Interactions of Nonsteroidal Anti-inflammatory Drugs with Rat Renal Organic Anion Transporter, OAT-K1

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ABSTRACT
We recently cloned and characterized the rat kidney-specific organic anion transporter, OAT-K1, which was suggested to mediate renal tubular transport of methotrexate. In this study, we investigated the interactions of nonsteroidal anti-inflammatory drugs (NSAIDs) with OAT-K1 by evaluating the effects of these drugs on renal distribution of methotrexate in vivo, and on methotrexate accumulation in the stably transfected LLC-PK1, cells expressing OAT-K1 (LLC-OAT-K1). NSAIDs such as indomethacin and ketoprofen had significant inhibitory effects on renal accumulation of methotrexate in rats after coadministration. Indomethacin and ketoprofen inhibited methotrexate accumulation by LLC-OAT-K1 cells in a competitive manner with the apparent inhibition constant values of 1.0 mM and 1.9 mM, respectively. Other NSAIDs including ibuprofen, flufenamate and phenylbutazone also showed potent inhibitory effects on methotrexate accumulation. However, indomethacin was not transported via OAT-K1. These results indicate that NSAIDs have potent inhibitory effects against the OAT-K1-mediated methotrexate transport, which suggests that the OAT-K1 may be one of interaction sites for methotrexate and NSAIDs in the kidney.

Methotrexate is widely used at high dosages in the treatment of malignancies and of other diseases such as psoriasis and rheumatoid arthritis at relatively low doses (Frei et al., 1975; Jackson, 1984; Bannwarth et al., 1996). In humans, methotrexate is eliminated almost entirely in an unchanged form in urine, which involves glomerular filtration and active tubular secretion (Shen and Azarnoff, 1978). Therefore, renal dysfunctions or drug interactions which reduce the clearance of methotrexate cause potential toxicity.

Methotrexate has been used effectively in combination with salicylates or NSAIDs for the treatment of various types of arthritis. However, interactions between methotrexate and NSAIDs have been reported with severe adverse effects after chemotherapeutic doses of methotrexate (Thysse et al., 1986; Brouwers and de Smet, 1994). The interactions may have been caused by protein binding displacement, inhibitory effects on the renal secretion of methotrexate and a decline of the glomerular filtration as a result of inhibition of prostaglandin synthesis (Tracy et al., 1992; Statkevich et al., 1993; Brouwers and de Smet, 1994; Kremer and Hamilton, 1995). Among these possible causes, the renal tubular secretion of methotrexate has been suggested as a major factor for the site of potential interactions (Frenia and Long, 1992). The tubular secretory pathway for methotrexate has been considered to be relatively nonspecific for a variety of organic anions including urate, p-aminophippurate, probenecid, salicylate and NSAIDs (Bourke et al., 1975; He et al., 1991; Statkevich et al., 1993). Nierenberg (1983) reported that several NSAIDs competitively inhibited the accumulation of methotrexate by rabbit kidney slices. Although transport system for weak organic acids has been considered for the tubular secretion of methotrexate, the transporter protein(s) responsible for the renal accumulation and/or secretion of methotrexate have not yet been identified.

We recently isolated cDNA encoding a rat kidney-specific organic anion transporter, designated OAT-K1 (Saito et al., 1996), which showed 72% amino acid identity with the oatp, an organic anion transporting polypeptide isolated from rat liver (Jacquemin et al., 1994). In the stably transfected renal epithelial cells expressing rat OAT-K1, methotrexate and folate, but not p-aminophippurate, were accumulated across the basolateral membranes (Saito et al., 1996). Nevertheless, the basolateral uptake of methotrexate by the rat OAT-K1-expressing cells was inhibited by typical substrates for the renal organic anion transport system, such as p-aminophippurate, probenecid, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (Saito et al., 1996). These findings suggest that diverse transporter proteins constitute the organic anion transport systems in the renal tubules, as for the hepatic

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; oatp, organic anion-transporting polypeptide.
anion transporters identified thus far, such as the Na\(^+\) gradient-dependent bile acid transporter (Hagenbuch et al., 1991; Meier, 1995) and the canalicular multispecific organic anion transporter (Paulusma et al., 1996), in addition to the oup.

In the present study, to clarify whether NSAIDs interact with the methotrexate transport via the OAT-K1 in the kidney, we examined the effects of NSAIDs on methotrexate accumulation by the stably transfected renal cells expressing OAT-K1.

Materials and Methods

Materials. [\(^{3}S,5^{7},7^{3}H\)]Methotrexate, sodium salt (285 GBq/mmol) was obtained from Amersham International (Buckinghamshire, UK). [\(^{14}C\)]Tetraethylammonium (124.3 MBq/mmol) and [\(^{14}C\)]indo- was obtained from Amersham International (Buckinghamshire, UK). [\(^{14}C\)]Tetraethylammonium (124.3 MBq/mmol) and [\(^{14}C\)]indo-

The radioactivity was determined in 5 ml of ACS II (Amersham) by liquid scintillation counting. The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Japan. All other chemicals used for the experiment were of the highest purity available.

