

Interaction and Transport of Methamphetamine and its Primary Metabolites by Organic Cation and Multidrug and Toxin Extrusion Transporters[§]

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ABSTRACT

Methamphetamine is one of the most abused illicit drugs with roughly 1.2 million users in the United States alone. A large portion of methamphetamine and its metabolites is eliminated by the kidney with renal clearance larger than glomerular filtration clearance. Yet the mechanism of active renal secretion is poorly understood. The goals of this study were to characterize the interaction of methamphetamine and its major metabolites with organic cation transporters (OCTs) and multidrug and toxin extrusion (MATE) transporters and to identify the major transporters involved in the disposition of methamphetamine and its major metabolites, amphetamine and *para*-hydroxymethamphetamine (*p*-OHMA). We used cell lines stably expressing relevant transporters to show that methamphetamine and its metabolites inhibit human OCTs 1–3 (hOCT1–3) and hMATE1/2-K with the greatest potencies against

hOCT1 and hOCT2. Methamphetamine and amphetamine are substrates of hOCT2, hMATE1, and hMATE2-K, but not hOCT1 and hOCT3. *p*-OHMA is transported by hOCT1–3 and hMATE1, but not hMATE2-K. In contrast, organic anion transporters 1 and 3 do not interact with or transport these compounds. Methamphetamine and its metabolites exhibited complex interactions with hOCT1 and hOCT2, suggesting the existence of multiple binding sites. Our studies suggest the involvement of the renal OCT2/MATE pathway in tubular secretion of methamphetamine and its major metabolites and the potential of drug-drug interactions with substrates or inhibitors of the OCTs. This information may be considered when prescribing medications to suspected or known abusers of methamphetamine to mitigate the risk of increased toxicity or reduced therapeutic efficacy.

Introduction

Methamphetamine is a widely abused illicit drug with approximately 1.2 million reported users in the United States (Volkow, 2013). Also known as meth, crystal, speed, or ice, methamphetamine is a potent and highly addictive central nervous stimulant that acts by inhibition and reversal of neurotransmitter transporters of dopamine, norepinephrine, and serotonin (Carvalho et al., 2012; Panenka et al., 2013). Illicit methamphetamine is sold as either a racemic mixture or the *d*-methamphetamine isomer since the dextro isomer is much more psychoactive (de la Torre et al., 2004). High or repeated doses of methamphetamine can affect multiple organ systems, leading to profound neurotoxicity, cardiotoxicity, acute renal failure, and pulmonary toxicity (Volkow et al., 2010; Carvalho et al., 2012).

Following oral, inhalation, or intranasal administration, methamphetamine is well-absorbed into the bloodstream (Harris et al., 2003; Schep et al., 2010) and is distributed into many organs with the highest uptake

occurring in lungs, liver, brain, and kidneys (Volkow et al., 2010). Methamphetamine is eliminated by both hepatic metabolism and renal excretion. In the liver, it is metabolized by the polymorphic enzyme cytochrome P450 2D6 to the *p*-hydroxylation metabolite, *para*-hydroxymethamphetamine (*p*-OHMA), and the *N*-demethylation product, amphetamine (Lin et al., 1997; Shima et al., 2008). Both metabolites have been reported to circulate in plasma of methamphetamine abusers up to the micromolar range (Shima et al., 2008). Amphetamine is also highly psychoactive and addictive with a mechanism of action similar to methamphetamine (Panenka et al., 2013). *p*-OHMA is not psychoactive but acts as a cardiovascular agent with hypertensive and adrenergic effects (Römhold et al., 2003). Concurrent use of CYP2D6 substrates or inhibitors with methamphetamine and related designer drugs represents a risk of potential drug interactions leading to toxicity (Wu et al., 1997; Pritzker et al., 2002; Newton et al., 2005).

Renal excretion is another major elimination pathway for methamphetamine and its metabolites. Approximately 37%–54% of methamphetamine is recovered unchanged in the urine although more may be eliminated renally in CYP2D6 poor metabolizers (Kim et al., 2004). The renal excretion rate of methamphetamine is highly dependent on urinary pH (Beckett and Rowland, 1965b,c; Cook et al., 1992, 1993). The fraction unbound (f_u) of methamphetamine is about 0.8 (de la Torre et al., 2004). The reported renal clearance of methamphetamine is highly variable (e.g., 67–371 ml/min) and much larger than the glomerular filtration rate in some individuals, suggesting that the drug is actively

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ABBREVIATIONS: DDI, drug-drug interaction; HBSS, Hanks' balanced salt solution; hMATE, human multidrug and toxin extrusion; hOAT, human organic anion transporter; hOCT, human organic cation transporter; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MATE, multidrug and toxin extrusion; OCT, organic cation transporter; *p*-OHMA, *para*-hydroxymethamphetamine.

secreted by the kidney (Beckett and Rowland, 1965b,c; Kim et al., 2004). Positron emission tomography imaging also revealed that methamphetamine is highly accumulated in the kidney (Volkow et al., 2010). Both metabolites (*p*-OHMA and amphetamine) also undergo urinary excretion with a possible active secretion component (Shima et al., 2006).

Little is currently known about the involvement of drug transporters in renal elimination and tissue distribution of methamphetamine and its metabolites. With a *pK_a* of ~9.9, methamphetamine and its primary metabolites exist predominantly as protonated cations at physiologic pH (de la Torre et al., 2004). The reported or calculated log *D* values of methamphetamine, amphetamine, and *p*-OHMA at 7.4 are −0.38, −0.62, and −1.11, respectively (Fowler et al., 2007), suggesting a low passive membrane diffusion for the protonated species. In rats, methamphetamine renal clearance was significantly reduced by cimetidine, a classic inhibitor of the renal organic cation secretion system (Kitaichi et al., 2003). In vitro studies have indicated that amphetamine is an inhibitor of human organic cation transporters (hOCTs) (Amphoux et al., 2006; Zhu et al., 2010). However, the inhibition potency, substrate specificity, and transport kinetics of methamphetamine and metabolites toward renal organic cation uptake and efflux transporters have not been comprehensively characterized. This information is important for understanding the mechanisms involved in the disposition and potential drug-drug interaction (DDI) of methamphetamine. The goals of this study were to characterize the interaction of methamphetamine and its major metabolites with hOCT1–3 and human multidrug and toxin extrusion (hMATE) transporters 1 and 2-K (hMATE1/2-K) and to identify the major transporters involved in renal secretion of methamphetamine, amphetamine, and *p*-OHMA.

