Quantitative Evaluation of the Drug-Drug Interactions between Methotrexate and Nonsteroidal Anti-Inflammatory Drugs in the Renal Uptake Process Based on the Contribution of Organic Anion Transporters and Reduced Folate Carrier

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ABSTRACT

The present study examined the possible role of transporters in the drug-drug interactions between methotrexate (MTX) and nonsteroidal anti-inflammatory drugs (NSAIDs) in the renal uptake process of MTX. MTX is recognized by reduced folate carrier (RFC-1) and rat organic anion transporters (rOat1 and rOat3) as a substrate. Uptake of MTX by kidney slices was saturable and inhibited potently by dibromosulfophthalein. Folate and benzylpenicillin (PCG) inhibited the uptake by 30 to 40% and 40 to 50% of the total saturable uptake of MTX by kidney slices, respectively, whereas the effect of p-aminohippurate (PAH) was minimal at the concentration selective for rOat1. In contrast, the uptake of 5-methyltetrahydrofolate by the kidney slices was inhibited by MTX, folate, and dibromosulfophthalein, but not by PAH and PCG. These results suggest that rOat3 and RFC-1 are almost equally involved in the uptake of MTX by the kidney slices, whereas RFC-1 is responsible for the renal uptake of 5-methyltetrahydrofolate. NSAIDs, except salicylate, were potent inhibitors of rOat3 (Ki of 1.3–19 μM), but weak inhibitors of RFC-1 (Ki of 70–310 μM). This is in a good agreement with the biphasic inhibition profiles of NSAIDs for the uptake of MTX by kidney slices. These results suggest that the renal uptake of MTX is not so greatly affected by NSAIDs as expected from the inhibition of rOat3-mediated transport.

Drug-drug interactions involving metabolism and/or excretion process prolong the plasma elimination half-lives, leading to the accumulation of drug in the body after repeated administration, and potentiate pharmacological/adverse effects. Investigating the mechanism of a drug-drug interaction is important as far as achieving safer chemotherapy is concerned. Transporters are one of the determinant factors governing drug disposition together with metabolic enzymes and have been regarded as important drug-drug interaction sites in the body (Ito et al., 1998). Recent progress in molecular biological research has shown that many types of transporters play important roles in the tissue uptake and/or subsequent secretion of drugs in the liver and kidney, and enables us to examine the incidence of drug-drug interactions involving membrane transport based on the contribution of the transporters to the membrane transport of the drugs in question.

Methotrexate (MTX) is an analog of natural folate and has been used not only for the treatment of neoplastic diseases (Evans et al., 1986) but also autoimmune diseases including rheumatoid arthritis (Giannini et al., 1992) and psoriasis (Chladek et al., 1998). Numerous case reports have indicated that coadministration of MTX with penicillin, probenecid, and nonsteroidal anti-inflammatory drugs (NSAIDs) cause drug-drug interactions, which affect the pharmacokinetic parameters of MTX (Furst, 1995). To date, several potential sites have been proposed for these drug-drug interactions: an increase in the unbound fraction of MTX (Tracy et al., 1992), a decrease in the urine flow rate resulting from the inhibition of prostaglandin synthesis (Kremer and Hamilton, 1995),

ABBREVIATIONS: MTX, methotrexate; NSAID, nonsteroidal anti-inflammatory drug; MDCK, Madin-Darby canine kidney; PAH, p-aminohippurate; PCG, benzylpenicillin; Oat/OAT, organic anion transporter; RFC-1, reduced folate carrier; 5-MTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; TCA, taurocholate; DBSP, dibromosulfophthalein; Mrp, multidrug resistance-associated protein.

MTX is mainly eliminated in the urine in metabolically unchanged form, and its pharmacokinetic profile shows non-linearity that is possibly associated with saturation of the reabsorption process at high dosages (Hendel and Nyfors, 1984). Thus, in the case of high-dose MTX chemotherapy, the contribution of renal tubular secretion to the net renal clearance may be greater than that in the case of low-dose therapy.

