Species Difference in the Inhibitory Effect of Nonsteroidal Anti-Inflammatory Drugs on the Uptake of Methotrexate by Human Kidney Slices

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Received February 14, 2007; accepted June 11, 2007

ABSTRACT

Simultaneous use of nonsteroidal anti-inflammatory drugs (NSAIDs), probenecid, and other drugs has been reported to delay the plasma elimination of methotrexate in patients. Previously, we have reported that inhibition of the uptake process cannot explain such drug-drug interactions using rats. The present study quantitatively evaluated the possible role of the transporters in such drug-drug interactions using human kidney slices and membrane vesicles expressing human ATP-binding cassette (ABC) transporters. The uptake of methotrexate by human kidney slices was saturable with a $K_m$ of 45 to 49 $\mu$M. Saturable uptake of methotrexate by human kidney slices was markedly inhibited by $p$-aminophenupurate and benzylpenicillin, but only weakly by 5-methyltetrahydrofolate. These transport characteristics are similar to those of a basolateral organic anion transporter (OAT) 3/SLC22A8. NSAIDs and probenecid inhibited the uptake of methotrexate by human kidney slices, and, in particular, salicylate, indomethacin, phenylbutazone, and probenecid were predicted to exhibit significant inhibition at clinically observed plasma concentrations. Among ABC transporters, such as BCRP/ABCG2, multidrug resistance-associated protein (MRP) 2/ABCC2, and MRP4/ABCC4, which are candidates for the luminal efflux of methotrexate, ATP-dependent uptake of methotrexate by MRP4-expressing membrane vesicles was most potently inhibited by NSAIDs. Salicylate and indomethacin were predicted to inhibit MRP4 at clinical plasma concentrations. Diclofenac-glucuronide significantly inhibited MRP2-mediated transport of methotrexate in a concentration-dependent manner, whereas naproxen-glucuronide had no effect. Inhibition of renal uptake (via OAT3) and efflux processes (via MRP2 and MRP4) explains the possible sites of drug-drug interaction for methotrexate with probenecid and some NSAIDs, including their glucuronides.

Drug-drug interactions involving metabolism and/or excretion processes prolong the plasma elimination half-lives leading to the accumulation of drugs in the body and potentiate pharmacological/adverse effects. 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 such transporters exhibit a broad substrate specificity with a degree of overlap, suggesting the possibility of transporter-mediated drug-drug interactions with other substrates (Shitara et al., 2005; Li et al., 2006).

Methotrexate (MTX) is an analog of natural folate and has been widely and successfully used for the treatment of neoplastic diseases and autoimmune diseases, including rheu-
matoid arthritis and psoriasis. However, when administered concomitantly with nonsteroidal anti-inflammatory drugs (NSAIDs) (Liegl et al., 1969; Ellison and Servi, 1985; Maiche, 1986; Thys et al., 1986; Ng et al., 1987; Tracy et al., 1992), penicillin antibiotics (Ronchera et al., 1993; Yamamoto et al., 1997; Titier et al., 2002), probenecid (Aherne et al., 1978), and ciprofloxacin (Dalle et al., 2002), the elimination of MTX from the systemic circulation was delayed or its pharmacokinetics was affected, sometimes resulting in severe adverse effects. Considering that MTX is largely excreted into the urine in unchanged form, the inhibition of renal tubular secretion has been considered as a site of drug-drug interactions.

Renal secretion of drugs is achieved by vectorial transport via the kidney epithelium of the proximal tubules, which consists of the uptake from blood via the basolateral membrane and the subsequent efflux into the lumen via the brush border membrane (BBM). MTX has been shown to be a substrate of basolateral organic anion transporters rOat1/Slc22a1 (Sekine et al., 1997; Hosoyaamada et al., 1999; Nozaki et al., 2004) and rOat3/hOAT3 (Slc22a8/SLC22A8) (Sekine et al., 1997; Hosoyamada et al., 1999; Nozaki et al., 2004) and rOat3/hOAT3 (Slc22a8/SLC22A8) (Cha et al., 2001; Nozaki et al., 2004). NSAIDs are inhibitors of rOat3 and exhibit significant inhibition of rOat3-mediated uptake at clinical plasma concentrations. We quantitatively investigated drug-drug interactions between MTX and NSAIDs using rat kidney slices (Nozaki et al., 2004). Unexpectedly, the MTX uptake was not markedly inhibited by NSAIDs in rat kidney slices because of the involvement of the NSAIDs-insensitive uptake mechanism, presumably reduced folate carrier (RFC)-1 (Cha et al., 2001; Nozaki et al., 2004). However, the possibility of interspecies differences could not be excluded. Indeed, the drug-drug interaction between famotidine and probenecid could not be reproduced in rodents because of an interspecies difference in the tissue distribution of OCT1 and the transport activity exhibited by hOAT3 (Tahara et al., 2005). Recently, we were able to obtain kidney slices from human intact renal cortical tissues removed from surgically nephrectomized patients with renal cell carcinoma and have demonstrated that they retain the transport activities of hOAT1 and hOAT3 (Nozaki et al., 2007). In the present study, the inhibitory effects of NSAIDs on the uptake of MTX by human kidney slices were examined to evaluate their clinical relevance.