Materials and Methods

Materials. *d*-Methamphetamine, *d*-amphetamine, and *p*-OHMA were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade. Currently, there is no evidence that organic cation transporters (OCTs) have stereo-selective interaction with cationic substrates (Yin et al., 2015). We focused our study on the dextro isoforms of methamphetamine and amphetamine because they are the psychoactive forms. In all our studies, methamphetamine and amphetamine refer to the dextro isoforms unless specified otherwise. Methamphetamine-*D*₁₁ and amphetamine-*D*₁₁ were purchased from Cerilliant Corporation (Round Rock, TX). [¹⁴C]Metformin (98 mCi/mmol) was purchased from Moravsek Biochemicals, Inc. (Brea, CA). [³H]Estrone sulfate (50 Ci/mmol), and [³H]para-aminohippurate (3 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Optima grade acetonitrile, water, and formic acid were purchased from Fisher Scientific (Waltham, MA). Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). All other chemicals were commercially available and of analytical grade or higher.

Uptake and Inhibition Assays in HEK293 Cells. Flp-in HEK293 cells stably expressing hOCT1, hOCT2, hOCT3, hMATE1, hMATE2-K, human organic anion transporter (hOAT)1, and hOAT3 were previously generated in our laboratory (Duan and Wang, 2010; Duan et al., 2015; Yin et al., 2015). The cells were cultured in high glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 150 µg/ml hygromycin B supplementation at 37°C with 5% CO₂ and high humidity. All cell culture plastic surfaces were coated with 0.1% poly-D-lysine to improve cell attachment. Uptake and inhibition assays were performed as previously described with modification for analysis of methamphetamine and its metabolites by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Duan and Wang, 2010; Duan et al., 2015; Yin et al., 2015). Briefly, cells were seeded in 96-well plates at 100,000 cells/well and grown overnight. Prior to incubation initiation, cells were washed with prewarmed Hanks' balanced salt solution (HBSS) and allowed to acclimate for 10 minutes at 37°C or preincubated with HBSS containing 30 mM ammonium chloride for 20 minutes for multidrug and toxin extrusion (MATE) experiments to acidify the intracellular compartment and drive MATE uptake (Tanihara et al., 2007). Media were removed and incubation initiated by addition of 100 µl of HBSS at pH 7.4

containing a substrate with or without inhibitor. Uptake was stopped by removal of media and washing the cells three times with ice cold HBSS. Cells were either lysed with 100 µl of 1 M NaOH and neutralized with 100 µl of 1 M HCl for incubations containing a radiolabeled substrate for measurement by liquid scintillation counting (Tri-Carb B3110TR; PerkinElmer, Waltham, MA) or permeabilized with 100 µl of methanol containing 100 nM stable labeled internal standard for analysis by LC-MS/MS. Protein content in the lysate in each well was measured by the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL) and the uptake in cells was normalized to their total protein concentrations. The inhibitory effect of methamphetamine, amphetamine, and *p*-OHMA on hOCT1, hOCT2, hOCT3, hMATE1, and hMATE2-K was assessed in transporter-expressing HEK293 cells using [¹⁴C]metformin, a well-established and clinically relevant probe substrate for these transporters (European Medicines Agency, 2012; Food and Drug Administration, 2012; Hillgren et al., 2013). The concentration of metformin in the inhibition experiments (11 µM, 1 µCi/ml) was selected to be much lower than its *K_m* values (780–1500 µM) for the transporters tested (Koepsell et al., 2007; Tanihara et al., 2007). Inhibition and kinetic experiments were performed during the initial rate period using a short incubation time as specified in the legends for Fig. 1, Fig. 2, Fig. 3, Fig. 4, and Fig. 5. Transport experiments were performed in triplicate and repeated three times independently. Uptake was performed in both empty vector- and transporter-transfected cells; and transporter-specific uptake was calculated by subtracting uptake in vector-transfected cells.

LC-MS/MS Analysis of Methamphetamine and its Metabolites. Methamphetamine, amphetamine, and *p*-OHMA levels were quantified using an LC-MS/MS system consisting of an API 4500 triple quadrupole mass spectrometer (AB-Sciex, Foster City, CA) coupled with an LC-20AD ultra-fast liquid chromatography system (Shimadzu Co., Kyoto, Japan). The Turbo Ion Spray interface was operated in positive ion mode. Ten microliters of cell lysate was injected onto an Agilent Eclipse Plus C18 column (1.8 µm; 4.6 × 50 mm) (Agilent, Santa Clara, CA) running with an isocratic method consisting of 0.28 ml/min 0.2% formic acid in water and 0.12 ml/min acetonitrile. Mass transitions (*m/z*) were 150 → 119, 136 → 91, 166 → 135, 161 → 97, and 147 → 98 for methamphetamine, amphetamine, *p*-OHMA, methamphetamine-*d*₁₁, and amphetamine-*d*₁₁, respectively. Data were analyzed using Analyst software version 1.6.2 (AB Sciex). Assay accuracy and precision were within 15% (20% for the lower limit of quantification).

Data Analysis. Transport experiments were performed in triplicate and repeated three times independently. Data representation and replicates with specific *n* numbers are detailed in each figure legend. The transport kinetics were fitted using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) for inhibitory interactions and uptake kinetics of hOCT1–3. WinNonLin Phoenix 6.4.0 (Certara, Princeton, NJ) was used for fitting hMATE apparent transport kinetics. The IC₅₀ values were calculated by fitting the log inhibitor concentration versus the transporter-specific uptake normalized to the vehicle control using the following equation:

$$v = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log(\text{IC}_{50}) - I)^{*H}}} \quad (1)$$

where *v* is the rate of uptake in the presence of the inhibitor; Bottom is the residual baseline value; Top is the rate of uptake in the absence of inhibitor; *I* is the inhibitor concentration; and *H* is the Hill coefficient. Two-site inhibition data were fitted using the following equation:

$$v = \text{Bottom} + (\text{Top} - \text{Bottom}) \left(\frac{\text{Fraction1}}{1 + 10^{I - \log \text{EC}_{50,1}}} + \frac{1 - \text{Fraction1}}{1 + 10^{I - \log \text{EC}_{50,2}}} \right) \quad (2)$$

One- and two-site inhibition equations were compared by an extra sum-of-squares *F* test using the data from all three independent experiments modeled simultaneously. Apparent hMATE1/2-K, *K_m*, and *V_{max}* values were obtained by simultaneously fitting the data to the Michaelis-Menten equation with a passive diffusion component in transporter-transfected cells and only the passive diffusion component in vector-transfected cells (Brouwer et al., 2013):

$$v = \frac{V_{\max} * S}{K_m + S} + P_{\text{diff}} * S \quad (3)$$

where *V* is the velocity of uptake; *V_{max}* is the maximum velocity of uptake; *S* is the substrate concentration; *K_m* is the Michaelis-Menten constant; and *P_{diff}* is the

nonsaturable passive diffusion rate constant. The sigmoidal saturation kinetics of *p*-OHMA hOCT2 transport were obtained by fitting transporter-mediated uptake to the Michaelis-Menten equation with a Hill slope for the substrate concentration and half-maximal transport concentration ($K_{1/2}$ in place of K_m) after inspection of the Eadie-Hofstee plot (Copeland, 2000):

$$v = \frac{V_{\max} * S^H}{K_{1/2}^H + S^H} \quad (4)$$

Amphetamine hOCT2 specific uptake kinetics were fit to a biphasic Michaelis-Menten equation:

$$v = \frac{V_{\max 1} * S}{K_{m1} + S} + \frac{V_{\max 2} * S}{K_{m2} + S} \quad (5)$$