Previous studies have demonstrated that p-aminohippurate (PAH), probenecid, and salicylate decrease the renal clearance of MTX in vivo (Liegener et al., 1969; Ahern et al., 1978) and organic anions, including NSAIDs, inhibit the uptake of MTX by kidney slices (Nierenberg, 1983), suggesting involvement of organic renal anion transporters. rOat1/hOAT1 (Slc22a6/SLC22A6) (Sekine et al., 1997; Sweet et al., 1997; Hosoyamada et al., 1999) and rOat3/hOAT3 (Slc22a8/SLC22A8) (Kusuhara et al., 1999; Cha et al., 2001), isoforms of the SLCC2A family (Oat/OATs; Slc22a8/SLC22A8), have been identified in rodent and human kidney and are suggested to be responsible for the renal uptake of various endogenous organic anions, xenobiotics, and drugs, including MTX (Hasegawa et al., 2002, 2003; Takeda et al., 2002). In addition to these organic anion transporters, reduced folate carrier, RFC-1 (Slc19a1), a transporter of reduced folate and its derivatives, has been known to accept MTX as a substrate. RFC-1 has been suggested to play an important role in intestinal folate absorption, folate uptake by proliferative tissues, and to be of pharmacological significance for MTX (Chiao et al., 1997; Sirotnak and Tolner, 1999). Recently, immunohistochemical analysis demonstrated the basolateral localization of mouse RFC-1 in the kidney (Wang et al., 2001), suggesting that RFC-1 is also involved in the renal uptake of MTX as well as organic anion transporters (rOat1 and rOat3).

According to inhibition studies using transfectants expressing hOAT1 and hOAT3, NSAIDs have been shown to be potent inhibitors of hOAT3, and inhibition of hOAT3 by certain NSAIDs such as salicylate, indomethacin, and phenylbutazone, are likely to occur at clinical doses (Takeda et al., 2002). However, to evaluate quantitatively whether the interaction is clinically relevant and to predict the interaction from in vitro studies using cDNA transfectants, the contribution of transporters to the renal uptake of MTX (rOat1, rOat3, and RFC-1) is an essential factor when multiple transporters are involved.

In the present study, the renal uptake of MTX via the basolateral membrane was investigated as a potential site of drug-drug interaction, especially focusing on the contribution of rOat1, rOat3, and RFC-1.

**Materials and Methods**

**Materials.** [3',5',7'-H]Methotrexate (25–29 Ci/mmol) and (65)-5-methyltetrahydro-[3',5',7'-H]folate (22.3 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled methotrexate and 5-methyltetrahydrofolate were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in the present study were commercially available and were reagent grade products.

**Animals.** Male Sprague-Dawley rats weighing 250 to 300 g (Charles River Japan, Kanagawa, Japan) were used throughout the studies. All animals were treated humanely.

**Establishment of Rat RFC-1-MDCKII.** The full coding region of rat RFC-1 was amplified from rat kidney cDNA by reverse transcription-polymerase chain reaction. Full-length rat RFC-1 was subcloned into the HindIII site of mammalian expression vector pEDNA3.1+ (Invitrogen, Carlsbad, CA). Cloned rat RFC-1 had three mutations (C22G, G24C, and T40C). T40C was a silent mutation, but C22G and G24C caused an amino acid change, Gly8Ala. However, direct sequence analysis revealed that the mutation was not a result of polymerase chain reaction accumulation, but derived from the original cDNA sequence. The cDNA was used for the construction of a stable expression system.

Parental MDCKII cells, a kind gift from Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands), were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Sigma-Aldrich) at 37°C in 5% CO2. The vector construct of RFC-1 was transfected into parental MDCKII cells by LipofectAMINE reagent (Invitrogen) following the manufacturer's instructions (RFC-1-MDCKII). After 2 weeks of G418 selection (800 μg/ml), positive clones were selected by Northern blot analysis.

**Transport Study.** rOat1 and rOat3 cDNA-transfected LLC-PK1 cells were established previously in our laboratory (Sugiyama et al., 2001). LLC-PK1 cells were grown in M199 supplemented with 10% fetal bovine serum at 37°C in 5% CO2. LLC-PK1 and MDCKII cells were seeded on 12-well plates at a density of 1.2 × 10⁶ cells/well. Cells were cultured for 48 h with the above-mentioned medium and for an additional 24 h with the culture medium supplemented with 5 mM sodium butyrate before starting the transport studies.

Transport studies were carried out as described previously (Sugiyama et al., 2001). Transport was initiated by adding the radiolabeled compounds to the medium in the presence or absence of inhibitors after cells had been washed twice and preincubated with Krebs-Henseleit buffer. 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 uptake was terminated at designated times by aspirating the incubation buffer and adding ice-cold Krebs-Henseleit buffer. Cells were washed twice with ice-cold buffer and dissolved in 500 μl of 0.2 N NaOH. The radioactivity associated with the specimens was determined in a liquid scintillation counter.

