Contribution of organic anion transporters to the renal uptake of anionic compounds and nucleoside derivatives in rat.

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Running title: Contribution of rOat1 and rOat3.

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The number of text pages: 33
The number of tables: 2
The number of figures: 10
The number of references: 31
The number of words in Abstract: 246
The number of words in Introduction: 792
The number of words in Discussion: 1782

Abbreviations: FBS, fetal bovine serum; PBS, phosphate buffered saline; OAT, organic anion transporter; MCT, monocarboxylate transporter; PAH, p-aminohippurate; PCG, benzylpenicillin; ACV, acyclovir; AZT, 3'-azido, 3'-deoxythymidine; 2,4-D, 2,4-dichlorophenoxyacetate; DHEAS, dehydroepiandrosterone sulfate; $K_m$, Michaelis-Menten constant; $K_i$, inhibition constant; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; RAF, relative activity factor; AIC, Akaike's Information Criterion

Section option: Gastrointestinal, Hepatic, Pulmonary and Renal
Abstract

Our previous kinetic analyses have shown that rat organic anion transporter 1 (rOat1; Slc22a6) and rOat3 (Slc22a8) are responsible for the renal uptake of p-aminohippurate (PAH) and pravastatin, respectively. In this study, their contribution to the renal uptake of organic anions and nucleoside derivatives was examined by investigating the uptake by rOat1- and rOat3-expressing cells and kidney slices. Transfection of rOat1 resulted in an increase of the uptake of temocaprilat (K_m = 0.56 µM), 2,4-dichlorophenoxyacetate (2,4-D; K_m = 10 µM) and 3'-azido, 3'-deoxythymidine (AZT; K_m = 43 µM). rOat3-expressing cells showed significant uptake of temocaprilat (K_m = 1.4 µM), estrone sulfate (K_m = 5.3 µM), dehydroepiandrosterone sulfate (DHEAS; K_m = 12 µM) and benzylpenicillin (PCG; K_m = 85 µM). All the test compounds were accumulated in kidney slices in a carrier-mediated manner although the saturable components of AZT and acyclovir were small. The K_m of 2,4-D uptake by kidney slices was comparable with that of rOat1, and the corresponding values of DHEAS and PCG were similar to those of rOat3. The uptake of estrone sulfate and temocaprilat by kidney slices consisted of two saturable components, with the K_m values of their high affinity components being similar to those for rOat3 (estrone sulfate), and rOat1 and rOat3 (temocaprilat), respectively. These results suggest that the renal uptake of 2,4-D is mainly accounted for by rOat1 and the uptake of PCG and DHEAS by rOat3, and rOat3 is partly involved in the renal uptake of temocaprilat and estrone sulfate.
Introduction

The kidney plays an important role in the urinary excretion of endogenous wastes, xenobiotics including drugs and their metabolites via glomerular filtration and tubular secretion as well as maintaining ionic and water homeostasis (Inui et al., 2000; Dresser et al., 2001; Kusuhara and Sugiyama, 2002; Russel et al., 2002). Cumulative evidence suggests that an exchanger with intracellular dicarboxylates is involved in the renal uptake of organic anions on the basolateral membrane of the proximal tubules (Pritchard and Miller, 1993). Rat organic anion transporter 1 (rOat1; Slc22a6) has been isolated from the kidney as a candidate for the classical renal organic anion transporter (Sekine et al., 1997; Sweet et al., 1997). rOat2 (Slc22a7) and rOat3 (Slc22a8) have been isolated by homology screening of the database and brain cDNA library (Sekine et al., 1998; Kusuhara et al., 1999), and human isoforms corresponding to rat isoforms have been already isolated and characterized (Hosoyamada et al., 1999; Lu et al., 1999; Cha et al., 2000). rOat2 is abundantly expressed in the liver and female kidney, but only moderately in male kidney (Buist et al., 2002; Kobayashi et al., 2002). The site of rOat2 expression in the kidney is the brush border membrane of the tubules in the medullary thick ascending loop of Henle and cortical and medullary collecting ducts (Kojima et al., 2002). However, human OAT2 (hOAT2) is localized on the basolateral membrane of the proximal tubules in human kidney (Enomoto et al., 2002). rOat3 is expressed in the liver, kidney but only weakly in the brain and eye of male rats (Kusuhara et al., 1999). rOat3 and hOAT3 is localized on the basolateral membrane of the proximal tubules (Cha et al., 2001; Hasegawa et al., 2002). In rats, basolateral
localization of rOat1 and rOat3 indicates their involvement in the renal uptake of organic anions. Both rOat1 and rOat3 have broad substrate specificity, which overlaps to a degree (Sekine et al., 2000; Russel et al., 2002). PAH, ochratoxin A, methotrexate and β-lactam antibiotics are their common substrates (Russel et al., 2002). rOat3 accepts amphipathic compounds, such as conjugated steroids, 17βestradiol-D-17β-glucuronide, estrone sulfate and an HMG-CoA reductase inhibitor, pravastatin, as well as the organic cation cimetidine (Kusuhara et al., 1999; Sugiyama et al., 2001). Recently, Sweet et al., have demonstrated that rOat3 is indirectly coupled to the Na⁺ gradient through Na⁺/dicarboxylate cotransport and functions as an organic anion/dicarboxylate exchanger as the case of rOat1 (Sweet et al., in press). Their results suggest that rOat3 is also a tertiary active transport system and that it is responsible for the energy-dependent uphill uptake of organic anions via the basolateral membrane of the proximal tubule. Our previous kinetic analyses have demonstrated that rOat1 and rOat3 play a major role in the renal uptake of PAH and pravastatin, respectively in male rats (Hasegawa et al., 2002). Together with these previous results, we suggested that rOat1 and rOat3 are responsible for the renal uptake of relatively small and hydrophilic organic anions and relatively hydrophobic organic anions, respectively. Recently, the mouse Oat3 (mOat3) knock-out mouse has been established (Sweet et al., 2002). The uptake of taurocholate and estrone sulfate by kidney slices was markedly reduced in the mOat3 knock-out mice (Sweet et al., 2002). In contrast to our results using rats, the uptake of PAH by kidney slices from mOat3 knock out mice was also reduced to half of that from the corresponding wild type, which may be
ascribed to a species difference (Sweet et al., 2002).