Effects of NSAIDs on distribution of methotrexate in rats. Male Wistar rats (220–240 g) were anesthetized and administered tracer amounts of [\(^{3}H\)]methotrexate (2 nmol/kg; 0.7 kBq/ml) with or without 40 μmol/kg of unlabeled methotrexate, indomethacin in ketoprofen, as a bolus via catheterized left femoral vein. Five minutes after the administration, blood and specimens such as liver, kidney cortex and kidney medulla were collected immediately after sacrificing the rats. The excised tissues were gently washed with 0.9% NaCl, and the wet weight was determined. After homogenizing the tissues in 3 volumes of 0.9% NaCl, an aliquot (100 μl) of each sample and plasma was solubilized in 0.5 ml of NCS II (Amersham). The protein content of the solubilized cell monolayers with 1 N HCl was determined.

To determine whether indomethacin was transported into LLC-OAT-K1 cells, kinetic analysis by Dixon plots was performed (Dixon, 1953). As illustrated in figure 2, ibuprofen, flufenamate and phenylbutazone were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used for the experiment were of the highest purity available.

Uptake measurements. The cellular uptake of radioactive drugs by the LLC-PK1 cells expressing rat OAT-K1, designated LLC-OAT-K1, was measured using monolayer cultures grown in a Transwell chamber (Costar, Cambridge, MA) as described (Saito et al., 1996).

Incubations were done in 2 ml of solution at both apical and basolateral sides (for 24-mm diameter) or in at the apical (0.5 ml) and basolateral (1 ml) side (for 12-mm diameter). The incubation medium for the uptake experiments was Dulbecco’s phosphate-buffered saline (PBS buffer, 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 1 mM CaCl2, 0.5 mM MgCl2, pH 7.4) with 5 mM D-glucose.

The protein content of the solubilized cell monolayers with 1 N HCl was determined by the method of Bradford (1976), with a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA) with the bovine γ-globulin as a standard. The protein contents of the intact monolayers were 0.19 to 0.24 and 0.9 to 1.1 mg/filter in the 12- and 24-mm filters, respectively.

Statistical analysis. Data were analyzed statistically by one-way analysis of variance followed by Fisher’s t test. Probability values less than 5% were considered significant.

Results

Effects of indomethacin and ketoprofen on renal accumulation of methotrexate in rats. NSAIDs such as indomethacin, ibuprofen and phenylbutazone were reported to inhibit the accumulation of methotrexate in rabbit kidney slices (Nierenberg, 1983), which suggests that NSAIDs would have inhibitory effects on the renal clearance of methotrexate in vivo. To examine interactions of NSAIDs with methotrexate in the rat liver and kidney was determined after a bolus administration of a tracer amount of [\(^{3}H\)]methotrexate with or without unlabeled methotrexate, indomethacin or ketoprofen. As summarized in table 1, the plasma concentration of [\(^{3}H\)]methotrexate was increased significantly with the coadministration of unlabeled methotrexate, but not of indomethacin and ketoprofen. The hepatic accumulation of [\(^{3}H\)]methotrexate was not significantly affected by these drugs. In contrast, the [\(^{3}H\)]methotrexate accumulations in the kidney cortex and medulla were significantly inhibited by unlabeled methotrexate and indomethacin. Ketoprofen also showed a weak inhibitory effect on the accumulation of [\(^{3}H\)]methotrexate. These results suggest that indomethacin has a potent in vivo inhibitory effect on the methotrexate accumulation in the rat kidney, but not in the liver.

Effects of NSAIDs on methotrexate accumulation in LLC-OAT-K1 cells. Next, we examined methotrexate accumulation by LLC-OAT-K1 cell monolayers in the absence and presence of NSAIDs. As shown in figure 1, the methotrexate accumulation across the basolateral membranes of the monolayers was markedly inhibited by the presence of indomethacin or ketoprofen. To determine whether the inhibitory effects of indomethacin and ketoprofen were a common feature of NSAIDs, the effects of other NSAIDs used in clinical treatments on methotrexate accumulation were studied. As shown in figure 2, ibuprofen, flufenamate and phenylbutazone, but not salicylate, also caused a significant decrease in the accumulation of methotrexate.

To clarify the manner of the inhibitory effects of indomethacin and ketoprofen on methotrexate accumulation by the LLC-OAT-K1 cells, kinetic analysis by Dixon plots was performed (Dixon, 1953). As illustrated in figures 3, A and B, both indomethacin and ketoprofen inhibited methotrexate accumulation in a competitive manner with the apparent inhibition constant values (Ki) of 1.0 mM and 1.9 mM, respectively.