Where indicated, the cells were incubated in the presence of unlabeled MTX (100 μM) for 1 h. Thereafter, the cells were washed twice with ice-cold buffer to remove extracellular unlabeled MTX, incubated at 37°C in 1 ml of buffer for 5 min to remove intracellular unbound MTX and then the uptake study was started.

**Intracellular Protein Binding of MTX in RFC-1-MDCKII.** Intracellular protein binding of MTX was assessed by determining the unbound fraction present in each cytosolic fraction prepared from RFC-1-transfected MDCKII cells using the ultrafiltration procedure (Ueda et al., 2001). RFC-1-transfected MDCKII cells were cultured with the above-mentioned Dulbecco's modified Eagle's medium for 48 h in 5% CO2 and for an additional 24 h with medium containing sodium butyrate. Where indicated, the cells were incubated in the presence of unlabeled MTX (100 μM) for 1 h, washed twice with ice-cold buffer to remove extracellular unlabeled MTX, and incubated at 37°C in 1 ml of buffer for 5 min to remove intracellular unbound MTX. Collected cells were suspended in 0.2 M potassium phosphate buffer, pH 6.2, and subsequently sonicated in the presence of protease inhibitor cocktail (Sigma-Aldrich) on ice. Homogenates were centrifuged at 100,000g for 1 h, and the supernatant was used to the assay of protein binding. NADPH was added to the supernatant to give a final concentration of 0.1 mM. Separation of free from protein-bound MTX was obtained by the filtration of free MTX through a YM-10 ultrafiltration membrane (diameter 14 mm; Millipore Corporation, Bedford, MA). No significant adsorption to the filtration equipment was observed. The ultrafiltrate (200 μl) was collected and the radioactivity was determined in a liquid scintillation counter.
Uptake of \[^{3}H\]Methotrexate by Rat Kidney Slices. Uptake studies by rat kidney slices were carried out as described previously (Hasegawa et al., 2002). Kidney slices (300 \mu m in thickness) from male Sprague-Dawley rats were kept in ice-cold buffer. The buffer for this study consists of 120 mM NaCl, 16.2 mM KCl, 1 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, and 10 mM NaH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} adjusted to pH 7.5. Two slices, each weighing 15 to 25 mg, were randomly selected and then incubated on a 12-well plate with 1 ml of oxygenated buffer in each well after preincubation of slices for 5 min. After incubating for the designated times, each slice was rapidly removed from the incubation buffer, washed in ice-cold buffer, blotted on filter paper, weighed, and dissolved in 1 ml of Soluene-350 (PerkinElmer Life Sciences, Boston, MA) at 50°C for 3 h. The radioactivity was determined in scintillation cocktail (Hionic Flour; PerkinElmer Life Sciences).

**Kinetic Analyses.** Kinetic parameters were obtained using the Langmuir equation for binding assay and the Michaelis-Menten equation for transport studies. Fitting was performed by the nonlinear least-squares method using the MULTI program (Yamaoka et al., 1981). The input data were weighed as the reciprocals of the observed values, and the Damping Gauss Newton method algorithm was used for fitting.

**Statistical Analysis.** Data are expressed as the mean ± S.E. Statistical differences were determined using a one-way analysis of variance with Dunnett’s post hoc test. Differences were considered to be significant at \( P < 0.05 \).

**Results**

**Characterization of RFC-1-Mediated Uptake of MTX.** The uptake of MTX was greater in RFC-1-expressing cells than in vector-transfected cells (Fig. 1A). The Eadie-Hofstee plot of the RFC-1-mediated uptake of MTX consisted of two saturable components with apparent \( K_m \) values for high- and low-affinity components of 0.319 ± 0.226 \mu M (\( V_{\text{max}} = 1.31 ± 0.86 \text{ pmol/mg protein/min} \)) and of 26.0 ± 11.7 \mu M (\( V_{\text{max}} = 58.1 ± 15.1 \text{ pmol/mg protein/min} \)), respectively (Fig. 1B).