In addition to the uptake process, it is also possible that NSAIDs and other inhibitors accumulate in the renal tubular cells by basolateral organic anion transporter(s) and inhibit the excretion of MTX across the BBM. To date, many kinds of transporters of organic anions have been identified on the apical side of the human kidney epithelium, including multidrug resistance-associated protein (MRP) 2, MRP4, breast cancer resistance protein (BCRP), OAT4, URAT1, and NPT1 (for review, see Russel et al., 2002). Hulot et al. (2005) identified a heterozygous mutation, which results in a loss of function of MRP2, in a patient who exhibited delayed MTX elimination. In addition, the pharmacokinetics of MTX was analyzed in Bcrp1 knockout mice. The area under the curve of the plasma concentration-time curve of MTX was approximately 2-fold higher in Bcrp1 knockout mice than in wild-type mice, whereas the amount of MTX excreted into the urine was unaltered (Breedveld et al., 2004). Therefore, the renal clearance of MTX, which is calculated by dividing the amount of MTX excreted into the urine by the area under the curve, was reduced in Bcrp1 knockout mice by approximately 50%. MRP4 is also expressed in the BBM of kidney proximal tubules and involved in the renal secretion of organic anions (Hasegawa et al., 2007; Imaoka et al., 2007). The present study examined the effect of NSAIDs and their glucuronide conjugates on the ATP-dependent uptake of MTX by MRP2-, BCRP-, and MRP4-expressing membrane vesicles.

Materials and Methods

Materials. [3H]MTX (25–29 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). [3H]-Aminohippurate (PAH; 4.1 Ci/mmol) and [3H]dehydropiandrostosterone sulfate (DHEAS; 60 Ci/mmoll were purchased from PerkinElmer Life Science (Boston, MA), and [14C]benzylpenicillin (PCG; 59 mCi/mmol) and [3H]-2,4-dichlorophenoxyacetate (2,4-D; 20 Ci/mmol) were obtained from GE Healthcare BioSciences (Waukesha, WI). Unlabeled MTX and 5-methyltetrahydrofolate (5-MTHF) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in the present study were of analytical grade and commercially available.

Preparation of Human Kidney Slices and Uptake of [3H]MTX by Human Kidney Slices. This study protocol was approved by the Ethics Review Boards at the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan) and Tokyo Women’s Medical University (Tokyo, Japan). All participants provided written informed consent.

Intact renal cortical tissues were obtained from five surgically nephrectomized patients with renal cell carcinoma at Tokyo Women’s Medical University between November, 2005 and January, 2006. Human kidney slices were prepared from kidney subjects; subsequently, the uptake of MTX and other substances by these human kidney slices was examined as described previously (Nozaki et al., 2007). The uptake of typical hOAT1 substrates (PAH and 2,4-D) and hOAT3 substrates (PCG and DHEAS) by human kidney slices was examined as positive controls and found to be comparable with previous results (Nozaki et al., 2007). Because the uptake of MTX by kidney slices apparently lasts for at least for 30 min (Fleck et al., 2002), the accumulation of MTX in human kidney slices for 15 min was used for the subsequent analyses.