Accumulation of indomethacin by LLC-OAT-K1 cells. To determine whether indomethacin was transported by OAT-K1, the accumulation of indomethacin by mock-transfected cells (LLC-pBK) and LLC-OAT-K1 cells were measured. As summarized in table 2, no enhanced accumu-

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control</th>
<th>Methotrexate</th>
<th>Indomethacin</th>
<th>Ketoprofen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>3.3 ± 0.2</td>
<td>5.8 ± 0.4***</td>
<td>3.6 ± 0.1</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>14.0 ± 0.4</td>
<td>14.3 ± 1.3</td>
<td>10.7 ± 1.1</td>
<td>14.7 ± 1.1</td>
</tr>
<tr>
<td>Kidney cortex</td>
<td>74.5 ± 3.7</td>
<td>18.7 ± 2.3***</td>
<td>38.2 ± 3.1***</td>
<td>40.4 ± 4.1***</td>
</tr>
<tr>
<td>Kidney medulla</td>
<td>40.3 ± 3.5</td>
<td>29.6 ± 2.9*</td>
<td>28.9 ± 2.3*</td>
<td>36.7 ± 1.0</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± S.E. of three rats. * P < .05; *** P < .001, significant differences from control values without inhibitor.
The accumulation by LLC-OAT-K1 cells was found for indomethacin, except for methotrexate, when compared with those by LLC-pBK cells.

**Effect of NSAIDs on tetraethylammonium accumulation.** To reveal that the interactions of these NSAIDs with methotrexate in the LLC-OAT-K1 cells were specific for the OAT-K1 effects of NSAIDs on the accumulation of tetraethylammonium across basolateral membranes of the monolayers. We reported previously that tetraethylammonium was taken up by LLC-PK1 cells through organic cation transport system localized at the basolateral membranes (Saito et al., 1992). As summarized in table 3, indomethacin and ketoprofen had no significant effects on the tetraethylammonium accumulation. These results indicated that NSAIDs examined could not interact with the basolateral organic cation transporter.

### Discussion

Recently, we cloned and characterized cDNA encoding a rat organic anion transporter expressed specifically in the kidney, which was revealed to recognize and translocate both methotrexate and folate in the basolateral membranes of the renal epithelial cells expressing OAT-K1 (Saito et al., 1996). Western blot analysis with the antiserum for rat OAT-K1 showed that an immunoreactive protein with the apparent molecular mass of 70 kDa was detected in the LLC-OAT-K1 cells.
plasma membrane fractions, but not in those of LLC-pBK and host LLC-PK1 (data not shown).

The use of methotrexate as a highly effective drug in the short- and long-term treatments of rheumatoid and psoriatic arthritis has increased (Frei et al., 1975; Jackson, 1984; Bannwarth et al., 1996). Recently, methotrexate was used in combination with salicylate or NSAIDs for rheumatoid arthritis. Because the renal clearance of methotrexate comprises a major portion of systemic clearance, interactions reducing the methotrexate secretion would cause significant decreases in its clearance (He et al., 1991). Nierenberg (1983) reported that many NSAIDs, including indomethacin, phenylbutazone and flufenamide, as well as weak organic acids, such as probenecid and p-aminohippurate competitively inhibit the accumulation of methotrexate by rabbit kidney slices. Therefore, interactions of NSAI with the OAT-K1-mediated accumulation of methotrexate imply a special significance for pharmacological role of the transporter in the kidney.

The mechanisms of the interactions between methotrexate and NSAI have not been fully elucidated. Statkevich et al. (1993) reported using the isolated perfused rat kidney that tubular secretion was significantly inhibited by NSAI, such as indomethacin and flurbiprofen, after concomitant administration of methotrexate. The organic anion transport systems have been considered as a potential site for the interactions, but the precise mechanisms involved in the NSAI-caused inhibition of methotrexate secretion remain to be clarified. In the present study, various NSAI, such as indomethacin, ketoprofen, flufenamide and ibuprofen, caused marked depression in methotrexate accumulation by the OAT-K1-expressing cells. Indomethacin and ketoprofen inhibited the methotrexate accumulation in a competitive manner (fig. 3). However, methotrexate was not recognized by OAT-K1 as a substrate (table 2), which suggests that indomethacin has “pseudo competitive inhibitory effect” on methotrexate transport. Therefore, it could be assumed that OAT-K1 is the potential site of the interactions between NSAI and methotrexate, but the transporter would not serve as a secretory pathway for NSAI.

In conclusion, NSAI had potential inhibitory effects on methotrexate transport via rat OAT-K1. These data suggest that the OAT-K1 is involved in the potential interaction sites between methotrexate and NSAI in the kidney.

References


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