Even if a compound is a good substrate, it is still unclear whether the transporter plays a major role in the membrane transport of the compounds. The contribution in the total membrane transport process should be examined to identify the transporter, which plays an important role in the disposition of drugs and endogenous substrates. The purpose of the present study is to investigate the importance of rOat1 and rOat3 in the total renal uptake of anionic compounds and nucleoside derivatives. In the present study, eight compounds were selected: temocaprilat, benzylpenicillin (PCG), 2,4-dichlorophenoxyacetate (2,4-D), salicylate, acyclovir (ACV), 3’-azido, 3’-deoxythymidine (AZT), estrone sulfate and dehydroepiandrosterone sulfate (DHEAS). These test compounds are known to be substrates of the organic anion transport system, and/or mainly excreted in the urine via tubular secretion (Bergeron et al., 1975; Patel et al., 1989; Burnette and de Miranda, 1994; Griffin et al., 1997; Dresser et al., 2001).

The kinetic parameters for the uptake by rOat1- and rOat3-expressing cells were compared with those determined for the uptake by kidney slices. In addition, to estimate the contribution of rOat1 and rOat3 to the total uptake by kidney slices, the uptake clearance was also examined using rOat1- and rOat3-expressing cells, and the uptake clearance by cDNA-transfected cells was compared with the corresponding uptake clearance by kidney slices.
Methods

Materials

\[^{3}H\]pravastatin (45.5 Ci/mmol), \[^{14}C\]temocapril (14.4 mCi/mmol) and unlabeled pravastatin and temocaprilat were kindly donated by Sankyo (Tokyo, Japan). \[^{3}H\]PAH (4.08 Ci/mmol), \[^{14}C\] salicylate (55.5 mCi/mmol), \[^{3}H\]estrone sulfate (60.0 Ci/mmol), \[^{3}H\]DHEAS (40.0 Ci/mmol) and \[^{3}H\] and \[^{14}C\] mannitol (19.9 Ci/mmol and 51 mCi/mmol, respectively) were purchased from PerkinElmer Life Sciences (Boston, MA). \[^{3}H\]PCG (20.0 Ci/mmoll) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). \[^{3}H\]AZT (15.7 Ci/mmoll) and \[^{3}H\]ACV (40.1 Ci/mmoll) were purchased from Moravec Biochemicals (Brea, CA). \[^{3}H\]2,4-D (20.0 Ci/mmoll) was purchased from American Radiolabeled Chemicals (St. Louis, MO). \[^{14}C\]temocaprilat was prepared by hydrolysis of \[^{14}C\]temocapril (5N NaOH for 5 hr)(Schwab et al., 1992). The radiochemical purity of \[^{14}C\]temocaprilat was checked by thin-layer chromatography (n-butanol : acetic acid : distilled water = 4 : 1 : 1), and was found to exceed 95 %. Unlabeled PAH, AZT, ACV, salicylate, estrone sulfate, DHEAS and 2,4-D were purchased from Sigma Chemical (St. Louis, MO), and unlabeled PCG was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and commercially available.

Cell culture.

rOat1- and rOat3-expressing LLC-PK1 cells were established as described previously (Sugiyama et al., 2001). Transfectants were grown in M199 (BRL, Gaithersburg, MD) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml)
and G418 (400 µg/ml) (BRL, Gaithersburg, MD) at 37 °C with 5% CO₂ and 95% humidity. Cells were seeded in 12-well plates at a density of 1.2 x 10⁵ cells/well. Cell culture medium was replaced with culture medium supplemented with sodium-butyrate (5 mM) 24 hr before the transport studies to induce the expression of rOat1 and rOat3.

**Transport studies**

Transport studies were carried out as described previously (Hasegawa et al., 2002). Uptake was initiated by adding medium containing radiolabeled ligands after cells were washed twice and preincubated with Krebs-Henseleit buffer at 37 °C for 15 min. The Krebs-Henseleit buffer consists of 118 mM NaCl, 23.8 mM NaHCO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose and 1.53 mM CaCl₂ adjusted to pH 7.4. The final concentration of [³H]AZT, [³H]ACV and [³H]PCG was 1.0 µM, that of [³H]2,4-D was 0.5 µM, that of [³H]estrone sulfate and [³H]DHEAS was 0.1 µM, and that of [¹⁴C]salicylate and [¹⁴C]temocaprilat was 5.0 µM and 3.4 µM, respectively. The uptake was terminated at a designed time by adding ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Then, cells were washed twice with 1 ml ice-cold Krebs-Henseleit buffer, dissolved in 500 µl 0.2 N NaOH and kept overnight. Aliquots (450 µl) were transferred to scintillation vials after adding 100 µl 1N HCl. The radioactivity associated with the cells and medium was determined by liquid scintillation counting. The remaining 50 µl of the aliquots of cell lysate were used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard. Ligand uptake is given by the cell-to-medium concentration ratio determined as the amount of ligand associated with the cells divided
by the medium concentration. Specific uptake was obtained by subtracting the uptake into vector-transfected cells from that into cDNA-transfected cells.