Transport Studies in hOAT1- and hOAT3-Transfected HEK293 Cells. hOAT1- and hOAT3-transfected HEK293 cells were established as described previously (Tahara et al., 2005). HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin at 37°C with 5% CO2 and 95% humidity. HEK293 cells were seeded on 12-well plates at a density of 1.2 × 105 cells/well. Cells were cultured for 48 h with the above-mentioned medium and for an additional 24 h with culture medium supplemented with 5 mM sodium butyrate before the transport studies. Transport studies were carried out as described previously (Tahara et al., 2005). Uptake was initiated by adding Krebs-Henseleit buffer (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, pH 7.4) containing radiolabeled compounds in the presence or absence of inhibitors after cells had been washed twice and preincubated with buffer. The uptake was terminated at designated times by aspirating the incubation buffer and adding ice-cold buffer. Cells were washed twice with ice-cold buffer and dissolved in 500 μl of 0.2 N NaOH. The aliquots neutralized with 2 N HCl were transferred to scintillation vials containing 2 ml of scintillation cocktail (Clearsol; Nacalai Tesque Inc., Kyoto, Japan), and the radioactivities associated with the specimens were determined in a liquid scintillation counter.
The remaining 50-μl aliquot of cell lysate was used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard.

**Vesicle Transport Studies.** Membrane vesicles were prepared from HEK293 cells, which were infected with human BCRP-, Mrp2-, and Mrp4-recombinant adenosviruses, as described previously (Hasegawa et al., 2007; Imaoka et al., 2007). In brief, HEK293 cells were infected with recombinant adenosviruses containing human Mrp4 (10 multiplicity of infection (MOI)) and BCRP (2 MOI). As negative controls, cells were infected with a virus containing green fluorescence protein cDNA (10 MOI). Cells were harvested 48 h after infection, and membrane vesicles were isolated by the hypotonic method (Hasegawa et al., 2007; Imaoka et al., 2007). Cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, at 4°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotin. The cell lysate was centrifuged at 100,000g for 30 min at 4°C, and the resulting pellet was suspended in 10 ml of isotonic TS buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C) and homogenized in a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top on a 38% (w/v) sucrose solution in 5 mM Tris-HEPES, pH 7.4, at 4°C) and stirred gently for 1 h in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotin. The cell lysate was centrifuged at 100,000g for 30 min at 4°C, and the resulting pellet was suspended in 10 ml of isotonic TS buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C) and homogenized in a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top on a 38% (w/v) sucrose solution in 5 mM Tris-HEPES, pH 7.4, at 4°C and centrifuged in a Beckman SW41 rotor centrifuge at 280,000g for 45 min at 4°C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000g for 30 min at 4°C. The resulting pellet was suspended in 400 ml of TS buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle using a syringe. They were finally frozen in liquid nitrogen and stored at −80°C until required.

Vesicle transport studies were carried out as described in a previous report. In brief, the transport buffer (10 mM Tris, 250 mM sucrose, and 0.1 M NaCl2, pH 7.4) then was passed through a 0.45-μm HA filter (Millipore Corp., Bedford, MA), and the filter was washed twice with 5 ml of ice-cold stop solution. The radioactivity retained on the filter was measured in a liquid scintillation counter. The ATP-dependent uptake of ligands was calculated by subtracting the ligand uptake in the presence of AMP from that in the presence of ATP.

**Preparation of Diclofenac- and Naproxen-Glucuronides.** β-1-O-Glucuronides of diclofenac and S-naproxen were prepared bio-synthetically in vitro from the respective parent drugs using rat liver microsomes according to published methods (Iwaki et al., 1995) with slight modifications. In brief, a mixture containing 10 mg/ml microsomal protein, 0.1 M Tris-HCl buffer, pH 6.9, 10 mM MgCl₂, 20 mM β-glucuronic acid-1,4-lactone, 2 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 1 mM diclofenac or naproxen, and 10 mM UDP-glucuronic acid was incubated for 1.5 h at 37°C. The reaction was terminated by the addition of 5 volumes of acetonitrile, acidified immediately with acetic acid, and then centrifuged. The obtained supernatant was evaporated to remove organic solvent under reduced pressure at 30°C, and the residual aqueous phase was freeze-dried. The residue was redissolved in a minimal volume of acetonitrile per 50 mM acetic acid (10:90, v/v). The glucuronides in this solution were purified by liquid chromatography (30 × 1.5-cm i.d., Cosmosil 75C18-PREP; Nacalai Tesque) using a stepwise gradient (acetonitrile per 50 mM acetic acid, 10:90, 20:80, 30:70, and 50:50). Eluted glucuronide fractions were collected and freeze-dried. The identities of the glucuronides were confirmed by cleavage to the respective parent drugs with β-1-glucuronidase and 1 N NaOH. The purity of the glucuronides obtained was determined by analytical high-performance liquid chromatography and found to be homogeneous (>96%) at a UV wavelength of 254 nm, with the remaining fraction consisting of polar impurities that did not yield the respective parent drugs.