Results

Inhibitory Effect of Methamphetamine and its Metabolites on hOCT1–3 and hMATE1/2-K. The transport activities of hOCT1, hOCT2, hOCT3, hMATE1, and hMATE2-K in the Flp-in HEK293 expression systems were first confirmed with metformin uptake in the presence or absence of the prototypical inhibitor cimetidine (Supplemental Fig. 1). Methamphetamine, amphetamine, and *p*-OHMA inhibited metformin uptake by hOCT1–3 and hMATE1/2-K in a concentration-dependent manner (Fig. 1; Supplemental Fig. 2). The IC_{50} values are summarized in Table 1. Methamphetamine and amphetamine were 4- to 20-fold more potent for hOCT1 and hOCT2 than for hOCT3, hMATE1, and hMATE2-K, with hOCT2 showing the greatest sensitivity to both psychostimulants (hOCT2 IC_{50} values of 15.0 ± 6.81 and $20.3 \pm 16.9 \mu M$, respectively). *p*-OHMA was a more potent inhibitor of hOCT1 than other transporters. Addition of the 4-hydroxyl

group to the aromatic phenyl ring (*p*-OHMA) greatly increased binding to hOCT3 but decreased its potency toward hOCT2 compared with methamphetamine. Interestingly, the Hill slope of methamphetamine and amphetamine inhibition of hOCT1 and hOCT2 was approximately 0.5 (Table 1). Conversely, the Hill slope of *p*-OHMA against hOCT2 was approximately 1.5. hMATE2-K also had steep Hill slopes ranging between 1.6 and 1.9 for methamphetamine and its metabolites. These Hill slopes suggest more complex interactions than simple competitive inhibition may be occurring with these transporters.

Inspection of methamphetamine and amphetamine dose-dependent inhibition of hOCT1 and hOCT2 revealed biphasic inhibition characteristics (Supplemental Fig. 3). A two-site inhibition model was compared with a one-site inhibition model using an extra sum-of-squares *F* test and a cutoff significance value of 0.05 by simultaneously fitting the data from three independent experiments each run in triplicate. The two-binding site model fit significantly better for methamphetamine inhibition of hOCT1 and hOCT2 as well as amphetamine inhibition of hOCT2 ($P < 0.0001$) but not hOCT1 (Table 2). The high-affinity EC_{50} values were in the low micromolar range (0.72 – $5.29 \mu M$), while the apparent low-affinity interactions appeared to be in the high micromolar range (58.2 – $400 \mu M$) for these transporters.

Uptake of Methamphetamine and Metabolites by hOCT1–3, hMATE1, and hMATE2-K. The substrate potential of methamphetamine, amphetamine, and *p*-OHMA was assessed by measuring the uptake of these compounds ($1 \mu M$) in control cells and transporter-expressing cells (Fig. 2). After 5-minute incubation, methamphetamine and amphetamine showed approximately 2-fold greater uptake in cells expressing hOCT2, hMATE1, and hMATE2-K. *p*-OHMA accumulated extensively in hOCT1, hOCT2, and hOCT3, and to a lesser degree in hMATE1, but did not accumulate at all in hMATE2-K-transfected cells

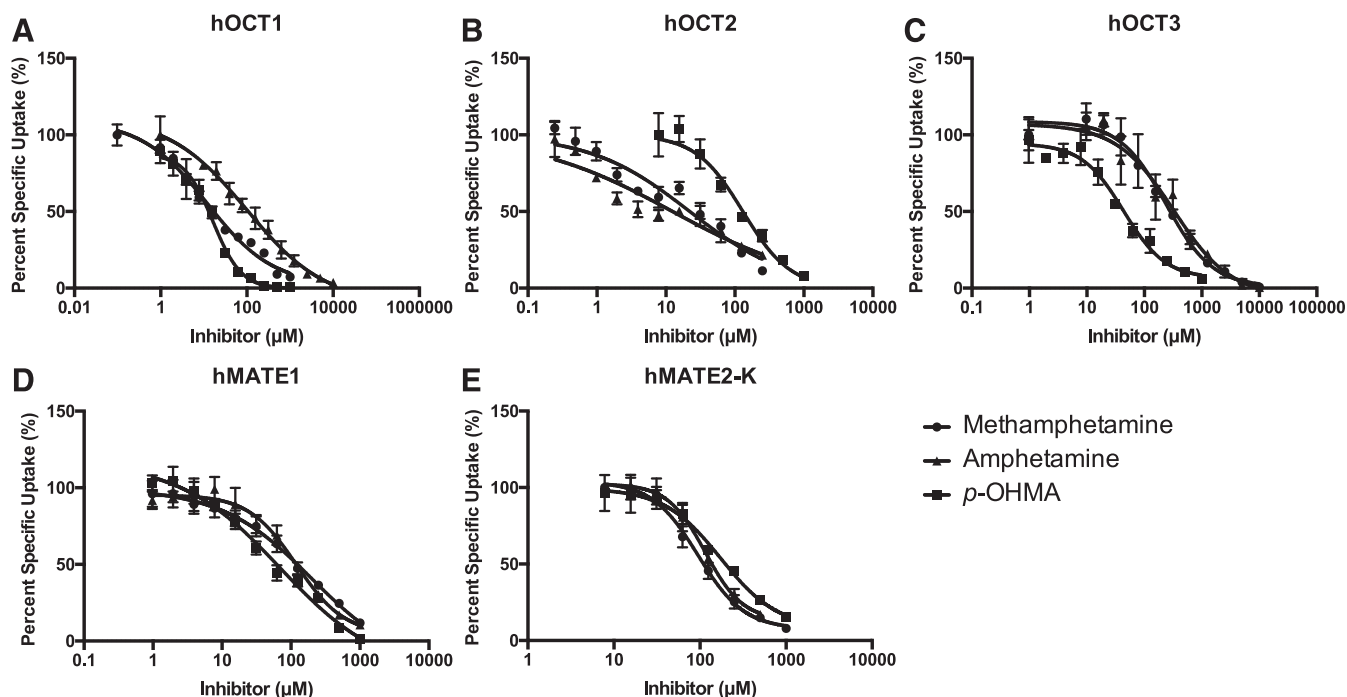


Fig. 1. Inhibition by methamphetamine, amphetamine, and *p*-OHMA of hOCT1, hOCT2, hOCT3, hMATE1, and hMATE2-K. Uptake of [^{14}C]metformin ($11 \mu M$) in the absence and presence of inhibitor was measured in both transporter-expressing and control human embryonic kidney cells. Transporter-specific uptake was obtained by subtracting the uptake in vector-transfected cells from the uptake in transporter-expressing cells. Incubations were performed at 2, 0.5, 2, 5, and 0.5 minutes for hOCT1 (A), hOCT2 (B), hOCT3 (C), hMATE1 (D), and hMATE2-K (E), respectively, which are within the linear initial rate of uptake. Activity in the absence of inhibitor (100%) corresponds to 28.2, 373, 60.2, 52.9, and 100 pmol/min/mg protein for hOCT1, hOCT2, hOCT3, hMATE1, and hMATE2-K, respectively. Each data point represents the mean $\pm$ S.D. from one representative experiment in triplicate. Curves from two additional independent repeats are displayed in Supplemental Fig. 2. The IC_{50} values shown in Table 1 are mean $\pm$ S.D. of the IC_{50} values from the three independent experiments.