![Fig. 1](image-url) Transport properties of MTX via RFC-1. A, time-profile of MTX in RFC-1-transfected cells (open circles) and control cells (closed squares) during a 30-min incubation. RFC-1-expressing cells were incubated at 37°C in Krebs-Henseleit buffer containing 50 nM \[^{3}H\]MTX. B, concentration dependence of RFC-1-mediated uptake of MTX. The initial uptake rates of MTX by control or RFC-1-transfected cells for 1 min were measured at different concentrations. RFC-1-mediated uptake was assessed by subtracting the transport velocity in control cells from that in RFC-1-transfected cells. C, inhibitory effect of MTX on RFC-1-mediated uptake of 5-MTHF. The uptake of 5-MTHF (100 nM) for 5 min was determined in the presence of unlabeled MTX at the concentrations indicated. The solid lines represent the fitted lines obtained by nonlinear regression analysis. Each data point represents the mean ± S.E. (\( n = 3 \)).

![Fig. 2](image-url) Transport and intracellular protein binding properties of MTX via RFC-1 after preloading. A, time profile of 50 nM MTX in RFC-1-transfected cells (open circles) and control cells (closed squares) during a 30-min incubation period after preloading. RFC-1-transfected cells and control cells were preloaded with unlabeled MTX (100 \mu M) at 37°C for 1 h, washed with buffer, and then the accumulated intracellular MTX was examined at the indicated time points. B, concentration dependence of RFC-1-mediated uptake of MTX. The initial uptake rates of MTX by control or RFC-1-transfected cells for 1 min were measured at different concentrations. RFC-1-mediated uptake was assessed by subtracting the transport velocity in control cells from that in RFC-1-transfected cells. Each data point represents the mean ± S.E. (\( n = 3 \)). C, specific binding of MTX to the cytosolic fraction of RFC-1-transfected cells prepared after preloading (closed squares) and untreated conditions (open circles). Separation of free from protein-bound \[^{3}H\]MTX was obtained by passing free \[^{3}H\]MTX through ultrafiltration membrane. The inset represents a magnification of the low concentration region. The solid lines represent the fitted lines obtained by nonlinear regression analysis.
MTX inhibited the RFC-1-mediated uptake of 5-MTHF, a typical substrate of RFC-1, and the $K_i$ value of MTX for 5-MTHF was determined to be 69.2 ± 10.5 μM (Fig. 1C).

To distinguish saturation of the transporter-mediated transport of MTX from that of intracellular protein binding, the uptake of MTX was measured after preincubation with unlabeled MTX (100 μM) for 1 h. RFC-1-MDCKII showed specific uptake of MTX even after they were preincubated with unlabeled MTX (Fig. 2A). Eadie-Hofstee plot indicated a marked reduction in the fraction of the high-affinity component, and the uptake of MTX by RFC-1-MDCKII cells is accounted for by one saturable component with a $K_m$ of 17.8 ± 1.8 μM ($V_{max} = 77.1 ± 7.1$ pmol/mg protein/5 min) (Fig. 2B). The cytosolic fraction of untreated RFC-1-MDCKII cells showed specific binding of MTX with a $K_m$ of 30.4 ± 5.0 μM, but this was not observed in that from unlabeled MTX-preloaded cells (Fig. 2C).

The effect of various inhibitors on the uptake of MTX by RFC-1-MDCKII was examined under two sets of conditions, preincubation of cells with unlabeled MTX (Fig. 3B) and no preincubation (Fig. 3A). In both cases, RFC-1-mediated uptake of MTX was preferentially inhibited by natural folate, such as 5-MTHF, folic acid, and leucovorin, inhibited the renal uptake of MTX by approximately 30%. PCG, a typical substrate of rOat3, inhibited the uptake of MTX by 50%, whereas the renal uptake of MTX was reduced to 70 to 80%.

Uptake of MTX by rOat1- and rOat3-Transfected Cells. The time-profiles of MTX in rOat1- and rOat3-transfected cells are shown in Fig. 4, A and C. The uptake of MTX by rOat1- and rOat3-expressing cells was significantly greater than that of vector-transfected cells. The Eadie-Hofstee plot of the uptake of MTX by rOat1-transfected cells revealed that rOat1-mediated transport of MTX consisted of two saturable components, a high-affinity component with a $K_m$ of 0.0866 ± 0.0274 μM ($V_{max} = 0.468 ± 0.087$ pmol/mg protein/5 min) and a low-affinity component with a $K_m$ of 14.9 ± 0.8 μM ($V_{max} = 28.8 ± 0.7$ pmol/mg protein/5 min) (Fig. 4B). As shown in Fig. 4D, the rOat3-mediated uptake of MTX also apparently consisted of two saturable components, but a good fit could not be obtained.