**Kinetic Analyses.** Kinetic parameters were obtained using the following Michaelis-Menten equations:

\[
\frac{v}{S} = \frac{V_{\max} \times S}{K_m + S}
\]  

Fig. 1. Concentration dependence of the uptake of MTX by human kidney slices. The concentration dependence of the uptake of MTX is shown as an Eadie-Hofstee plot. The uptake of MTX was measured at concentrations between 0.1 and 10,000 μM for 15 min at 37°C. A and B, data for human kidney slices prepared from subjects 1 and 2, respectively. Each point represents the results from one slice. Solid lines represent the fitted lines obtained by nonlinear regression analysis.

Fig. 2. Inhibitory effect of PAH, PCG, and 5-MTHF on the uptake of MTX by human kidney slices. The uptake of [3H]MTX (0.1 μM) was determined in the presence and absence of unlabeled PAH (open circles), PCG (closed circles), and 5-MTHF (open squares) for 15 min at 37°C. The values are shown as a percentage of the uptake in the absence of inhibitors. The present data were taken from those of subjects 3 and 4. Each point represents the mean ± S.E. (n = 6 slices).
one saturable, and one nonsaturable component,

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{dif}} \times S \]  

(2)

where \( v \) is the uptake velocity of the substrate (nanomoles per gram of kidney per 15 min or picomoles per milligram of protein per minute), \( S \) is the substrate concentration of medium (micromolar), \( K_m \) is the Michaelis constant (micromolar), \( V_{\text{max}} \) is the maximal uptake velocity (nanomoles per gram of kidney per 15 min or picomoles per milligram of protein per minute), and \( P_{\text{dif}} \) is the nonsaturable uptake clearance (milliliters per gram of kidney per 15 min).

The degree of inhibition (\( R \)) is expressed by the following equation:

\[ R = \frac{\text{CL}_{\text{inhibitor}}}{\text{CL}} = \frac{1}{1 + I/K_i} \]  

(3)

where \( \text{CL} \) represents the uptake clearance and \( \text{CL}_{\text{inhibitor}} \) represents the uptake clearance in the presence of inhibitor. \( I \) represents the concentration of inhibitor (micromolar). The substrate concentration was low compared with its \( K_m \) in the inhibition studies.

Fitting was performed by the nonlinear least-squares method using the MULTI program (Yamaoka et al., 1981). The input data were weighted as the reciprocals of the observed values, and the Damping Gauss Newton Method algorithm was used for fitting.

### Results

**The Uptake of Typical hOAT1 and hOAT3 Substrates by Human Kidney Slices.** The saturable uptake clearance

![Inhibitory effect of NSAIDs and other drugs on the uptake of MTX by human kidney slices. The uptake of MTX (0.1 μM) was determined in the presence and absence of unlabeled salicylate (A), diclofenac (B), indomethacin (C), ketoprofen (D), naproxen (E), phenylbutazone (F), probenecid (G), and ciprofloxacin (H) for 15 min at 37°C. The data of salicylate, indomethacin, probenecid, and ciprofloxacin were taken from subject 1, that of ketoprofen was from subject 3, that of diclofenac was from subject 4, and those of naproxen and phenylbutazone were from subject 5. Values are shown as a percentage of the uptake in the absence of inhibitors. Solid lines represent the fitted lines obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).**

**TABLE 1**

Quantitative evaluation of drug-drug interactions with MTX using human kidney slices

