \alpha KG \), 8.3 (50 μM in versus 6 μM out) in vesicles incubated in uptake buffer containing 5 μM \( \alpha KG \), and 1-fold in vesicles incubated in uptake buffer containing 50 μM \( \alpha KG \) (50 μM in versus 50 μM out). Uptake was stopped by adding ice-cold stop solution containing the following (in mM): 300 sucrose, 3 Tris base (titrated to pH 7.4 with HEPES), and 0.2 ibuprofen (OAT 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 were 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 \( \alpha KG \) to Human Plasma Proteins.** \( \alpha KG \) 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 \( \alpha KG \) binds to human plasma proteins. In plasma from three separate donors, binding of \( \alpha KG \) 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 \( \alpha KG \) and ABC123 was 100%, indicating that neither compound bound nonspecifically to the equilibrium dialysis apparatus.

**Effect of Exogenous \( \alpha KG \) 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 \( \alpha KG \) 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 \( \alpha KG \) on transport of two structurally unrelated OAT1 substrates, PAH (a carboxylate) and cidofovir (a phosphonate). The uptakes of \(^3\)H)PAH and \(^3\)H)cidofovir were reduced 14.5- and 18-fold, respectively, when 100 μM \( \alpha KG \) was added to the transport buffer (Fig. 1A). Figure 1B shows the inhibitory kinetic profile of \( \alpha KG \) against transport of PAH and cidofovir by OAT1. Despite their structural differences, \( \alpha KG \) was an equi-effective 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 \( \alpha KG \) (8–12 μM) and its limited plasma protein binding (data presented in this work), the \( IC_{50} \) value of \( \alpha KG \) against OAT1 is near its physiologic unbound plasma concentration. Thus, subsequent experiments to assess the mechanism by which \( \alpha KG \) cis inhibits OAT1 and its effects on the potency with which compounds inhibit its activity were conducted using
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 (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. Exogenous

![Image](image_url)
αKG (5 μM) caused a significant rightward shift in IC_{50} values (reduced potency) for some drugs, but not others (Fig. 5; Table 2). The greatest fold increase in IC_{50} 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 IC_{50} 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 IC_{50} values determined for each of the drugs tested. Inclusion of αKG (5 μM) in the transport buffer caused a ∼2-fold significant increase in IC_{50} value for all inhibitors tested (Fig. 6; Table 2). A comparison between the control IC_{50} data in which PAH was the substrate and literature IC_{50} 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 IC_{50} values to shift above the line of unity, and the correlation between literature IC_{50} values and IC_{50} 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

**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>J_{max} (pmol × mg protein^{-1} × min^{-1})</th>
<th>K_{m} (μM)</th>
<th>T_{eff} (μl × mg protein^{-1} × min^{-1})</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 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
A 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.
The effect of extracellular αKG (5 μM) on potency of OAT1 inhibition

Data are mean ± S.D. of four to six experiments. Significant differences in IC50 values (between control and +5 μM αKG) was determined by unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control</th>
<th>+5 μM αKG</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>Ibuprofen</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>Cefalothin</td>
<td>283 ± 30</td>
<td>789 ± 140***</td>
<td>2.8</td>
</tr>
<tr>
<td>Celebrexione</td>
<td>391 ± 134</td>
<td>958 ± 99.7**</td>
<td>2.4</td>
</tr>
</tbody>
</table>

IC50 against PAH

IC50 against Cidofovir

ND, not determined.
*P < 0.05; **P < 0.01; ***P < 0.001.

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

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 Burchardt (2012). The dashed line is the line of unity (slope = 1). The inset figures show the linear regression analysis of the data. For cephalothin and ceftriaxone 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 outwardly directed αKG gradient, thus stimulating 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 $K_m$ 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 $K_I$ and $K_m$ 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, $K_I$ and $K_m$ values should increase 2-fold, and this fold change should increase further under conditions in which plasma αKG levels are elevated. Accordingly, the $K_m$ values for both PAH and cidofovir increased approximately 2-fold in the presence of a αKG concentration, approximating its $K_I$ 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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