Interactions of *n*-Tetraalkylammonium Compounds and Biguanides with a Human Renal Organic Cation Transporter (hOCT2)

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INTRODUCTION

Many clinically used drugs are transported in the liver and kidney by organic cation transporters (OCT) (1). To date, three organic cation transporters in the OCT family have been cloned and characterized (OCT1-3) (2). Of these, OCT1 appears to be an important transporter in the liver and OCT2 appears to be a major transporter in the kidney. With the availability of the cloned transporters, it is now possible to begin investigating their roles in renal and hepatic drug elimination.

The human transporters, hOCT1 and hOCT2, share 70% sequence identity, and their predicted secondary structures, based on hydropathy analysis, are essentially the same. This might suggest that paralogous organic cation transporters such as hOCT1 and hOCT2 have similar functional characteristics and are functionally redundant. However, recent chimeric and mutagenesis studies of transporters have shown that changes in even one or two amino acids can dramatically alter specificity (3). Therefore, it is reasonable to propose that these two organic cation transporter homologs serve different functions *in vivo*.

The goal of this study was to compare the substrate and inhibition profiles of hOCT2 and hOCT1 to determine whether these transporters are functionally distinct. Differences in their specificities may provide insights into organ-specific elimination of organic cations. We examined the interactions of *n*-tetraalkylammonium (nTAA) compounds and biguanides with hOCT2 and compared our results with our previous results for hOCT1 (4,5). Substantial differences between hOCT2 and hOCT1 in their interactions with nTAAs were found, whereas their interactions with the biguanides, metformin and phenformin, were similar. This report demonstrates that there are compound-dependent differences in the specificities of hOCT1 and hOCT2; these differences may contribute to organ-specific elimination of drugs.

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MATERIALS AND METHODS

Xenopus Oocyte Expression and Tracer Uptake Measurements

Transport of ^{3}H-1-methyl-4-phenylpyridinium (MPP^+) (82 Ci/mmol) or ^{3}H-cimetidine (15 Ci/mmol) was measured in healthy stage V and VI *Xenopus laevis* oocytes 2 to 7 days after injection of hOCT1 or hOCT2 cRNA, as described previously (6). Tracer uptakes were performed as follows: groups of seven to nine oocytes were incubated for 1 h in Na^+ buffer (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.2) containing ^{3}H-MPP^+ (1 μM: 0.1 μM ^{3}H-MPP^+ and 0.9 μM unlabeled MPP^+) or ^{3}H-cimetidine (1 μM). For inhibition and kinetic studies, unlabeled compounds were added to the reaction solutions as needed. To stop the uptake experiments, oocytes were washed five times with 3 mL of ice-cold Na^+ buffer. The radioactivity associated with each oocyte was then determined by scintillation counting.

In the *trans*-stimulation studies, groups of five to seven hOCT2-expressing or uninjected oocytes were washed three times with K^+ buffer (2 mM NaCl, 100 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.2). The oocytes were then rapidly injected with 50 nL of K^+ buffer containing an unlabeled compound. Oocytes injected with 50 nL of K^+ buffer without compound were used for controls. After injections, the oocytes were quickly transferred to a small disposable borosilicate glass culture tube; any remaining buffer was aspirated off, and 85 μL of MPP^+ (1 μM: 0.1 μM ^{3}H-MPP^+ and 0.9 μM unlabeled MPP^+) in K^+ buffer was added. ^{3}H-MPP^+ uptake was arrested after 10 min by washing the oocytes five times with 3 mL of ice-cold K^+ buffer. The radioactivity associated with each oocyte was then determined by scintillation counting.

Electrophysiology Studies

Electrophysiology experiments were performed at room temperature (21–23°C) 5–10 days post injection. Steady-state ligand-induced currents were measured with a two-electrode voltage clamp. Oocytes were voltage clamped at −50 mV and superfused with Na^+ buffer 2 min before and after 30 s ligand superfusions. Recordings were obtained in a 25-μL recording chamber at flow rates of 3 mL/min. Uninjected oocytes were used as controls.

Data Analysis

Values are expressed as mean ± standard error (SE) or mean ± standard deviation (SD) as indicated in the legends. All experiments were repeated at least once using different batches of oocytes, unless indicated otherwise in the legends. IC_{50} values were determined as described previously (5). The unpaired *t* test or analysis of variance (for multiple comparisons) was used to test for statistically significant differences where $p < 0.05$ was considered significant.