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Clinical Concentration</th>
<th>( I_u )</th>
<th>( K_i ) (μM)</th>
<th>( R ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>1100–2200μM</td>
<td>55–440</td>
<td>18.4 ± 8.6</td>
<td>0.040–0.25</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>3.6μM</td>
<td>&lt;0.018</td>
<td>26.6 ± 5.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.84–84μM</td>
<td>0.084–8.4</td>
<td>3.11 ± 1.58</td>
<td>0.27–0.97</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>12μM</td>
<td>0.0096</td>
<td>1.85 ± 0.96</td>
<td>1.0</td>
</tr>
<tr>
<td>Naproxen</td>
<td>&gt;517μM</td>
<td>0.851</td>
<td>14.3 ± 5.3</td>
<td>0.96</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>162–786μM</td>
<td>6.3–19.0</td>
<td>4.87 ± 1.4</td>
<td>0.20–0.44</td>
</tr>
<tr>
<td>Probenecid</td>
<td>170μM</td>
<td>18.7</td>
<td>0.171 ± 0.8</td>
<td>0.009</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>7.6μM</td>
<td>4.5</td>
<td>&gt;1000</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\( ^a \) Takeda et al. (2002).

\( ^b \) Riess et al. (1978).

\( ^c \) Brunton et al. (2006).
(milliliter per gram of kidney per 15 min, mean of duplicate determinations) of the typical substrates in subject 1 (PAH, 2.88; 2,4-D, 8.28; PCG, 3.87; DHEAS, 8.21), subject 2 (PAH, 1.69; 2,4-D, 6.19; PCG, 1.97; DHEAS, 5.10), subject 3 (PAH, 1.98; 2,4-D, 6.10; PCG, 1.95; DHEAS, 7.78), subject 4 (PAH, 1.25; 2,4-D, 9.11; PCG, 2.31; DHEAS, 5.18), and subject 5 (PAH, 1.48; 2,4-D, 5.83; PCG, 2.40; DHEAS, 7.55) was comparable with those in a previous report (Nozaki et al., 2007).

Characterization of the Uptake of MTX by Human Kidney Slices. The concentration dependence of the uptake of MTX was examined using human kidney slices, which were prepared from two different batches (Fig. 1, A and B). The uptake of MTX by two batches of human kidney slices consists of one saturable and one nonsaturable component, with $K_m$ values of 48.9 ± 17.3 and 44.6 ± 23.4 μM, $V_{max}$ of 70.2 ± 23.1 and 48.5 ± 24.1 μM, and $P_{dif}$ of 0.51 ± 0.048 and 0.515 ± 0.065 ml/g kidney per 15 min, respectively (mean ± S.D.).

Figure 2 describes the inhibitory effect of PAH, PCG, and 5-MTHF on the uptake of MTX by human kidney slices. PAH, PCG, and 5-MTHF (typical inhibitors of hOAT1, hOAT3, and RFC-1, respectively) inhibited the uptake of MTX in a concentration-dependent manner. PAH and PCG inhibited the saturable component of MTX uptake (49.5 ± 1.2 and 45.0 ± 4.8% of control at 1 mM, respectively), whereas the inhibitory effect of 5-MTHF was weak (65.0 ± 4.0% of control at 1 mM) (Fig. 2).

Inhibitory Effect of NSAIDs on the Uptake of MTX by Human Kidney Slices and hOAT1 and hOAT3. The effect of NSAIDs and other drugs was examined with regard to the uptake of MTX in human kidney slices (Fig. 3). Except for ciprofloxacin, the inhibitors inhibited MTX uptake in a concentration-dependent manner. The $K_i$ values are summarized in Table 1. The unbound plasma concentrations ($I_u$) at clinical dosages are taken from the literature, and, based on the $K_i$ values, the degree of inhibition in clinical situations ($R$) was predicted (Table 1). The inhibitory effect of NSAIDs on hOAT1- and hOAT3-mediated uptake was also examined, and the $K_i$ values of NSAIDs for hOAT1 and hOAT3 are summarized in Table 2.

ATP-Dependent Uptake of MTX by Human BCRP-, MRP2-, and MRP4-Expressing Vesicles. The uptake of MTX by human BCRP-, MRP2-, and MRP4-expressing vesicles and control vesicles was examined in the presence of ATP or AMP. The ATP-dependent uptake of MTX was significantly greater in BCRP-, MRP2-, and MRP4-expressing vesicles than that in control vesicles (Fig. 4, A–C, respectively). The concentration dependence of BCRP-, MRP2-, and MRP4-mediated transport of MTX was examined (Fig. 4, D–F, respectively), and their $K_m$ values were 5210 ± 500, 1540 ± 250, and 103 ± 5 μM, and their $V_{max}$ values were 74.1 ± 7.6, 21.2 ± 2.8, and 1.33 ± 0.06 nmol/mg protein per 5 min, respectively.