**Uptake by kidney slices**

Uptake studies were carried out as described in a previous report (Hasegawa et al., 2002). Slices (0.3 mm thick) of whole kidneys from male Sprague-Dawley rats (7 – 8 weeks old, SLC Co., Ltd, Shizuoka, Japan) were placed in ice-cold oxygenated incubation buffer. The incubation buffer consists of 120 mM NaCl, 16.2 mM KCl, 1 mM CaCl$_2$, 1.2 mM MgSO$_4$ and 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ adjusted to pH 7.5. Two slices, each weighing 10-20 mg, were randomly selected and then incubated in a 12-well plate with 1 ml oxygenated incubation buffer in each well after preincubation for 5 min. The uptake study was carried out at 37 °C. After incubating for an appropriate time, each slice was rapidly removed from the incubation buffer, washed in ice-cold saline, blotted on filter paper, weighed, and dissolved in 1 ml soluene-350 (Packard Instruments, Downers Grove, IL) at 50 °C for 3 hr. The radioactivity was determined in a liquid scintillation counter after adding 10 ml scintillation fluid (Hionic Flour; Packard Instruments).

**Kinetic analyses**

Kinetic parameters were obtained using the following equation:

One-saturable (two-saturable) component,

$$ v = \frac{V_{\text{max 1}} \times S}{(K_{\text{m 1}} + S)} + \frac{V_{\text{max 2}} \times S}{(K_{\text{m 2}} + S)} $$

One-saturable and one non-saturable component,
\[ v = \frac{V_{\text{max}} \times S}{(K_m + S)} + CL_{\text{non}} \times S \]

where \( v \) is the uptake velocity of the substrate, \( S \) is the substrate concentration in the medium, \( K_m \) is the Michaelis-Menten constant, \( V_{\text{max}} \) is the maximum uptake rate and \( CL_{\text{non}} \) is the non-saturable uptake clearance. The number of components involved in the uptake by kidney slices was determined based on Akaike’s Information Criterion (AIC) values (Yamaoka et al., 1981). Inhibition constants (\( K_i \) values) of a series of compounds were obtained by examining their inhibitory effects on rOat1- and rOat3-mediated uptake assuming competitive inhibition using the following equation:

\[ CL_{+I} = \frac{CL}{1 + I / K_i} \]

where \( CL \) represents the uptake clearance in the absence of inhibitor and the subscript \(+I\) represents the value in the presence of an inhibitor. \( I \) represents the concentration of inhibitor. The substrate concentration used for the transport experiment was low enough compared with its \( K_m \) value in the inhibition study.

Fitting was performed by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981) and the Damping Gauss Newton Method algorithm was used for fitting.

**Estimation of uptake clearance in kidney slices from cDNA-transfectants**

Recently, several prediction approaches for assessing the contribution of multiple cytochrome P-450 enzymes (CYPs) to certain metabolic reactions in human liver microsomes have been reported (Nakajima et al., 2002). Crespi proposed the relative Activity Factor (RAF), as the ratio of human liver microsomal activity divided by CYP
activity for an isoform-specific reaction (Crespi, 1995). Based on this concept, we estimated the contribution of rOat1 and rOat3 to the total uptake by kidney slices. Since the renal uptake of PAH and pravastatin was predominantly accounted for by rOat1 and rOat3, respectively (Hasegawa et al., 2002), they were used as reference compounds for rOat1 and rOat3. The uptake clearances by cDNA-transfected cells (CL_{rOat1} and CL_{rOat3}) was divided by R_{rOat1} and R_{rOat3}, respectively, as described in the following equation.

\[
R_{rOat1} = \frac{CL_{PAH, \text{slice}}}{CL_{PAH, rOat1}}
\]

\[
R_{rOat3} = \frac{CL_{prav, \text{slice}}}{CL_{prav, rOat3}}
\]

\[
CL_{\text{test, slice}} = CL_{\text{test, rOat1}} \cdot R_{rOat1} + CL_{\text{test, rOat3}} \cdot R_{rOat3}
\]

Statistical analysis

Statistical differences were determined using one-way ANOVA followed by Fisher’s least significant difference method. Differences were considered significant at \( P < 0.05 \).
Results

Uptake of anionic compounds and nucleoside derivatives by transfectants

The time-profiles of the uptake of anionic compounds and nucleoside derivatives are shown in Figure 1. Transfection of rOat1 resulted in an increase in the uptake of [14C]temocaprilat, [3H]2,4-D, [3H]AZT, [3H]ACV and [14C]salicylate compared with vector-transfected cells (Figure 1A – 1C, 1G, 1H), and a slight increase in the uptake of [3H]estrone sulfate was observed in rOat1-expressing cells (Figure 1D). The uptake of [3H]estrone sulfate, [3H]ACV and [14C]salicylate was not high enough for further analysis (Figure 1D, 1G, 1H). The uptake of [3H]DHEAS and [3H]PCG by rOat1-expressing cells was very close to that exhibited by vector-transfected cells (Figure 1E, 1F), and no statistically significant difference was observed. Significant uptake of [14C]temocaprilat, [3H]estrone sulfate, [3H]DHEAS and [3H]PCG was observed in the rOat3-expressing LLC-PK1 cells (Figure 1A, 1D, 1E, 1F). Statistically difference was observed in the uptake of [3H]2,4-D and [3H]AZT by rOat3-expressing cells (Figure 1B, 1C), however, the transport activity was much lower than that of other substrates and was not high enough for further analysis. No statistically difference was observed in the uptake of [3H]ACV and [14C]salicylate by rOat3-expressing cells compared with vector-transfected cells over the linear range (Figure 1G, 1H).