Characterization of Renal Uptake of MTX. Figure 5A shows the time profiles of MTX and 5-MTHF in rat kidney slices. MTX showed higher transport activity than 5-MTHF, and the uptake of MTX and 5-MTHF was linear during the initial 30-min period. For the following kinetic studies, a 15-min incubation period was chosen for both MTX and 5-MTHF. Eadie-Hofstee plot analysis indicated that the renal uptake of MTX consisted of three components, high- and low-affinity components with $K_m$ values of 0.118 ± 0.028 μM ($V_{max} = 0.409 ± 0.094$ nmol/g kidney/15 min) and 76.9 ± 18.3 μM ($V_{max} = 112 ± 24$ nmol/g kidney/15 min), respectively, and a nonsaturable component (nonsaturable uptake clearance, 0.346 ± 0.037 ml/g kidney/15 min) (Fig. 5B).

Figure 6, A and B, describes the inhibitory effect of various compounds on the uptake of MTX and 5-MTHF by kidney slices. DBSP was the most potent inhibitor, and probenecid was a moderate or relatively weak inhibitor of MTX. Natural folate, such as 5-MTHF, folic acid, and leucovorin, inhibited the renal uptake of MTX by approximately 30%. PCG, a typical substrate of rOat3, inhibited the uptake of MTX by 50%, whereas the renal uptake of MTX was reduced to 70 to 80%.

The $K_m$ and $K_i$ values for the uptake by rOat1- and rOat3-LLC-PK1 cells, and by RFC-1-MDCKII cells was determined by nonlinear regression analysis. Compounds in parentheses represent substrates for determination of $K_i$ values.

### Table 1

<table>
<thead>
<tr>
<th>Substrate/Inhibitor</th>
<th>rOat1</th>
<th>rOat3</th>
<th>RFC-1</th>
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<tbody>
<tr>
<td>PAH</td>
<td>$K_m$ = 47.0 μM, 59.8 μM</td>
<td>$K_m$ = 398 μM (PCG)</td>
<td>$K_m$ &gt; 1000 μM (MTX)</td>
</tr>
<tr>
<td>PCG</td>
<td>$K_m$ = 418 μM, 800 μM</td>
<td>$K_m$ = 82.6 μM (PAH)</td>
<td>$K_m$ &gt; 1000 μM (MTX)</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>$K_i$ &gt; 1000 μM (MTX)</td>
<td>$K_i$ &gt; 1000 μM (MTX)</td>
<td>$K_i$ = 5.03 ± 3.05 μM (MTX)</td>
</tr>
</tbody>
</table>

* Nagata et al., 2002.
* Hasegawa et al., 2002.
* Mean ± S.D.
80% of the control value by PAH, TCA, and cimetidine. More detailed kinetic analysis revealed that PCG and PAH inhibited the uptake of MTX by kidney slices in a concentration-dependent manner with $K_i$ values of 90.1 ± 52.7 and 630 ± 125 μM, respectively (Fig. 6C). On the other hand, the uptake of 5-MTHF by kidney slices was completely inhibited by 1 mM MTX, folic acid, and leucovorin (Fig. 6B). DBSP inhibited the uptake of 5-MTHF by approximately 50% at 1 mM but was insensitive or only minimally sensitive to the other compounds used in this study.

**Inhibitory Effect of NSAIDs on rOat1-, rOat3-, and RFC-1-Mediated Uptake, and the Uptake of MTX by Kidney Slices.** We examined the inhibitory effect of NSAIDs on rOat1-, rOat3-, and RFC-1-mediated transport of typical substrates (Fig. 7; Table 2). Although MTX was transported by rOat1 and rOat3, they showed relatively low transport activity for MTX (Fig. 4). Therefore, PAH and PCG were used as test compounds to obtain $K_i$ values of NSAIDs for rOat1 and rOat3, respectively. All NSAIDs, except salicylate, were potent or moderate inhibitors of rOat1 and rOat3, but weak inhibitors of RFC-1, whereas salicylate was a low-affinity inhibitor of rOat1 and rOat3, but weak inhibitors of RFC-1. Their $K_i$ values are summarized in Table 2. rOat1 and rOat3 were inhibited by NSAIDs with similar $K_i$ values, except sulindac and phenylbutazone, which preferentially inhibited rOat3 compared with rOat1 (Table 2).