Inhibitory Effect of NSAIDs and Other Drugs on ATP-Dependent Transport of MTX via BCRP, MRP2, and MRP4. We examined the inhibitory effect of NSAIDs and other drugs on the ATP-dependent transport of MTX via BCRP, MRP2, and MRP4 (Fig. 5, A–C, respectively). BCRP-mediated transport of MTX was partially inhibited by indomethacin, phenylbutazone, diclofenac, and probenecid (Fig. 5A). MRP2-mediated transport of MTX was inhibited only by probenecid and stimulated in the presence of 1 μM phenylbutazone (Fig. 5B). Compared with BCRP and MRP2, MRP4 was more sensitive to the tested inhibitors (Fig. 5C), and indomethacin, ketoprofen, ibuprofen, naproxen, phenylbutazone, and salicylate inhibited the MRP4-mediated transport of MTX in a concentration-dependent manner, with $K_i$ values of 2.95 ± 0.76, 23.3 ± 6.8, 73.3 ± 20.9, 75.3 ± 19.7, 354 ± 54, and 218 ± 29 μM, respectively (mean ± S.D.) (data not shown). The clinical concentrations, plasma unbound concentrations, $K_i$ values calculated from in vitro vesicle transport studies, and $R$ values of inhibitors are summarized in Table 3. The inhibitory effect of diclofenac and naproxen glucuronides on BCRP-, MRP2-, and MRP4-mediated transport of MTX was also examined (Fig. 5, D–F, respectively). Diclofenac glucuronide significantly inhibited MRP2-mediated transport of MTX in a concentration-dependent manner, whereas BCRP and MRP4 were inhibited slightly or not at all by diclofenac and naproxen glucuronides.

Discussion

NSAIDs, penicillin, and other drugs have been reported to inhibit the renal tubular secretion of MTX, leading, in some cases, to lethal toxicity. The underlying mechanisms of the interactions remain to be elucidated. We previously investigated this interactions focusing on the uptake process using rat kidney slices and reported that the inhibitory effect of NSAIDs on the uptake of MTX by rat kidney slices was too weak to account for the drug-drug interactions by inhibition of the uptake process (Nozaki et al., 2004). In the present study, we re-evaluated the drug-drug interactions using human kidney slices and membrane vesicles expressing human ATP-binding cassette transporters.

The uptake of MTX by human kidney slices was saturable (Fig. 1). Nonlinear regression analysis revealed that the uptake of MTX in human kidney slices consists of one saturable component and one nonsaturable component, whereas the uptake in rat kidney slices consisted of three components (two saturable components and one nonsaturable component) (Nozaki et al., 2004). The $K_m$ value of MTX uptake in human kidney slices was comparable with that of the low-affinity component in rat kidney slices (77 μM). To identify the candidate transporter involved, inhibition studies were carried out. Although PAH and PCG exhibited different potentials.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>$K_i$ (μM)</th>
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<tbody>
<tr>
<td>Salicylate</td>
<td>407 ± 82</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>1.52 ± 0.07</td>
</tr>
<tr>
<td>Sulindac</td>
<td>77.8 ± 11.1</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>6.72 ± 2.2</td>
</tr>
<tr>
<td>Etoracolac</td>
<td>102 ± 22</td>
</tr>
<tr>
<td>Tolmetin</td>
<td>5.08 ± 0.48</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1.38 ± 0.48</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>0.890 ± 0.400</td>
</tr>
<tr>
<td>Naproxen</td>
<td>1.18 ± 0.60</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>71.6 ± 7.1</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.
with regard to the uptake of OAT1 and OAT3 substrates in human kidney slices (Nozaki et al., 2007), they inhibited the uptake of MTX with a similar potency in human kidney slices (Fig. 2). In addition, 5-MTHF weakly inhibited MTX uptake in comparison with PAH and PCG. It should be noted that PAH and PCG did not fully inhibit the saturable uptake of MTX by human kidney slices. Saturable uptake accounted for 75 and 67% of the net uptake, whereas almost 50% of the uptake remained in the presence of 1 mM of PAH or PCG.