Kinetic study of the uptake of anionic compounds and nucleoside derivatives by rOat1-expressing LLC-PK1 cells

Eadie-Hofstee plots of [14C]temocaprilat, [3H]2,4-D and [3H]AZT are shown in Figure 2. A non-saturable component was observed in the Eadie-Hofstee plot, even for the
specific uptake of [14C]temocaprilat and [3H]AZT by rOat1 (Figure 2A, C), which was determined by subtraction of the uptake by vector-transfected cells from that by rOat1-expressing cells. The K_i values of estrone sulfate, ACV, DHEAS, PCG and salicylate on PAH uptake by rOat1-expressing LLC-PK1 cells were examined in the rOat1-expressing LLC-PK1 cells (Figure 3), and kinetic parameters are summarized in Table 1. Furthermore, inhibition studies were carried out to examine the mode of inhibition of the ligands, whose uptake in rOat1-expressing cells was detected, but which was not high enough to allow for further characterization. rOat1-mediated uptake of [3H]PAH was measured at several substrate concentrations in the presence or absence of estrone sulfate, ACV and salicylate (Figure 4). All of these compounds increased the apparent K_m values but not affect the V_max values (data not shown), suggesting that these weak substrates of rOat1 inhibit the uptake of [3H]PAH in a competitive manner.

Kinetic study of the uptake of anionic compounds and nucleoside derivatives by rOat3-expressing LLC-PK1 cells

Eadie-Hofstee plots of [14C]temocaprilat, [3H]PCG, [3H]estrone sulfate and [3H]DHEAS are shown in Figure 5. The affinity of 2,4-D, AZT, ACV and salicylate for rOat3 was examined as the K_i value for [3H]pravastatin uptake by rOat3-expressing cells (Table 1; Figure 6). Furthermore, inhibition studies were carried out to test the mode of inhibition of the 2,4-D and AZT, whose uptake in rOat3-expressing cells was minimally detected. Eadie-Hofstee plot analyses demonstrated that these two compounds inhibited rOat3-mediated uptake of [3H]pravastatin in a competitive manner.
Uptake of anionic compounds and nucleoside derivatives by kidney slices

The time-profiles for the uptake of anionic compounds and nucleoside derivatives by kidney slices are shown in Figure 8. Eadie-Hofstee plots of the uptake of $[^3\text{H}]2,4$-D, $[^3\text{H}]$DHEAS, $[^3\text{H}]$PCG, $[^1\text{C}]$temocaprilat, $[^1\text{C}]$salicylate and $[^1\text{H}]$estrone sulfate are shown in Figure 9. One saturable and one non-saturable component were observed in the uptake of $[^3\text{H}]2,4$-D, $[^3\text{H}]$PCG and $[^3\text{H}]$DHEAS (Figure 9A - C). Two saturable components were observed in the uptake of $[^1\text{C}]$temocaprilat (Table 1; Figure 9D). Two saturable and one non-saturable components were observed in the uptake of $[^1\text{C}]$salicylate (Figure 9E). Kinetic parameters were calculated with the $K_m$ value of the low affinity component ($K_{m2}$) fixed as 550 µM, which was the $K_i$ value of salicylate for rOat1. In the uptake of $[^3\text{H}]$estrone sulfate, two saturable and one non-saturable components were also observed (Table 1; Figure 9F). $K_m$ and $V_{\text{max}}$ values of the uptake of anionic compounds are shown in Table 1. The concentration-dependence of the uptake of $[^3\text{H}]$AZT and $[^3\text{H}]$ACV is shown in Figure 9G. Even in the presence of 3 mM unlabeled ligands, the uptake of $[^3\text{H}]$AZT and $[^3\text{H}]$ACV was reduced to only 80 % and 60 % of the controls, respectively (Figure 9G).

Estimation of uptake clearance in kidney slices from cDNA-transfectants

Comparison of the observed and estimated renal uptake clearance is shown in Figure 10. The absolute values showing the predicted rOat1-mediated uptake ($CL_{\text{test}, \text{rOat1} \cdot R_{\text{rOat1}}}$), rOat3-mediated uptake ($CL_{\text{test}, \text{rOat3} \cdot R_{\text{rOat3}}}$), the sum of the rOat1- and rOat3-mediated uptake ($CL_{\text{test, slice}}$) and observed uptake clearance are summarized in...
Table 2. The observed and predicted uptake of PCG, temocaprilat, estrone sulfate and DHEAS were similar to each other. The observed values of salicylate and ACV were 7- and 18- fold higher than the predicted values, while that of 2,4-D was smaller than the predicted value.
Discussion

In the present study, we examined the contribution of rOat1 and rOat3 to the renal uptake of anionic compounds and nucleoside derivatives by examining the uptake by rOat1- and rOat3-expressing cells and kidney slices in terms of their affinity and transport activity.