The inhibitory effect of salicylate, diclofenac, indomethacin, phenylbutazone, ketoprofen, and naproxen on the uptake of MTX by kidney slices was examined (Fig. 8). These compounds inhibited the renal uptake of MTX in

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**Fig. 4.** Transport properties of rOat1- and rOat3-mediated uptake of MTX. Left, time profiles of 500 nM MTX in rOat1- (A) and rOat3- (C)-transfected cells. Open circles and closed squares represent rOat1/ rOat3-transfected and vector control cells, respectively. The right figures show the concentration dependence of rOat1- (B) and rOat3- (D)-transfected cells. Specific uptake of MTX was obtained by subtracting the transport velocity in vector control cells from that in rOat1- or rOat3-expressing cells. The inset represents a magnification of the low concentration region. The solid line represents the fitted lines obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).

**Fig. 5.** Transport properties of the uptake of MTX and 5-MTHF by kidney slices. A, uptake of MTX (500 nM, open circles), 5-MTHF (500 nM, open triangles), and mannitol (tracer, closed squares) was examined at 37°C. B, concentration dependence of MTX is shown as Eadie-Hofstee plots. The initial uptake rates of MTX by kidney slices for 15 min were measured at different concentrations. The solid line represents the fitted line obtained by nonlinear regression analysis. Inset, a magnification of the low concentration region. Each data point represents the mean ± S.E. (n = 3).
a concentration-dependent manner with two inhibition curves at relatively low concentrations and high concentrations of NSAIDs.

**Discussion**

In the present study, the drug-drug interactions between MTX and NSAIDs involving the renal uptake process were investigated focusing on the contribution of transporters, such as rOat1, rOat3, and RFC-1, all of which accept MTX as a substrate.

Significant uptake of MTX was observed in RFC-1-transfected cells, compared with vector controls (Fig. 1A). Eadie-Hofstee plot analysis revealed that RFC-1-mediated uptake of MTX apparently consisted of two saturable components (Fig. 1B), but the $K_i$ value of MTX for RFC-1-mediated up-

**Fig. 6.** Inhibitory effect of various compounds on the uptake of MTX (A) and 5-MTHF (B) by rat kidney slices. The uptake of [3H]MTX (500 nM) and [3H]5-MTHF (500 nM) by kidney slices for 15 min was determined in the presence and absence of inhibitors. The values are expressed as a percentage of the uptake in the absence of inhibitors. C, inhibitory effect of PCG (closed squares) and PAH (open circles) on the uptake of MTX by kidney slices. The uptake of [3H]MTX (500 nM) by kidney slices was determined in the presence of PCG and PAH at designated concentrations. The solid lines represent the fitted lines obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3). *, $P < 0.05$; **, $P < 0.01$, significantly different from control.

**Fig. 7.** Inhibitory effect of NSAIDs on the uptake of PAH, PCG, and MTX by rOat1, rOat3, and RFC-1, respectively. The uptake of [3H]PAH (500 nM), [3H]PCG (500 nM), and [3H]MTX (500 nM) by rOat1 (closed circles), rOat3 (open squares) and RFC-1 (open triangles), respectively, was examined in the presence and absence of unlabeled salicylate (A), diclofenac (B), indomethacin (C), phenylbutazone (D), ketoprofen (E), and naproxen (F). The uptake by rOat1 and rOat3 was determined at 5 min and RFC-1 at 1 min. The values are expressed as a percentage of the uptake in the absence of inhibitors. Solid lines represent the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).
The intracellular protein binding of MTX (Fig. 2C). The labeled MTX, which caused a significant reduction in the mediated transport of MTX separately from intracellular possible that the high-affinity component involves saturation low-affinity component (Fig. 1, B and C). Because MTX represented the mean $K$ take of 5-MTHF was comparable with the $K$ of RFC-1, and RFC-1-transfected cells, respectively, were examined. The $K$ values were determined by nonlinear regression analysis. Data were taken from Fig. 7. All values represent the mean ± S.D.