The effect of NSAIDs and other drugs, all of which have caused drug-drug interactions with MTX in clinical situations, was examined using human kidney slices (Fig. 3). All the tested compounds, except ciprofloxacin, inhibited the uptake of MTX in human kidney slices in a concentration-dependent manner. Using \( K_i \) values determined in this study and the plasma unbound concentrations at clinical dosages, the degree of inhibition (\( R \) value) was predicted (Table 1). Among the tested compounds, the \( R \) values of salicylate, phenylbutazone, and probenecid were less than 1, suggesting that their inhibition is clinically relevant. In particular, probenecid is predicted to markedly inhibit the uptake of MTX in the kidney. Indomethacin has also the potential to inhibit the renal uptake of MTX at high clinical concentrations. It should be noted that the degree of inhibition by ketoprofen and probenecid was smaller than that by other drugs. Fifty percent of the uptake remained as the noninhibitable fraction for ketoprofen and probenecid, whereas the saturable fraction was almost completely inhibited by the other drugs (Fig. 3). Together with the partial inhibition by PAH and PCG, this suggests an involvement of multiple transporters in the uptake of MTX in human kidney slices, which exhibited different sensitivities to ketoprofen and probenecid. Because probenecid is a potent inhibitor of OAT1 and OAT3 (Tahara et al., 2005), the degree of inhibition by probenecid suggests a contribution of OAT1 and OAT3 to the net uptake. This is also supported by the fact that the inhibitable fraction by probenecid was comparable with that by PAH and PCG (Figs. 2 and 3G). Unlike the typical substrates (Nozaki et al., 2007), the inhibition profiles by PAH and PCG were similar and failed to clearly indicate the isoform involved in MTX uptake. Considering that the \( K_m \) value determined in the human kidney is similar to that for OAT3 (21 \( \mu \)M) rather than OAT1 (550 \( \mu \)M) (Takeda et al., 2002), it is likely that OAT3 makes a more significant contribution to the net uptake process.

There was an interspecies difference in the potency of inhibition by NSAIDs for the uptake of MTX in human and rat kidney slices. Unlike rodents, some drugs are predicted to inhibit significantly the renal uptake process of MTX in clinical situations. Two factors can account for this interspecies difference. Firstly, the contribution of OATs to the net uptake is greater in human than in rat kidney. Indeed, the PAH- and PCG-inhibitable fraction was greater in human kidney slices than in rat kidney slices (50 versus 30% in human and rat kidney slices).
kidney slices, respectively) (Nozaki et al., 2004; this study). Secondly, the NSAIDs, except for ketoprofen, inhibited the unknown transporter more potently in human kidney slices than in rat kidney slices. In particular, the $K_i$ value of salicylate determined in human kidney slices was smaller than that for OAT3 (Tables 1 and 2). These NSAIDs may be more potent inhibitors of this unknown transporter than OAT3. As suggested in rodents, RFC-1 is a candidate transporter. In addition, recently, proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) was also identified as a novel MTX transporter, which is also expressed in the kidney, at least, at the mRNA level (Qiu et al., 2006). This transporter may be another candidate transporter. Further studies are required to elucidate their importance.

Human kidney slice studies also suggested that diclofenac, ketoprofen, and naproxen do not inhibit the uptake of MTX at clinical concentrations, although they have caused drug-drug interactions with MTX in clinical situations (Thyss et al., 1986; Ng et al., 1987; Tracy et al., 1992; Davies and Anderson, 1997a). Because renal tubular secretion involves excretion into the lumen through the BBM of the proximal tubules, inhibition of apical efflux transporters can also serve as an alternative interaction site. Therefore, the effect of NSAIDs was examined for the ATP binding cassette transporters, such as MRP2, BCRP, and MRP4, which accept MTX as a substrate. ATP-dependent transport of MTX was observed in BCRP-, MRP2-, and MRP4-expressing vesicles (Fig. 4, A–C). The $K_m$ values of MTX for BCRP, MRP2, and MRP4 were consistent with previously reported values (Bakos et al., 2000; Mitomo et al., 2003; Volk and Schneider, 2003). The