Materials

The nTAA compounds and biguanides were purchased from Sigma (St. Louis, MO, USA). All other reagents were purchased from either Sigma or Fisher (Pittsburgh, PA, USA)

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or as indicated. ^{3}H-MPP$^+$ (82 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA, USA), and ^{3}H-cimetidine (15 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, IL, USA)

RESULTS

Inhibition of hOCT2-Mediated Uptake by nTAA Compounds

The IC_{50} values for inhibition of ^{3}H-MPP$^+$ uptake (1 μM) by the four nTAA compounds, tetramethylammonium (TMA), tetraethylammonium (TEA), tetrapropylammonium (TPrA), and tetrabutylammonium (TBA), in inhibiting MPP$^+$ uptake by hOCT2-expressing oocytes were not statistically different from one another, suggesting that these compounds have essentially the same affinity for hOCT2 (Table I). There were notable differences between hOCT1 and hOCT2 in their affinities (IC_{50}'s) for several nTAAs. For example, TMA, the smallest nTAA compound, had an 82-fold higher affinity for hOCT2 compared to hOCT1, whereas TBA, the largest nTAA, had a 4-fold higher affinity for hOCT1 compared to hOCT2 (Table I).

Efflux of nTAAs from Oocytes Expressing hOCT2

Trans-stimulation studies were performed under depolarized conditions (e.g., in K$^+$ containing buffer) to minimize the effects of membrane potential on ^{3}H-MPP$^+$ (1 μM) uptake. Previous studies have shown that the resting membrane potential in oocytes incubated in K+ buffer is approximately –10 mV (6). Marked differences in the function of hOCT2 and hOCT1 were revealed by these studies (Fig. 2A). In oocytes expressing hOCT2, ^{3}H-MPP$^+$ uptake was *trans*-stimulated only by TMA and was *trans*-inhibited by TPrA and TBA. In contrast, ^{3}H-MPP$^+$ uptake by hOCT1 was *trans*-stimulated by TEA and TPrA. The remaining nTAAs had little effect on hOCT1-mediated ^{3}H-MPP$^+$ uptake.

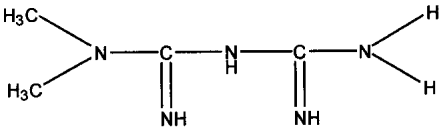
Interactions of Biguanides with hOCT2 and hOCT1

Metformin and phenformin (2.5 mM, Fig. 1) inhibited ^{3}H-cimetidine transport mediated by hOCT1 and hOCT2 (data not shown). Both of the biguanides interacted with hOCT2 and hOCT1 with similar potencies (Table I). Both compounds *trans*-stimulated ^{3}H-MPP$^+$ influx mediated by hOCT1 and hOCT2, suggesting that they are substrates of these transporters (Fig. 2B). Electrophysiologic-based studies indicated that TEA (1 mM) and metformin (0.5 mM) induced significant currents in hOCT2-expressing oocytes (Fig. 3). In contrast, quinidine (200 μM), a potent inhibitor of organic cation transporters, did not induce a current.

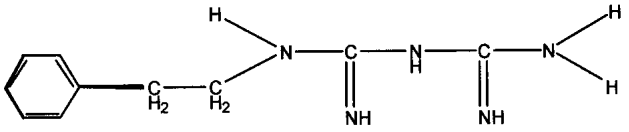
Table I. IC_{50} Values (μM) of *n*-Tetraalkylammonium (nTAA) Compounds and Biguanides in Inhibiting MPP$^+$ or Cimetidine Uptake Mediated by hOCT2

Compound[b]	hOCT2	hOCT1[a]
TMA	150 ± 37[c]	12400 ± 1280
TEA	156 ± 78	158 ± 40.5
TPrA	128 ± 32	102 ± 13.0
TBA	120 ± 52[c]	29.6 ± 3.6
Metformin	1700 ± 960	2010 ± 220
Phenformin	65 ± 11[c]	10 ± 6.9

[a] Data obtained from (4).
[b] Inhibition of ^{3}H-MPP$^+$ uptake was measured in IC_{50} studies with the nTAA compounds; inhibition of ^{3}H-cimetidine uptake was measured with the biguanides.
[c] $p < 0.05$ vs. hOCT1 (unpaired *t* test). The IC_{50} values of the nTAA compounds for hOCT2 were not statistically different (analysis of variance).