TABLE 1
IC₅₀ values of methamphetamine, amphetamine, and *p*-OHMA for hOCT1–3 and hMATE1/2-K determined by one-binding site fitting

Results represent mean $\pm$ S.D. of three independent experiments each run in triplicate.

Transporter	One-Binding Site					
	Methamphetamine		Amphetamine		<i>p</i> -OHMA	
	IC ₅₀	Hill Slope	IC ₅₀	Hill Slope	IC ₅₀	Hill Slope
	μM		μM		μM	
hOCT1	21.1 $\pm$ 8.8	0.55 $\pm$ 0.02	96.7 $\pm$ 37	0.61 $\pm$ 0.28	12.0 $\pm$ 3.4	1.17 $\pm$ 0.14
hOCT2	15.0 $\pm$ 6.8	0.56 $\pm$ 0.07	20.3 $\pm$ 16.9	0.44 $\pm$ 0.1	83.8 $\pm$ 22.3	1.55 $\pm$ 0.32
hOCT3	300 $\pm$ 139	1.42 $\pm$ 0.68	363 $\pm$ 56.4	1.1 $\pm$ 0.2	44.4 $\pm$ 25.5	1.1 $\pm$ 0.26
hMATE1	107 $\pm$ 38	0.79 $\pm$ 0.09	94.0 $\pm$ 25.3	0.89 $\pm$ 0.2	59.1 $\pm$ 14.3	0.84 $\pm$ 0.12
hMATE2-K	84.3 $\pm$ 12.9	1.63 $\pm$ 0.14	158 $\pm$ 48	1.9 $\pm$ 0.6	234 $\pm$ 86.8	1.21 $\pm$ 0.11

when compared with control cells (Fig. 2). These data suggest that renal secretion of methamphetamine and its primary metabolites may involve the hOCT2/hMATE pathway.

Interaction of Methamphetamine and Metabolites with Renal hOAT1 and hOAT3. While hOCT2 and hOAT1/3 mediate renal secretion of organic cations and organic anions, respectively, some substrate and inhibitor overlap between hOCT and hOATs has been reported (Lai et al., 2010). We then investigated if methamphetamine and metabolites interact with hOAT1 and hOAT3 (Fig. 3, A and B). hOAT1- and hOAT3-mediated *para*-aminohippurate or estrone sulfate uptake was completely suppressed by the reference inhibitor probenecid. In contrast, methamphetamine and amphetamine showed no inhibitory effect on hOAT1 or hOAT3 at 1 mM. Only *p*-OHMA showed significant inhibition of hOAT1 and hOAT3 at 1 mM with 47 $\pm$ 17 and 38 $\pm$ 28% inhibition, respectively. Uptake studies showed that none of the compounds were substrates of hOAT1 or hOAT3 (Fig. 3, C–E), suggesting a primary role of the hOCT2/hMATE pathway in active renal secretion of these compounds.

Methamphetamine and Metabolites Uptake Kinetics by Cation Transporters. The kinetics of hOCT1, hOCT2, hOCT3, hMATE1, and hMATE2-K in transporting methamphetamine, amphetamine, and *p*-OHMA was assessed by determining concentration-dependent transport rates. The specific uptake was obtained for hOCT1–3 by subtracting uptake in the control cells and Eadie-Hofstee plots were evaluated to identify the type of interaction. Due to high passive diffusion at high concentrations, methamphetamine kinetics can only be accurately determined at a low concentration range (0–15 μM). Within this range, methamphetamine displayed saturable kinetics with a K_m value of 2.09 $\pm$ 0.88 μM (Fig. 4A; Supplemental Fig. 4; Table 3), which is very close to the high-affinity, half-inhibitory concentration (1.21 $\pm$ 0.19 μM) observed in Supplemental Fig. 2B. For amphetamine, we were able

to cover a wider concentration range (0–600 μM). As shown in Fig. 4B, biphasic transport kinetics were observed, and the K_m values for the apparent high- and low-affinity binding sites were determined to be 0.830 $\pm$ 0.55 and 534 $\pm$ 350 μM , respectively. Interestingly, *p*-OHMA displayed sigmoidal kinetics for hOCT2 as clearly revealed by the Eadie-Hofstee plot (Fig. 4D). The *p*-OHMA hOCT2 half-maximal transport concentration ($K_{1/2}$) is 31.8 $\pm$ 9.3 μM and the Hill slope is 1.64 $\pm$ 0.15. For *p*-OHMA transport by hOCT1 and hOCT3, no apparent sigmoidal or biphasic pattern was observable in the Eadie-Hofstee plot. Fitting to a standard Michaelis-Menten equation yielded apparent K_m values of 14.5 $\pm$ 8.7 and 53.3 $\pm$ 6.2 μM for hOCT1 and hOCT3, respectively (Fig. 4; Supplemental Fig. 4; Table 4).

hMATE1/2-K transport studies were conducted after intracellular acidification to provide an outwardly directed proton gradient to drive substrate uptake because the MATE transporters function as proton/organic cation exchangers. Under this condition, we observed very high uptake of methamphetamine and metabolites in vector-transfected cells, likely due to a pH effect on passive diffusion. The high uptake in vector-transfected cells makes it difficult to discern transporter-specific uptake at high substrate concentrations. Therefore, we fitted the concentration-dependent uptake in transporter-expressing cells to a Michaelis-Menten equation with a non-saturable passive diffusion component (Fig. 5; Supplemental Fig. 5; Table 4). This simultaneous fitting of both carrier- and noncarrier-mediated uptake allowed for an estimate of the apparent K_m values for hMATE1/2-K in the presence of a high-passive permeability component (Table 4).

Discussion

In spite of the major role of renal clearance in methamphetamine disposition, the molecular mechanisms underlying the tubular secretion

TABLE 2

EC₅₀ values of methamphetamine and amphetamine for hOCT1 and hOCT2 determined by two-binding site fitting

The EC₅₀ values were obtained by fitting inhibition data in Fig. 1 and Supplemental Fig. 2 using eq. 2 described in *Materials and Methods* with the fit shown in Supplemental Fig. 3. The last column lists the *P* values obtained by comparing eqs. 1 and 2 with an extra sum-of-squares *F* test. Results represent mean $\pm$ S.D. of three independent experiments each run in triplicate.