**Table 3**
Quantitative evaluation of drug-drug interactions between MTX and NSAIDs via MRP4

Inhibitory effect of NSAIDs on MRP4-mediated transport of MTX was examined, and $K_i$ values were determined by nonlinear regression analysis. All $K_i$ values represent the mean ± S.D. Plasma unbound concentrations of the inhibitors ($I_u$) were calculated from the total plasma concentrations and unbound fractions. All $K_i$ values represent the mean ± S.D. Plasma unbound concentrations of the inhibitors ($I_u$) were calculated from the total plasma concentrations and unbound fractions.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Clinical Concentration</th>
<th>$I_u$</th>
<th>$K_i$</th>
<th>$R$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicylate</td>
<td>1100–2200</td>
<td>55–440</td>
<td>218</td>
<td>0.33–0.80</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>3.6</td>
<td>&lt;0.018</td>
<td>&gt;100</td>
<td>1.0</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.84–8.4</td>
<td>0.084–8.4</td>
<td>2.95</td>
<td>0.26–0.97</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>48.5</td>
<td>&lt;0.485</td>
<td>73.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>12</td>
<td>0.0096</td>
<td>23.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Naproxen</td>
<td>&gt;217</td>
<td>0.651</td>
<td>75.3</td>
<td>0.99</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>162–788</td>
<td>6.3–19.0</td>
<td>354</td>
<td>0.95–0.98</td>
</tr>
</tbody>
</table>

$a$ Takeda et al. (2002).

$b$ Riess et al. (1978).
effect of NSAIDs, probenecid, and PCG on the BCRP-, MRP2-, and MRP4-mediated transport of MTX was examined (Fig. 5, A–C, respectively). NSAIDs showed only a weak or minimal effect on MRP2 (Fig. 5B), which is consistent with a previous report (Horikawa et al., 2002). Because NSAIDs are mainly excreted into the urine as the glucuronide-conjugated form (Davies and Anderson, 1997a,b), we evaluated the inhibitory effect of diclofenac and naproxen glucuronide, which were prepared biosynthetically in vitro, on MRP2-mediated transport of MTX in a concentration-dependent manner (Fig. 5E). Therefore, this drug–drug interaction may involve inhibition of MRP2 by the glucuronide conjugate, but not the parent compound, although the clinical relevance of this inhibition remains unknown.

BCRP-mediated transport of MTX was significantly inhibited by 100 μM indomethacin and phenylbutazone and 1000 μM probenecid (Fig. 5A). However, such inhibition was not clinically relevant considering their unbound plasma concentrations in clinical situations. MRP4 is more susceptible to NSAIDs compared with BCRP and MRP2 (Fig. 5C), which agrees with very recently published results (El-Sheikh et al., 2007). Salicylate, indomethacin, ibuprofen, ketoprofen, naproxen, and phenylbutazone inhibited MRP4-mediated transport of MTX in a concentration-dependent manner, and the K_{i} values of these NSAIDs for MRP4 were generally comparable with some previous results with some exceptions. Salicylate exhibited no inhibition of MRP4-mediated MTX transport at a concentration of 100 μM (Fig. 5). Addition of experimental points at higher concentrations gave K_{i} values of 218 μM, although the K_{i} value was 7-fold smaller than the previously reported values for some unknown reason. Based on R values (Table 3), salicylate can be expected to inhibit MRP4-mediated transport at clinical doses, and indomethacin also has a potential to inhibit MRP4 at high clinical concentrations. Because several NSAIDs are substrates of OAT1 (Apiwattanakul et al., 1999), it is possible that NSAIDs, concentrated in the renal tubular cells by basolateral organic anion transporter(s), may exhibit a greater inhibition than expected from the plasma unbound concentrations. It must be kept in mind that the impact of the inhibition of MRP4 by salicylate and/or indomethacin on the renal elimination of MTX totally depends on the contribution of MRP4 to the net efflux across the BBM. It is required to evaluate the contribution of apical efflux transporters in the future for more reliable prediction.

In conclusion, the present study suggests that drug–drug interactions between MTX and salicylate, indomethacin, phenylbutazone, and probenecid involve inhibition of the uptake mediated by OAT3 and other unknown transporters. The transport studies using human kidney slices demonstrated an interspecies difference in the inhibition potencies of NSAIDs, indicating the importance of using human materials for the quantitative prediction of drug–drug interactions. As far as MRP4 is concerned, salicylate and indomethacin were predicted to have a significant effect in clinical situations. In addition to the parent compounds, drug–drug interactions may involve the inhibition of apical ATP-binding cassette transporters (MRP2 and MRP4) by glucuronide conjugates of NSAIDs.

References
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