Transfection of rOat1 resulted in a significant increase in the uptake of temocaprilat, 2,4-D, AZT, ACV and salicylate, and a slight increase in the uptake of estrone sulfate was observed in rOat1-expressing cells compared with the uptake by vector-transfected cells (Figure 1). In rOat3-expressing cells, a significant increase in the uptake of temocaprilat, estrone sulfate, DHEAS and PCG was observed, and the uptake of 2,4-D and AZT was slightly greater than that by vector-transfected cells (Figure 1). The difference in the spectrum of substrate specificity of rOat1 and rOat3 was clearly observed except for temocaprilat, a common substrate of rOat1 and rOat3 with similar transport activity and $K_m$ values (Figure 1; Table 1). The $K_i$ values of 2,4-D, ACV and salicylate, determined for the uptake of pravastatin by rOat3, were similar to those for rOat1, and the $K_i$ value of AZT for rOat3 was approximately 3-fold greater than that for rOat1 (Table 1). Although their uptake by rOat3-expressing cells was quite small or zero, their affinity was very close to that of rOat1. Since mutual inhibition study suggested that 2,4-D and AZT share the substrate-binding site with pravastatin in rOat3 molecules (Figure 7), the $K_i$ values can be used to estimate $K_m$ values for rOat3. It is possible that the relatively low uptake of 2,4-D and AZT by rOat3 is ascribed to the efficacy of the translocation process. However, the $K_i$ values of
estrone sulfate, DHEAS and PCG for rOat1 were 7~10 fold greater than their $K_m$ values for rOat3 (Table 1), suggesting that their lower affinity for rOat1 is one explanation of the low uptake by rOat1-expressing cells. Although significant uptake of PCG was not detected in rOat1-expressing LLC-PK1 cells (Figure 1), significant uptake of PCG was detected in rOat1-expressing *Xenopus laevis* oocytes compared with that by control oocytes (Jariyawat et al., 1999). Thus, PCG will be a substrate of rOat1 with low transport activity, like estrone sulfate.

In order to determine the importance of the transporter(s) in the renal uptake of xenobiotics including drugs and endogenous substrates, the contribution of rOat1 and rOat3 to the total renal uptake needs to be investigated. The renal uptake of test compounds was investigated using kidney slices. All test compounds significantly accumulated in kidney slices compared with mannitol used for the correction of adherent water volume. The uptake of 2,4-D, estrone sulfate and DHEAS was relatively high followed by temocaprilat, salicylate and PCG (Figure 8). The uptake of nucleoside derivatives by kidney slices was relatively low compared with the uptake of other test compounds (Figure 8). The concentration-dependence of the uptake of test compounds was investigated (Figure 9). Kinetic analyses revealed that a single saturable component was involved in the uptake of 2,4-D, DHEAS and PCG, while two were involved in the uptake of temocaprilat, salicylate and estrone sulfate. The $K_m$ value of 2,4-D for the uptake by kidney slices was comparable with that for rOat1 (Table 1), while those of DHEAS and PCG were comparable with those of rOat3 (Table 1). Taking the results of the transport studies using cDNA-transfected cells into
consideration, it is possible that the renal uptake of 2,4-D is mainly mediated by rOat1, and that of DHEAS and PCG is mediated by rOat3. The uptake of temocaprilat and estrone sulfate consisted of two saturable components and/or one non-saturable component (Figure 9). The high affinity components of temocaprilat and estrone sulfate account for 60% and 50% of the total uptake by kidney slices, respectively (Table 1). The $K_m$ values for the high affinity components of the uptake of temocaprilat and estrone sulfate were not necessarily comparable with those of rOat1 and/or rOat3 (Table 1). The kinetic parameters of the high affinity components, which are determined by the non-linear least-squares method, tends to be low when multiple saturable components are involved. Taking this into consideration, the difference in the $K_m$ values will be in the range of experimental deviation. It may be that rOat1 and rOat3 mediate the high affinity component of temocaprilat, while the high affinity component of estrone sulfate uptake by kidney slices is mediated by rOat3. The uptake of estrone sulfate was significantly reduced in kidney slices from mOat3 knockout mice, but still greater than the uptake in the presence of inhibitors such as probenecid and BSP (Sweet et al., 2002). This observation was consistent with our kinetic analysis, and the remaining uptake by kidney slices in mOat3 knockout mice may be partly accounted for by rOat1, since the $K_m$ value of the low affinity component in kidney slices in this study was similar to the $K_i$ value for rOat1 (Table 1).

The $K_m$ value of salicylate for the uptake by kidney slices was much smaller than the $K_i$ value for rOat1 or Oat3 (Table 1). Even though we assumed the presence of a low affinity component using the $K_i$ value of low affinity component fixed as that for...
rOat1 and rOat3, the contribution of the low affinity component was approximately 10% of the total saturable component, suggesting involvement of another transporter. Among the members of the rOat family, rOat2 has been shown to accept salicylate as a substrate (Morita et al., 2001). However, it is not localized on the basolateral membrane of the proximal tubules (Kojima et al., 2002), and the $K_m$ value for rOat2 (82 $\mu$M) is much greater than the $K_m$ value determined in kidney slices (Morita et al., 2001). Member(s) of the monocarboxylate transporter family are alternative candidate transporters (Takanaga et al., 1995; Eladari et al., 1999).