<table>
<thead>
<tr>
<th>NSAID</th>
<th>rOat1</th>
<th>rOat3</th>
<th>RFC-1</th>
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<tr>
<td></td>
<td>$\mu$M</td>
<td>$\mu$M</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>Salicylate</td>
<td>2110 ± 190</td>
<td>519 ± 132</td>
<td>2930 ± 660</td>
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<td>Diclofenac</td>
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N.D., not determined.

take of 5-MTHF was comparable with the $K$ value for the low-affinity component (Fig. 1, B and C). Because MTX strongly binds to intracellular dihydrofolate reductase, it is possible that the high-affinity component involves saturation of the intracellular binding of MTX. To evaluate the RFC-1-mediated transport of MTX separately from intracellular binding, RFC-1-transfected cells were preincubated with unlabeled MTX, which caused a significant reduction in the intracellular protein binding of MTX (Fig. 2C). The $K_m$ value calculated under preloaded conditions (18 $\mu$M) was almost the same as that of the low-affinity component (Figs. 1B and 2B). These results strongly support the hypothesis that the high-affinity component comes from intracellular protein binding, whereas the low-affinity component represents saturation of RFC-1-mediated transport of MTX. This phenomenon is not specific to MDCKII cells because two saturable components were also observed in the uptake by rOat1- and rOat3-transfected cells, and by kidney slices (Figs. 4, B and D, and 5B).

To demonstrate the inhibitor selectivity, the effect of folate derivatives and several organic anions on RFC-1-mediated transport of MTX was examined (Fig. 3, A and B). Under both untreated and preloaded conditions, the uptake of MTX by RFC-1+MDCKII cells was selectively inhibited by folate, whereas it had no effect on rOat1- and rOat3-mediated transport (data not shown). As summarized in Table 1, PAH has a higher affinity for rOat1 than rOat3, whereas PCG preferentially inhibits rOat3. Neither POG nor PAH showed any significant inhibitory effect on RFC-1 up to 1 mM (Fig. 3, A and B). In contrast to the report by Honscha et al. (2000), TCA did not show any inhibitory effect on RFC-1-mediated uptake of MTX (Fig. 3). Preincubation with nonradiolabeled MTX did not affect the inhibition profiles (Fig. 3, A and B), suggesting that the inhibitors used in this study do not affect the intracellular binding of MTX. Thus, the degree of inhibition by folate, PAH and PCG suggests a contribution of RFC-1, rOat1, and rOat3 to renal uptake of MTX.

To evaluate the contribution of each transporter to the renal uptake of MTX, the transport studies using kidney slices were carried out. Kidney slices provide us with great insight into renal physiology because it was previously suggested that uptake by kidney slices occur through the basolateral membrane (Wedeen and Weiner, 1973). Saturable uptake of MTX was observed in rat kidney slices, which exhibited a greater transport activity than 5-MTHF (Fig. 5A). 5-MTHF, folate, and leucovorin inhibited the uptake of MTX by kidney slices by 30% (Fig. 6A). Furthermore, MTX uptake by kidney slices was significantly inhibited by PCG and moderately by PAH with the $K$ values similar to their $K_m$ values for rOat3 (Table 1). Cimetidine was also used as a selective inhibitor to distinguish rOat3-mediated transport from rOat1-mediated transport because the uptake of PAH by rOat1 is not inhibited by cimetidine up to 1 mM (Nagata et al., 2002). Cimetidine also significantly inhibited the renal uptake of MTX by 30% at 1 mM. These results suggest that RFC-1 and rOat3 are involved in the uptake of MTX with their contribution being 30% and 30 to 50%, respectively, and rOat1 accounts for only a limited contribution. Considering that twenty-percent of the total MTX uptake remained even in the presence of 1 mM unlabeled MTX (Fig. 6A), the remaining fraction may be accounted for by passive diffusion and/or adsorption.

Unlike the uptake of MTX, the renal uptake of 5-MTHF was specifically inhibited by MTX and folate but not by PCG, cimetidine, or PAH, indicating the major contribution of RFC-1. The present study revealed that RFC-1 contributes to supplying plasma folate to the kidney epithelium and partially to the renal secretion of MTX. Although 5-MTHF and MTX have very similar chemical structures, the contribution of rOat3 to the renal uptake of 5-MTHF is minimal, if any. MTX exists as a divalent organic anion under physiological conditions, whereas 5-MTHF, which has two carboxyl groups and one amino group, exists as a zwitterion. The different charged conditions of folate derivatives may affect their recognition by rOat3.

Comparison of $K$ values of NSAIDs revealed that rOat1 and rOat3 exhibited a smaller $K$ values to all NSAIDs, except for salicylate, than RFC-1 (Table 2). A high concentration of salicylate was required to inhibit the transport activity by rOat1, rOat3, and RFC-1. NSAIDs, such as diclofenac, indomethacin, ketoprofen, phenylbutazone, and naproxen, which have been reported to cause severe drug-drug interactions with MTX (Adams and Hunter, 1976; Elinson and Servi, 1985; Thyss et al., 1986; Ng et al., 1987), showed a potent inhibitory effect on rOat3 but had only a minimal effect on RFC-1.