Inhibitor	Transporter	Two-Binding Site		Two-Binding versus One-Binding Site
		EC ₅₀ Value		
		High Affinity	Low Affinity	
		μM	μM	
Methamphetamine	hOCT1	5.29 ± 0.66	400 ± 229	$P < 0.0001$
	hOCT2	1.21 ± 0.19	58.2 ± 23.4	$P < 0.0001$
Amphetamine	hOCT1	NA	NA	$P = 0.55$
	hOCT2	0.72 ± 0.29	145 ± 104	$P < 0.0001$

NA, not applicable.

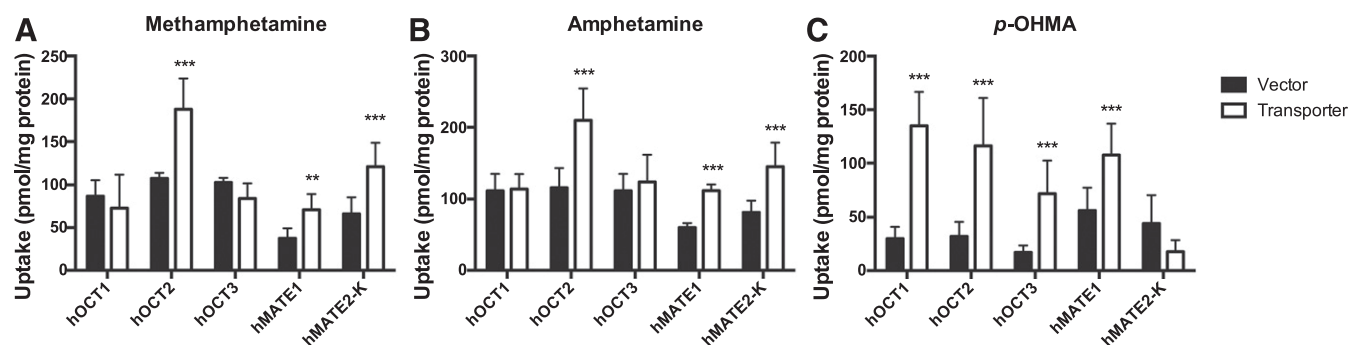


Fig. 2. Uptake of 1 μ M methamphetamine, amphetamine, and *p*-OHMA by hOCT1, hOCT2, hOCT3, hMATE1, and hMATE2-K. Uptake was measured after 5-minute incubation at 37°C. Data are illustrated as the mean $\pm$ S.D. from three independent experiments performed in triplicate. Uptake in transporter-expressing cells was compared with that in control cells (** P < 0.01; *** P < 0.001).

of methamphetamine and its major metabolites had not been fully elucidated (Caldwell et al., 1972; Kim et al., 2004; Carvalho et al., 2012). Here, we showed methamphetamine and its metabolites interact with hOCT1–3 and hMATE1/2-K at clinically relevant concentrations (Melega et al., 2007; Shima et al., 2009). We further demonstrated that methamphetamine and amphetamine are substrates of hOCT2, hMATE1, and hMATE2-K, but not hOCT1 or hOCT3. Interestingly, *p*-OHMA was a substrate of hOCT1–3 as well as hMATE1, but not hMATE2-K. Methamphetamine and its metabolites do not interact with renal hOAT1 or hOAT3. Methamphetamine and its metabolites

demonstrated complex inhibitory and substrate kinetics with hOCT2. Our data suggest that the hOCT2/hMATE pathway is involved in renal secretion of methamphetamine and its metabolites, and that inhibition of hOCT2 and hMATEs by methamphetamine may lead to potential DDIs for drugs that are eliminated by the hOCT2/hMATE pathway.

The importance of renal elimination of methamphetamine has long been known; however, the exact molecular mechanisms of renal secretion had not been identified (Beckett and Rowland, 1965c; Caldwell et al., 1972). Here, we identified the hOCT2/hMATE pathway as being involved in the active renal secretion of methamphetamine and