Metformin



Phenformin

Fig. 1. Chemical structures of the biguanides metformin and phenformin.

DISCUSSION

A number of recent studies have begun to address the question of whether OCT1 and OCT2 are functionally redundant or distinct. In particular, studies with rOCT1, rOCT2, and hOCT3 have shown that these transporters have slight differences in their substrate and inhibitor selectivities (7–10). Recent work also suggests OCT1 and OCT2 may function primarily in different tissues (i.e., the liver and kidney, respectively) (11–13). Therefore, it is possible that differences in the specificities of OCT1 and OCT2 could lead to organ-specific elimination of organic cations.

The goal of this study was to compare the substrate and inhibitor selectivities of the human organic cation transporter isoforms, hOCT2 and hOCT1, by conducting *cis*-inhibition, *trans*-stimulation, and electrophysiology studies with nTAA compounds and/or biguanides. Inhibition studies give information regarding the inhibitor selectivities of a particular transporter, but do not test whether or not a compound is a true substrate. In contrast, *trans*-stimulation studies are a means of testing whether two compounds share a common translocation pathway. Studies have shown that compounds which *trans*-stimulate transport of a known substrate are usually substrates of the same transport system. Then TAA compounds, a group of organic cations, have been used extensively to study the functional characteristics of organic cation transporters (4,5,14). Inhibition and *trans*-stimulation studies revealed substantial differences in the interactions of the nTAAs with the two organic cation transporters. For example, the smallest nTAA, TMA, inhibited hOCT2 80-fold

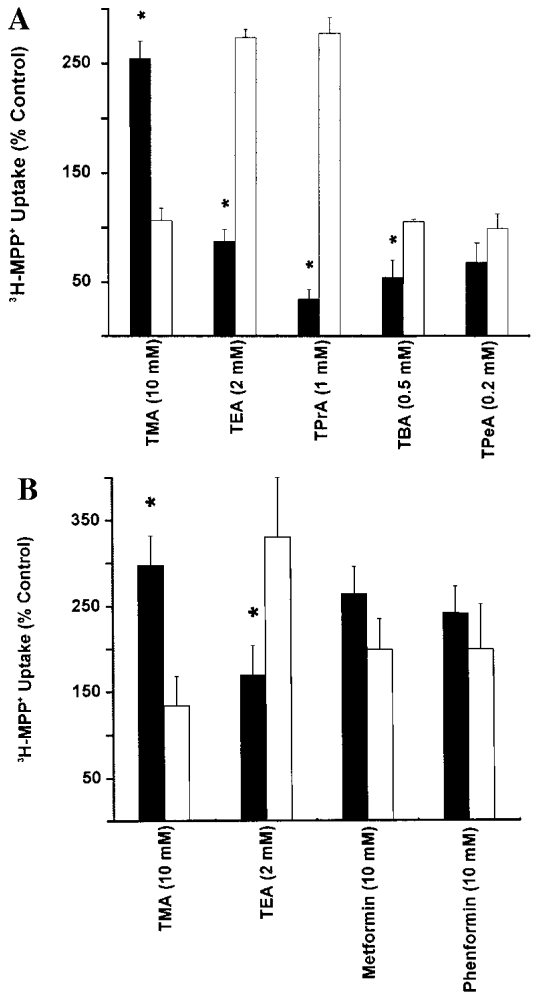


Fig. 2. Effect of *trans* (A) nTAA and (B) biguanide compounds on the influx of ^{3}H-MPP$^+$ in *Xenopus laevis* oocytes expressing hOCT2 or hOCT1. Depolarized oocytes, expressing either hOCT2 (dark bars) or hOCT1 (open bars) were injected with 50 nL of an unlabeled *n*-tetraalkylammonium (A) or biguanide (B) compound dissolved in K^+ buffer to produce the final intracellular *n*-tetraalkylammonium concentrations indicated in the figure. The 10-min uptake of ^{3}H-MPP$^+$ (1 μM) in K^+ buffer was then measured. The mean control uptake (*trans*-zero) was taken as 100% (control oocytes were injected with 50 nL of K^+ buffer). In studies with the biguanides, tetramethylammonium was used as a positive control for hOCT2 and tetraethylammonium was used as a positive control for hOCT1. Data represent mean ± SE from three separate experiments; five to nine oocytes were used per compound per experiment. *$p < 0.05$ vs. hOCT1 (unpaired *t* test). hOCT1 data were previously published (4).