The uptake of nucleoside derivatives was saturated at high substrate concentrations, but the saturable components can account for the limited fraction of the total uptake, approximately 20% and 40% of the total uptake of AZT and ACV, respectively, as far as the concentrations examined are concerned (Figure 9). The uptake of AZT did not exhibit any saturation at a concentration sufficient to saturate rOat1- and rOat3-mediated uptake, while the uptake of ACV by kidney slices was significantly reduced at a concentration similar to the $K_m$ and $K_i$ values for rOat1 and rOat3, respectively. These results suggest that another transporter with lower affinity and/or passive diffusion mediates the uptake of AZT, while the uptake of ACV is mediated by rOat1, and possibly, by rOat3, at least in part.

In addition to the comparison of the $K_m$ values, the contribution was evaluated in terms of the relative transport activity. Crespi proposed the Relative Activity Factor (RAF) as the ratio of the human liver microsomal metabolic activity divided by the CYP activity for an isoform-specific reaction (Crespi, 1995). According to the RAF concept,
the uptake clearance of test compounds by kidney slices is compared with the values obtained from the transport activity by cDNA-transfected cells (Figure 10; Table 2). Also, PAH and pravastatin were used as reference compounds for rOat1 and rOat3, respectively, assuming the kinetics of transport determined in cDNA-transfected cells, in particular, that the relative transport activities are comparable with those in the kidney slices. A nice correlation was observed between the observed - and predicted uptake clearance except for 2,4-D, where the uptake clearance by kidney slices was smaller than the expected value (Figure 10). Based on this result, we propose the contribution of rOat1 and rOat3 to the total renal uptake of the test compounds is as follows; 1) rOat1 plays a major role in the renal uptake of 2,4-D, 2) rOat3 accounts for the uptake of PCG and DHEAS, and for the high affinity component of estrone sulfate uptake by kidney slices, 3) both rOat1 and rOat3 can account for the high affinity component of temocaprilat uptake by kidney slices, however, the uptake clearance corrected by the RAF value for rOat3 was greater than that for rOat1, suggesting that the contribution of rOat3 will be greater than rOat1, and 4) the uptake of ACV by rOat1 was too low to account for the uptake of ACV by kidney slices.

The uptake of 2,4-D by kidney slices was lower than the predicted value (Figure 10; Table2). There are two possibilities to account for this. Firstly, diffusion limited the uptake when the ligand is extensively taken up by kidney slices. Secondly, rOat1-expressing LLC-PK1 cells are not an appropriate model to investigate the renal uptake process in a quantitative manner in terms of the driving force and local membrane environment which affects the transport activity and substrate specificity of
the transporter. Since the intrinsic transport activity of 2,4-D by rOat1 was 6-fold greater than that of PAH, it is possible that the uptake of 2,4-D by kidney slices is diffusion-limited.

Although the $K_i$ value of estrone sulfate for rOat1 was comparable with the $K_m$ value for the low affinity component of the uptake by kidney slices (Table 1), the uptake corrected by the RAF value was too low. Therefore, the contribution of rOat1 to the low affinity component of estrone sulfate uptake by kidney slices will be minor.

Finally, the number of rOat1 substrates for which renal uptake is supposed to be mediated by rOat1 was limited in this study. Further studies are required to examine whether the RAF concept can be applied to rOat1-mediated uptake or not by increasing the number of rOat1 substrates the renal uptake of which is mainly mediated by rOat1.

The present study addressed the importance of investigating the contribution made by each transporter to the total uptake process in addition to the transport studies using cDNA-transfected cells. Even although a compound is a good substrate, it is possible that the contribution of the transporter, supposed to be involved, is not the major mechanism. A good correlation between the observed and predicted values suggests that the RAF concept can be applied; at least, as far as rOat3 substrates are concerned. This approach is useful for predicting the contribution by transport studies using cDNA-transfected cells once the absolute value of the uptake of the transporter-specific ligand by the target organ has been determined. Further studies are necessary to establish this approach by investigating the rationale of the in vitro model and by examining the effect of selective inhibitors of each transporter.
In conclusion, we have demonstrated that the kidney is equipped with an uptake system for amphipathic organic anions, which is mainly mediated by rOat3. Although rOat1 transports certain hydrophobic compounds, its contribution to the total renal uptake is minor. It is also suggested that there is an additional uptake system(s) involved in the renal uptake of salicylate, temocaprilat, estrone sulfate and ACV.
Acknowledgement

We would like to thank Sankyo (Tokyo, Japan) for providing labeled and unlabeled pravastatin, labeled temocapril and unlabeled temocaprilat, and Mr. Daisuke Sugiyama for his help in cellular uptake studies using rOat1- and rOat3-expressing LLC-PK1 cells.
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Footnotes

This work was supported by CREST (Core Research for Evolutilional Science and Technology) of Japan Science and Technology Corporation.
Figure legends

Figure 1. Time-profiles of the uptake of organic anions by rOat1- and rOat3-expressing LLC-PK1 cells. The uptake of [14C]temocaprilat (3.4 µM) (A), [3H]2,4-D (0.5 µM) (B), [3H]AZT (1.0 µM) (C), [3H]estrone sulfate (0.1 µM) (D), [3H]ACV (1.0 µM) (E), [3H]DHEAS (0.1 µM) (F), [3H]PCG (1.0 µM) (G) and [14C]salicylate (5.0 µM) (H) by cDNA transfected cells was examined at 37 °C. Squares, circles and triangles represent the uptake by rOat1- and rOat3-expressing cells and vector-transfected LLC-PK1 cells, respectively. Each point represents the mean ± S.E. (n=3).