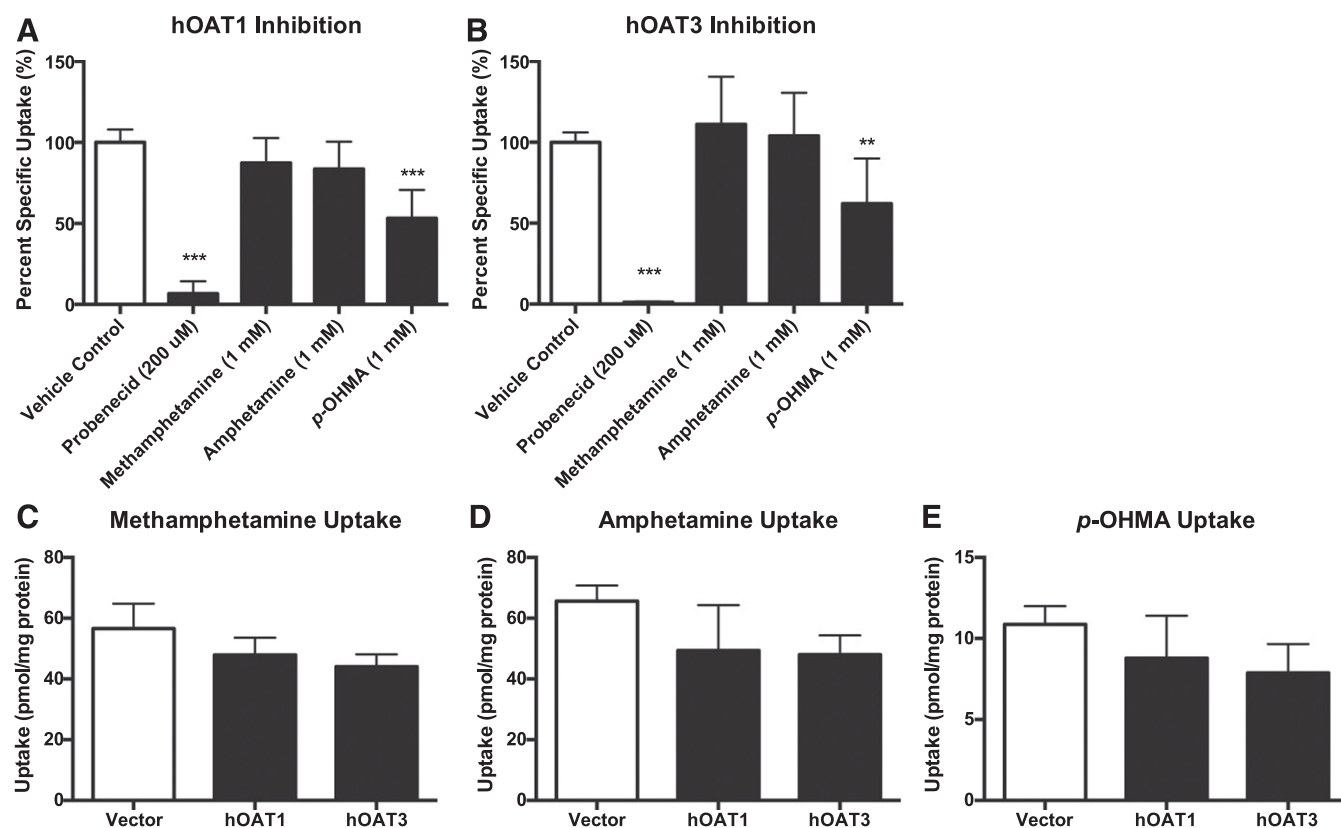


Fig. 3. Interactions of methamphetamine, amphetamine, and *p*-OHMA with renal hOAT1 and hOAT3. Effect of methamphetamine, amphetamine, and *p*-OHMA on *para*-aminohippurate (PAH) (1 μ M) uptake by hOAT1 (A) and estrone sulfate (0.06 μ M) uptake by hOAT3 (B) was measured at 1 minute after incubation at 37°C. Transporter-specific uptake was obtained by subtracting the uptake in vector-transfected cells from the uptake in transporter-expressing cells. The classic organic anion transporter inhibitor probenecid was used as the control. Activity in the absence of an inhibitor (100%) corresponded to 36.6 and 2.1 pmol/min/mg protein for PAH and estrone sulfate uptake, respectively. Uptake of methamphetamine (C), amphetamine (D), and *p*-OHMA (E) by hOAT1 and hOAT3 was measured after 5-minute incubation at 37°C. Data are illustrated as the mean $\pm$ S.D. from three independent experiments performed in triplicate. Uptake in transporter-expressing cells was compared with that in control cells (** P < 0.01; *** P < 0.001).

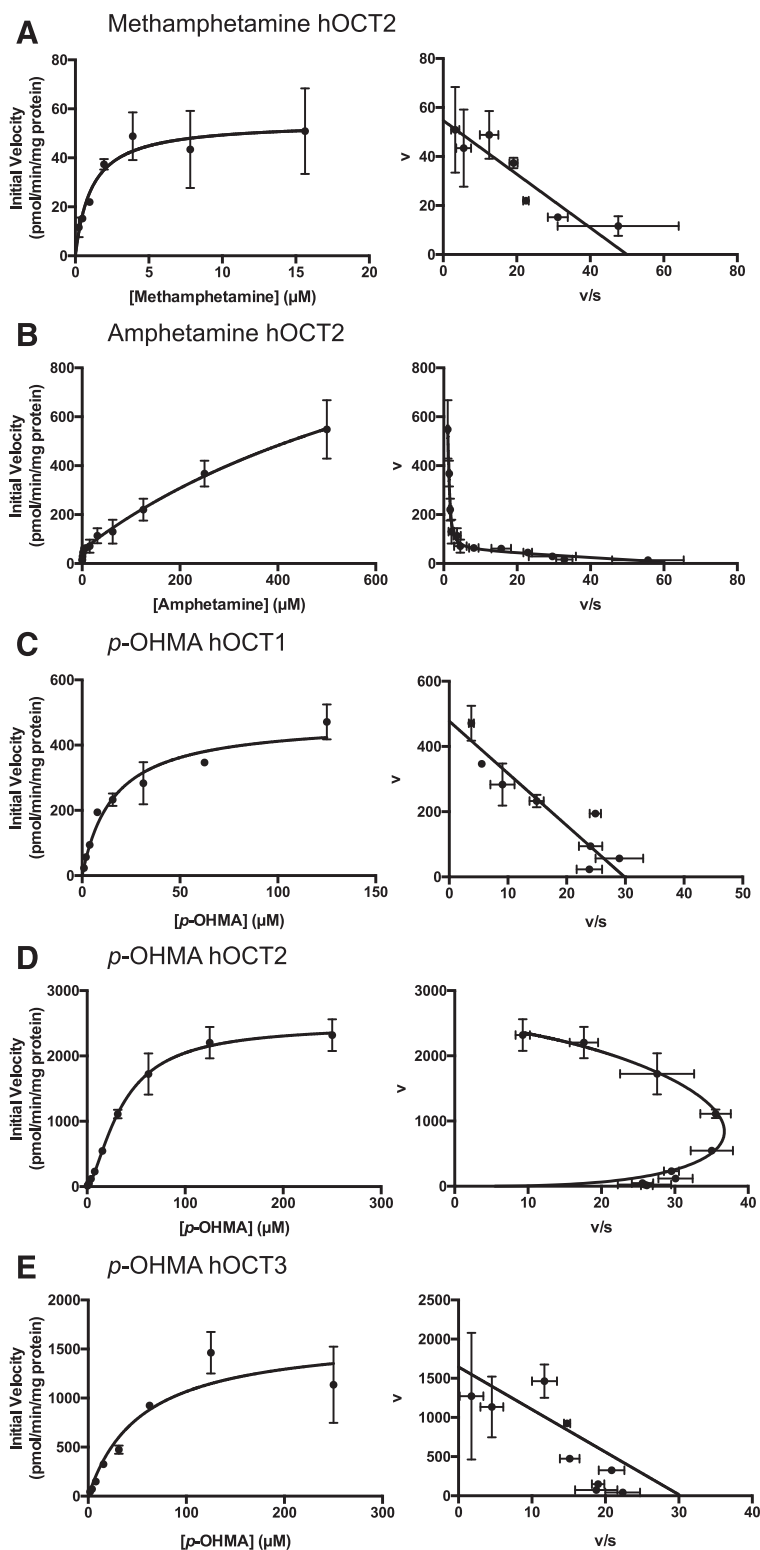


Fig. 4. Methamphetamine, amphetamine, and *p*-OHMA uptake kinetics by hOCTs. Concentration-dependent uptake of substrate was measured in both transporter-expressing and control cells at 37°C after 1-minute incubations. Transporter-specific uptake was obtained by subtracting the uptake in vector-transfected cells from the uptake in transporter-expressing cells. Panels display saturation curves (*v* vs. *s*) and Eadie-Hofstee transformations (*v* vs. *v/s*) for the kinetic data. Based on the Eadie-Hofstee plots, the kinetics for hOCT2-mediated methamphetamine transport (A) and hOCT- and hOCT3-mediated *p*-OHMA transport (C and E) were fitted with the standard Michaelis-Menten equation. hOCT2-mediated amphetamine transport (B) was fitted to a biphasic Michaelis-Menten equation (eq. 5). hOCT2-mediated *p*-OHMA transport (D) was fitted to the Michaelis-Menten equation with a Hill slope (eq. 4). Each data point represents the mean $\pm$ S.D. from one representative experiment in triplicate. Curves from two additional independent repeats are displayed in Supplemental Fig. 4. The kinetic parameters in Table 3 are mean $\pm$ S.D. of the values from three independent experiments.