more potently than hOCT1 (Table I) and *trans*-stimulated MPP$^+$ uptake by hOCT2, but not by hOCT1. These results suggest that the kidney transporter, hOCT2, prefers smaller hydrophilic substrates in contrast to the liver transporter, hOCT1, which may interact with larger, more hydrophobic compounds (Fig. 2A and Table I). Interestingly, the rank order of *trans*-inhibition by TPrA, TBA, and TPeA for hOCT2 was strikingly similar to what we previously found for the mouse and rat OCT1 isoforms (4). Hence, it is possible that the elements involved in the binding or translocation of these compounds at the *trans*-side of the transporters might be conserved among hOCT2, mOCT1, and rOCT1, but not hOCT1.

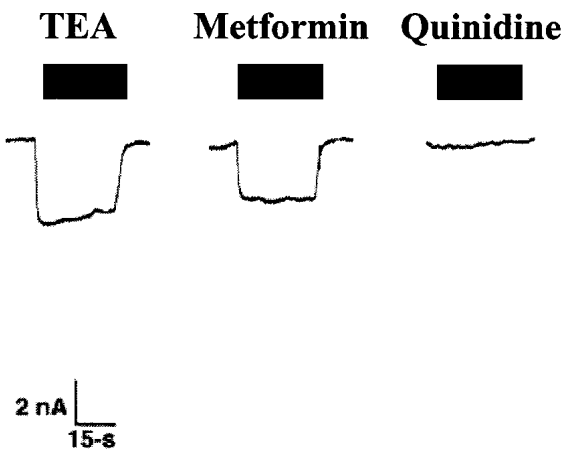


Fig. 3. Representative recordings of ligand-induced inward currents in hOCT2-expressing oocytes. An hOCT2-expressing oocyte was voltage-clamped at −50 mV in sodium buffer. The dark bars above the traces indicate 30-s applications of ligands: tetraethylammonium (1 mM), metformin (0.5 mM), and quinidine (100 μM). Between applications, the ligands were removed by washing in sodium buffer; the original baseline current was restored before applying a different ligand. Similar results were obtained in other hOCT2-expressing oocytes (n = 2). Ligand-induced currents were not observed in control (i.e., uninjected) oocytes (n = 2).

Metformin, a biguanide used in the treatment of non-insulin-dependent diabetes mellitus, is eliminated by active secretion in the kidney, and it is likely that one or more organic cation transporters are involved in this process (15–17). We observed that both metformin and phenformin interact with hOCT1 and hOCT2, but that both transporters had a much higher affinity for phenformin compared to metformin. *Cis*-inhibition studies revealed that metformin and phenformin inhibited ^{3}H-cimetidine uptake mediated by hOCT1 and hOCT2, suggesting that hOCT1 and hOCT2 may be the molecular site of the previously characterized metformin-cimetidine drug-drug interaction (15). The data demonstrating that both metformin and phenformin *trans*-stimulated ^{3}H-MPP$^+$ influx mediated by the transporters suggest that the compounds are substrates of both hOCT1 and hOCT2. Furthermore, metformin induced a current in voltage-clamped oocytes expressing hOCT2, providing more evidence that metformin is a substrate of hOCT2. Because hOCT2 is present in greater abundance in the kidney, it is likely that it plays a greater role in the renal elimination of metformin than does hOCT1.

In summary, the substrate and inhibitor specificities of hOCT1 and hOCT2 were studied using nTAA compounds and biguanides. The biguanides have similar affinities for hOCT1 and hOCT2 (Table I), whereas significant differences were found between hOCT1 and hOCT2 in their interaction with nTAA compounds. These results suggest that there are significant differences in the binding sites of these paralogous transporters. Studies ascertaining the critical domains and amino acid residues required for substrate recognition and translocation will shed light on the molecular mechanisms responsible for the distinct substrate selectivities of the hOCT isoforms.

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