Figure 2. Eadie-Hofstee plots of the uptake of [14C]temocaprilat, [3H]2,4-D and [3H]AZT by rOat1-expressing LLC-PK1 cells. The concentration-dependence of the uptake of [14C]temocaprilat (A), [3H]2,4-D (B) and [3H]AZT (C) by rOat1 is shown as Eadie-Hofstee plots. The uptake of [14C]temocaprilat for 5min, [3H]2,4-D for 1min and [3H]AZT for 2min was determined at various concentrations (temocaprilat; 0.7~300 µM, 2.4-D; 0.5~100 µM, AZT; 1~3000 µM). Solid lines represent the fitted line obtained by non-linear regression analysis. Each point represents the mean ± S.E. (n=3).

Figure 3. Inhibitory effect of estrone sulfate, ACV, DHEAS, PCG and salicylate on the uptake of [3H]PAH by rOat1-expressing LLC-PK1 cells. The uptake of [3H]PAH (1 µM) for 2min by rOat1 was determined in the presence and absence of unlabeled
estrone sulfate (A), ACV (B), DHEAS (C), PCG (D) and salicylate (E) at the designed concentrations. The values are expressed as a percentage of the uptake in the absence of any unlabeled compounds. The rOat1-mediated transport was obtained by subtracting the transport velocity in vector-transfected cells from that in rOat1-expressed cells. Solid lines represent the fitted line obtained by non-linear regression analysis. Each point represents the mean ± S.E. (n=3).

Figure 4. Inhibitory effect of estrone sulfate, ACV and salicylate on the uptake of PAH by rOat1-expressing LLC-PK1 cells. The concentration-dependence of the uptake of \[^{3}\text{H}]\text{PAH}\) by rOat1 is measured in the presence (circles and triangles) and absence (squares) of estrone sulfate (A), ACV (B) and salicylate (C) and the results are shown as Eadie-Hofstee plots. The uptake of \[^{3}\text{H}]\text{PAH}\) for 2 min was determined at various concentrations (1 ~ 1000 µM). The concentration of estrone sulfate was 50 µM (A), that of ACV was 980 µM (B), and that of salicylate was 300 and 900 µM (C). Each point represents the mean ± S.E. (n=3).

Figure 5. Eadie-Hofstee plots of the uptake of \[^{14}\text{C}]\text{temocaprilat}, [\[^{3}\text{H}]\text{PCG}, [\[^{3}\text{H}]	ext{estrone sulfate} and [\[^{3}\text{H}]\text{DHEAS}\) by rOat3-expressing LLC-PK1 cells. The concentration-dependence of the uptake of \[^{14}\text{C}]\text{temocaprilat}\) (A), \[^{3}\text{H}]\text{PCG}\) (B), \[^{3}\text{H}]	ext{estrone sulfate}\) (C) and \[^{3}\text{H}]\text{DHEAS}\) (D) by rOat3 is shown as Eadie-Hofstee plots. The uptake of \[^{14}\text{C}]\text{temocaprilat}\) and \[^{3}\text{H}]\text{PCG}\) for 5 min and \[^{3}\text{H}]	ext{estrone sulfate}\) and \[^{3}\text{H}]\text{DHEAS}\) for 1 min was determined at various concentrations (temocaprilat; 0.7~300
μM, PCG; 1~3000 μM, estrone sulfate; 0.1~1000 μM, DHEAS; 0.1~500 μM). Solid lines represent the fitted line obtained by non-linear regression analysis. Each point represents the mean ± S.E. (n=3).

Figure 6. Inhibitory effect of 2,4-D, AZT, ACV and salicylate on the uptake of [3H]pravastatin (0.5 μM) for 5min by rOat3-expressing LLC-PK1 cells. The uptake of [3H]pravastatin (0.5 μM) by rOat3 was determined in the presence and absence of unlabeled 2,4-D (A), AZT (B), ACV(C), and salicylate (D) at the designed concentrations. The values are expressed as a percentage of the uptake in the absence of any unlabeled compounds. Solid lines represent the fitted line obtained by non-linear regression analysis. Each point represents the mean ± S.E. (n=3).

Figure 7. Inhibitory effect of AZT and 2,4-D on the uptake of pravastatin by rOat3-expressing LLC-PK1 cells. The concentration-dependence of the uptake of [3H]pravastatin by rOat3 is measured in the presence (circles and triangles) and absence (squares) of 2,4-D (A) and AZT (B) and the results are shown as Eadie-Hofstee plots. The uptake of [3H]pravastatin for 5 min was determined at various concentrations (0.5 ~ 300 μM). The concentration of 2,4-D was 5 and 20 μM (A) and that of AZT was 140 μM (B). Each point represents the mean ± S.E. (n=3).

Figure 8. Time-profiles of the uptake of organic anions and nucleoside derivatives by kidney slices. The uptake of [14C]temocaprilat (0.5 μM) (A), [3H]2,4-D (0.5 μM) (B),
[3H]AZT (1.0 µM) (C), [3H]estrone sulfate (0.1 µM) (D), [3H]ACV (1.0 µM) (E), [3H]DHEAS (0.1 µM) (F), [3H]PCG (1.0 µM) (G) and [14C]salicylate (1 µM) (H) by kidney slices was examined at 37 °C. Each point represents the mean ± S.E. (n=3).