amphetamine. Methamphetamine may be a potential victim of DDIs by inhibitors (e.g., cimetidine, zalcitabine, dolutegravir) of OCT2 and/or MATE transporters, which could reduce its renal clearance and increase exposure (Jung et al., 2008; Reese et al., 2013). Located at the apical membrane of renal proximal tubule cells, the MATE transporters function as proton/organic cation exchangers, which rely on the transmembrane proton gradient to drive organic cation secretion into the

urine (Otsuka et al., 2005). The pH dependence of methamphetamine and amphetamine renal excretion rates has long been known, where urine acidification increases renal excretion while urine alkalization has an opposite effect (Beckett and Rowland, 1965a,b,c). The effect of urinary pH on methamphetamine or amphetamine renal excretion has been mostly attributed to the pH effect on ionization and membrane partitioning, which affects tubular reabsorption of these weak bases

TABLE 3

Kinetic parameters of methamphetamine and metabolites determined from modeling the data in Fig. 4 and Supplemental Fig. 4

Models were chosen based on examination of Eadie-Hofstee plots. Methamphetamine was fit to a standard Michaelis-Menten equation. Amphetamine uptake kinetics was fit to a biphasic Michaelis-Menten equation (eq. 5). *p*-OHMA hOCT1- and hOCT3-mediated transport were fit to a standard Michaelis-Menten equation. Sigmoidal kinetics of hOCT2-mediated *p*-OHMA transport was obtained by fitting transporter-mediated uptake to the Michaelis-Menten equation with a Hill slope (eq. 5) for the substrate concentration and half-maximal transport concentration ($K_{1/2}$ in place of K_m). Results represent mean $\pm$ S.D. of three independent experiments each run in triplicate.

Compound	Transporter	K_{m1}	V_{max1}	K_{m2}	V_{max2}
		μM	$pmol/mg/min$	μM	$pmol/mg/min$
Methamphetamine	hOCT2	2.09 ± 0.88	49.7 ± 12.2	ND	ND
Amphetamine	hOCT2	0.830 ± 0.55	34.6 ± 23.7	534 ± 350	853 ± 474
<i>p</i> -OHMA	hOCT1	14.5 ± 8.7	312 ± 163	NA	NA
	hOCT2	$K_{1/2}: 31.8 \pm 9.3; H: 1.64 \pm 0.15$	1780 ± 718	NA	NA
	hOCT3	53.3 ± 6.2	1290 ± 830	NA	NA

NA, not applicable; ND, not determined (due to high diffusion).

(Beckett and Rowland, 1965a,b,c). Here, our data suggest that renal secretion of methamphetamine and amphetamine involves the pH-dependent MATE transporters. Therefore, the increased excretion rates observed with acidic urine could be due to a combined effect of acidic pH in reducing partition-mediated reabsorption along with increasing MATE-mediated tubular secretion.

The liver is the major site of methamphetamine metabolism. Intriguingly, methamphetamine and amphetamine were not substrates of hOCT1, the major OCT isoform responsible for hepatic uptake of organic cations. Therefore, hepatic uptake of methamphetamine and amphetamine may be facilitated by other transporters yet to be identified or be driven by passive diffusion. Interestingly, *p*-OHMA was transported by hOCT1, suggesting that the *para*-hydroxyl group may be important for OCT1 transport selectivity of substituted amphetamines. hOCT1 may thus be involved in hepatic transport of *p*-OHMA.

In this study, we used metformin as the probe substrate because it is recommended as an in vitro and in vivo probe substrate for evaluating hOCT2, hMATE1, and hMATE2-K interaction studies by the International Transporter Consortium (Hillgren et al., 2013), Food and Drug Administration (2012), and European Medicines Agency (2012). Substrate-dependent inhibition has previously been demonstrated for OCTs with a number of substrates and inhibitors (Moaddel et al., 2005; Gorbunov et al., 2008; Minuesa et al., 2009; Hacker et al., 2015; Yin et al., 2016). For example, inhibition potencies of several clinical drugs toward hOCT2 were reported to be approximately 10-fold more potent when atenolol was used as the substrate compared with metformin (Yin et al., 2016). Due to the observed complex interactions, the apparent inhibition potencies of amphetamines may be highly dependent on the substrate. As an illicit drug, abusers may use methamphetamine while taking prescription medications. Testing the inhibition potencies with the specific hOCT substrate drugs used by methamphetamine abusers

may be warranted to determine the likelihood of clinically relevant interactions.

Particularly high levels of methamphetamine abuse are reported in individuals receiving treatment of human immunodeficiency virus and hepatitis who may be receiving multiple medications for treatment (Panenka et al., 2013; Volkow, 2013; Bracchi et al., 2015). Importantly, numerous antiretrovirals (e.g., lamivudine, zalcitabine) interact with OCTs and rely on these transporters for cellular uptake into human immunodeficiency virus-infected CD4 cells (Zhou et al., 2006; Jung et al., 2008). Methamphetamine and its metabolites inhibited the active transport of the probe substrate metformin by hOCT1–3 and hMATE1/2-K (Table 1) within the concentration range reported in abusers of methamphetamine (Melega et al., 2007; Shima et al., 2009). The free plasma concentrations of methamphetamine in some abusers have been reported to be in the tens of micromolar range and even $130 \mu M$ in one individual, indicating the potential to reach inhibitory concentrations of hOCT1–3 and hMATE1/2-K in vivo (de la Torre et al., 2004; Shima et al., 2008). Inhibition of hOCT1 and hOCT2 may reduce intracellular levels of some antiretrovirals in human immunodeficiency virus-infected CD4 cells, reducing their effective concentration and efficacy at the site of action (Minuesa et al., 2008, 2009; Wagner et al., 2016). These potential distributional DDIs are of particular concern with drugs of abuse since patients may not be willing to reveal their use of illicit drugs.

Methamphetamine and its metabolites demonstrated complex interactions with hOCT1 and hOCT2, suggesting they may have multiple binding sites on these transporters. The structural basis of the complex kinetic interactions between amphetamines and hOCTs is currently unclear since the crystal structures of these transporters have not been obtained. Amphetamine showed biphasic hOCT2 uptake kinetics with an apparent high affinity ($0.830 \pm 0.55 \mu M$) and low affinity ($534 \pm 350 \mu M$) aligning with the observed high- and low-affinity inhibitory

TABLE 4

Apparent kinetic transport parameters for methamphetamine, amphetamine, and *p*-OHMA for hMATE1 and hMATE2-K from simultaneously modeling active and passive accumulation

Results represent Mean $\pm$ S.D. of three independent experiments each run in triplicate (Fig. 5; Supplemental Fig. 5).