The inhibitory effect of several NSAIDs on the uptake of MTX by kidney slices was also examined (Fig. 8). In all cases, inhibition curves showed biphasic profiles. Focusing on indomethacin, it inhibited the uptake of MTX by kidney slices significantly over a concentration range of 0 to 10 $\mu$M and above 100 $\mu$M (Fig. 8C). Considering the results that indomethacin inhibited rOat3- and RFC-1-mediated transport with a $K_i$ of 1.3 and 490 $\mu$M, respectively (Table 2), it is conceivable that the biphasic profiles are ascribed to the inhibition of rOat3 and RFC-1, respectively. This interpretation can be applied to the inhibition curves for other NSAIDs, except naproxen, which exhibited a relatively larger $K_i$ value than expected from the $K_i$ value for rOat3 (Fig. 8F).

To examine whether the inhibition is clinically relevant, it is important to compare the $K_i$ of inhibitors with the clinical unbound concentrations. Because NSAIDs are highly bound to serum proteins, the unbound plasma concentrations of NSAIDs are very low, e.g., salicylate, 431 $\mu$M; ketoprofen, 0.0096 $\mu$M; phenylbutazone, and 12.5 $\mu$M; indomethacin, 8.4 $\mu$M (Tukeda et al., 2002). The $K_i$ of salicylate (520 $\mu$M),
phenylbutazone (8.5 μM), and indomethacin (1.3 μM) for rOat3 are comparable with the unbound concentration of these drugs, suggesting that these three drugs could inhibit rOat3 as well as hOAT3 in clinical situations (Takeda et al., 2002). Other NSAIDs are unlikely to inhibit the renal uptake of MTX in clinical situations although diclofenac, ketoprofen and naproxen have been reported to cause severe drug-drug interactions with MTX (Thyss et al., 1986; Ng et al., 1987).

The present study demonstrated that RFC-1, which is insensitive to NSAIDs, also contributes to the renal uptake of MTX, concomitantly with rOat3. Therefore, a dramatic reduction in the renal uptake of MTX may not be expected even in the presence of high concentrations of NSAIDs, such as salicylate, indomethacin, and phenylbutazone. Thus, whether RFC-1 is involved in the renal uptake of MTX in humans is an essential point when considering the mechanism of drug-drug interactions between MTX and NSAIDs. Further studies are necessary to reveal the contribution of individual human transporters.

Renal tubular secretion involves uptake from blood via the basolateral membrane followed by excretion into the lumen through the brush-border membrane of the proximal tubules. Therefore, inhibition of apical efflux transporters by NSAIDs may serve as an alternative interaction site with MTX. Moreover, several NSAIDs have been shown to be substrates of rOat1, suggesting that they are actively taken up by renal tubular cells (Apiwattanakul et al., 1999). It is possible that NSAIDs accumulate in renal tubular cells via basolateral organic anion transporter(s) and inhibit the excretion of MTX across the brush-border membrane, even although they do not affect the basolateral uptake of MTX. To date, OAT-K1, OAT-K2, Oatp1, Mrp2, and Mrp4 have been identified in the apical membrane of the kidney epithelium, and all of these have been reported to accept MTX as a substrate except Oatp1 (Russel et al., 2002). Recently, Reid et al. (2003) reported that MRP4-mediated transport of estradiol 17-β-D-glucuronide was inhibited by several NSAIDs, including indomethacin, ketoprofen, and diclofenac with a relatively high affinity, suggesting that MRP4 is a possible site of drug-drug interactions. Further studies are required to identify the transporter(s) responsible for the luminal efflux of MTX and to examine the inhibitory effect of NSAIDs on the luminal efflux of MTX.

In conclusion, the renal uptake of MTX is accounted for by rOat3 and RFC-1 with an equal contribution. rOat3/hOAT3-mediated uptake is a potential drug-drug interaction site for NSAIDs, and salicylate, indomethacin, and phenylbutazone could cause significant inhibition of the renal uptake of drugs predominantly accounted for by rOat3/hOAT3. In the case of MTX, RFC-1 is insensitive to NSAIDs as far as the clinical concentrations are concerned, and inhibition of renal uptake of MTX by NSAIDs is expected not to be extensive.

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References