Figure 9. Eadie-Hofstee plots of the uptake of [3H]2,4-D, [3H]DHEAS, [3H]PCG, [14C]temocaprilat, [14C]salicylate and [3H]estrone sulfate by kidney slices, and the concentration-dependence of the uptake of AZT and ACV. The concentration-dependence of [3H]2,4-D (A), [3H]DHEAS (B), [3H]PCG (C), [14C]temocaprilat (D), [14C]salicylate (E) and [3H]estrone sulfate (F) is shown as Eadie-Hofstee plots. The uptake of [3H]PCG, [14C]temocaprilat and [14C]salicylate for 15 min and [3H]2,4-D, [3H]DHEAS and [3H]estrone sulfate for 10 min was determined at various concentrations (2,4-D; 0.2~300 µM, DHEAS; 0.1~500 µM, PCG; 1~10000 µM, temocaprilat; 0.5~300 µM, salicylate 1~10000 µM, estrone sulfate; 0.1~1000 µM). The concentration-dependence of the uptake of [3H]AZT and [3H]ACV is shown as a percentage of the uptake at the minimum concentration (G). The uptake of [3H]AZT and [3H]ACV for 3 min was determined at various concentrations. Adherent water was determined by the uptake of mannitol, and that was subtracted from the distribution volume of the substrates. Each point represents the mean ± S.E. (n=3). *P < 0.05, significant difference from each control at 1 µM.

Figure 10. Relationship between the observed and predicted renal uptake clearance. The predicted values represent the sum of the rOat1- and rOat3-mediated transport pathways.
corrected by the relative transport activity of test compounds with the reference compounds. PAH and pravastatin were used as reference compounds for rOat1 and rOat3, respectively. The details of the experiments are described in the Methods section. Key: PAH (open square); pravastatin (closed square); ACV (open diamond); AZT (closed diamond); 2,4-D (open triangle); PCG (closed triangle); temocaprilat (open inverted triangle); salicylate (closed inverted triangle); estrone sulfate (open circle); DHEAS (closed circle).
Table 1

Kinetic parameters of the uptake of anionic compounds and nucleoside derivatives by kidney slices, rOat1- and rOat3-expressing LLC-PK1 cells.

Data shown in the Figures 2, 3, 5, 6 and 9 were used to determine the $V_{\text{max}}$, $K_m$ and $K_i$ values for the uptake of organic anions by cDNA-transfected cells and kidney slices. PAH and pravastatin were used as substrate to determine the $K_i$ values for the rOat1- and rOat3-expressing cells, respectively.

Each value represents the mean ± S.D.

<table>
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<th>Compound</th>
<th>rOat1-LLC</th>
<th>rOat3-LLC</th>
<th>Kidney slice</th>
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<tr>
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<td>$K_m$ (µM)</td>
<td>$V_{\text{max}}$ (pmol/min/mg protein)</td>
<td>$K_m$ (µM)</td>
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<tr>
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<td>0.556 ± 0.376*</td>
<td>3.71 ± 0.85</td>
<td>30.1 ± 10.6</td>
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<tr>
<td>estrone sulfate</td>
<td>30 ± 20.4</td>
<td>-</td>
<td>30.6 ± 5.9</td>
</tr>
<tr>
<td>DHEAS</td>
<td>511 ± 167</td>
<td>-</td>
<td>145 ± 34</td>
</tr>
<tr>
<td>salicylate</td>
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<td>paclitaxel</td>
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<td>-</td>
<td>204 ± 24</td>
</tr>
</tbody>
</table>

* $K_m$ value
Table 2

Comparison of uptake clearances in kidney slices with predicted values from the uptake study using rOat1- and rOat3-expressing LLC-PK1 cells.

The observed values represent the intrinsic transport activity by kidney slices (V<sub>max</sub>/K<sub>m</sub>). The intrinsic transport activity of the component which K<sub>m</sub> value is comparable with that for the uptake in cDN- transfected cells was used for the uptake of estrone sulfate, temocaprilat and salicylate. The inhibitable portion was used for the observed value for AZT and ACV uptake. The details of the experiments are described in the Methods.

<table>
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<th>rOat1-mediated uptake (ml/min/g kidney)</th>
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<th>observed value (ml/min/g kidney)</th>
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<tr>
<td>ACV</td>
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<td>-</td>
<td>0.0048</td>
<td>0.090</td>
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<tr>
<td>salicylate</td>
<td>0.014</td>
<td>-</td>
<td>0.014</td>
<td>0.091</td>
</tr>
</tbody>
</table>
Figure 1

A  temocaprilat
B  2.4-D
C  AZT
D  estrone sulfate

E  DHEAS
F  PCG
G  ACV
H  salicylate
Figure 2

A  temocaprilat

B  2.4-D

C  AZT
Figure 3

A  estrone sulfate

B  ACV

C  DHEAS

D  PCG

E  salicylate
Figure 4

A

B

C

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

A  temocaprilat

B  PCG

C  estrone sulfate

D  DHEAS

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 6

A  2.4-D

uptake (% of control) vs. inhibitor conc. (µM)

B  AZT

uptake (% of control) vs. inhibitor conc. (µM)

C  ACV

uptake (% of control) vs. inhibitor conc. (µM)

D  salicylate

uptake (% of control) vs. inhibitor conc. (µM)
Figure 7
Figure 8

A  temocaprilat

B  2.4-D

C  AZT

D  estrone sulfate

E  ACV

F  DHEAS

G  PCG

H  salicylate
Figure 9

A  2.4-D  

B  DHEAS  

C  PCG  

D  temocaprilat  

E  salicylate  

F  estrone sulfate  

G

<table>
<thead>
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<th>substrate conc. (µM)</th>
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Figure 10