Transporter	Methamphetamine		Amphetamine		<i>p</i> -OHMA	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
	μM	$pmol/mg/min$	μM	$pmol/mg/min$	μM	$pmol/mg/min$
hMATE1	20.6 ± 4.5	86.6 ± 54	14.1 ± 4.9	238 ± 141	49.8 ± 26	257 ± 190
hMATE2-K	18.1 ± 11	97.7 ± 25.3	16.4 ± 12.2	92.9 ± 8.5	NA	NA

NA, not applicable.

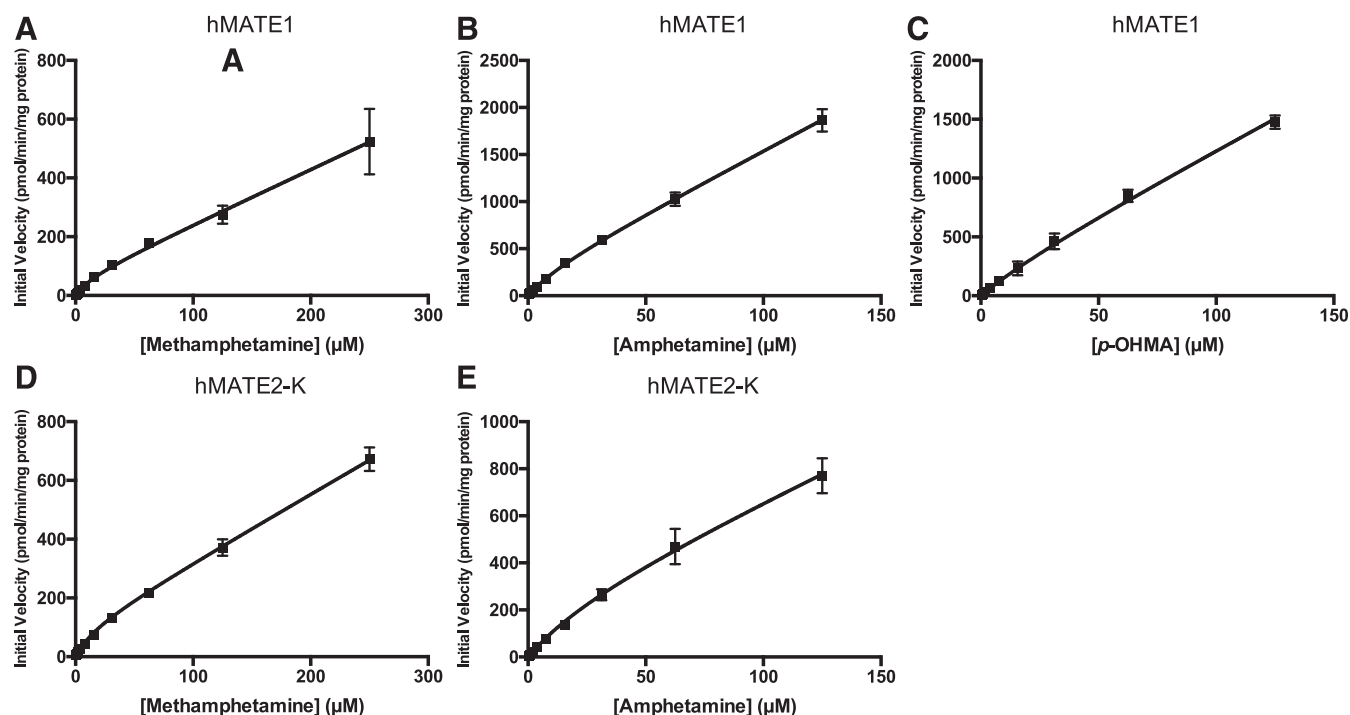


Fig. 5. Methamphetamine and metabolite uptake kinetics by hMATE1 and hMATE2-K. Methamphetamine, amphetamine, and *p*-OHMA uptake in hMATE1-expressing cells was performed during the initial linear uptake time at 5, 2, and 2 minutes, respectively (A–C). Methamphetamine and amphetamine uptake in hMATE2-expressing cells was performed during initial linear uptake time at 5 minutes (D and E). Incubations were performed at 37°C and data were fitted with a Michaelis-Menten equation with a nonsaturable passive diffusion component (eq. 3). Each data point represents the mean $\pm$ S.D. from one representative experiment in triplicate. Curves from two additional independent repeats are displayed in Supplemental Fig. 5. The kinetic parameters in Table 4 are mean $\pm$ S.D. of the values from three independent experiments.

interactions. Conversely, *p*-OHMA had a relatively steep hOCT2 inhibition Hill slope (1.55 ± 0.32) and demonstrated sigmoidal uptake kinetics (Fig. 4; Table 3). Sigmoidal uptake kinetics may be characteristic of homotropic activation (Segel, 1976; Atkins, 2005). Both a large binding pocket in the outward facing cleft allowing for spatially distinct binding as well as a distal allosteric binding site have been proposed for OCTs based on kinetics and biochemical analyses (Gorboulev et al., 1999; Harper and Wright, 2013; Koepsell, 2015). The possible distal allosteric binding site has demonstrated very high-affinity interactions with no transport observed in the concentration range (e.g., 6–41 pM for 1-methyl-4-phenylpyridinium) (Moaddel et al., 2005; Gorbunov et al., 2008; Minuesa et al., 2009; Koepsell, 2015). Binding within the transportable region is generally a lower-affinity interaction (e.g., 0.87–12.3 μM for 1-methyl-4-phenylpyridinium) with known substrates (Moaddel et al., 2005; Gorbunov et al., 2008; Minuesa et al., 2009; Koepsell, 2015). Recent developments have also suggested the possibility of simultaneous binding of two substrates within the transport site (Harper and Wright, 2013; Koepsell, 2015). The apparent affinities observed for methamphetamine and metabolites across their transportable concentration ranges suggest that both binding sites may reside within the transport region. More studies are needed to understand the complex kinetic behaviors of the OCTs and their structure-function relationships.

In summary, our study determined the molecular mechanisms involved in transport and disposition of methamphetamine and its metabolites. Moreover, our studies showed that methamphetamine has the potential to inhibit hOCT and hMATE transporters at clinically relevant concentrations. Finally, we identified complex kinetic interactions between amphetamines and hOCT2. Our findings provide useful information that may be considered when prescribing medications to methamphetamine users to mitigate the risk of DDIs that may potentially compromise therapeutic efficacy and drug safety.

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Authorship Contributions

Participated in research design: Wagner, Isoherranen, Wang.

Conducted experiments: Wagner, Sager, Duan.

Contributed new reagents or analytic tools: Isoherranen.

Performed data analysis: Wagner, Wang.

Wrote or contributed to the writing of the manuscript: Wagner, Sager, Isoherranen, Wang.

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