Inhibition of Biliary Excretion of Methotrexate by Probenecid in Rats: Quantitative Prediction of Interaction from in Vitro Data

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ABSTRACT

This study was designed to establish a strategy to predict drug interactions involving biliary excretion. The interaction between methotrexate and probenecid was examined as an interaction model since this interaction has already been clinically reported. Coadministration of probenecid reduced the biliary clearance of methotrexate in a dose-dependent manner in rats. This inhibition by probenecid was confirmed in vivo both in the uptake and excretion processes of methotrexate across sinusoidal and canalicular membranes, respectively. That is, both hepatic uptake clearance, assessed in integration plot analysis, and steady-state biliary clearance defined with respect to hepatic unbound methotrexate, were reduced in the presence of probenecid. Probenecid inhibited the active transport of methotrexate both in isolated hepatocytes and canalicular membranes, confirming the interaction at those two membranes. The degree of inhibition of the uptake and excretion processes found in vivo was comparable with the predicted values using the inhibition constant assessed in isolated hepatocytes and canalicular membranes, respectively. This suggests that the interaction at each membrane transport process can be quantitatively estimated from in vitro data. We have also proposed the method to predict the degree of inhibition of the net excretion from circulating plasma into the bile, the predicted values being also comparable with the inhibition actually found in vivo. The present analysis demonstrates a strategic rationale for predicting drug interactions involving biliary excretion using in vitro systems to avoid any false negative predictions.

The occurrence of drug interactions is one of the key factors in the clinical use of therapeutic agents. It is, therefore, of great importance to be able to predict such interactions by using simple experimental systems in vitro. Many attempts have been reported involving an extrapolation to predict a drug interaction via P450 metabolism in the liver based on data from in vitro microsomal studies (Bertz and Graneman, 1997; Ito et al., 1998a; Lin and Lu, 1998). However, there is little information about predicting drug interactions via the hepatic transporters, which are responsible for drug uptake and subsequent excretion in the liver. Recently, a variety of different types of xenobiotics and drugs (e.g., anticancer agents, angiotensin-converting enzyme inhibitors, quinolone antibiotics, endothelin antagonists, and 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) have been shown to be actively taken up and/or secreted in the liver (Stieger and Meier, 1998; Konig et al., 1999; Suzuki and Sugiyama, 1999). This suggests that drug interactions involving these transporters may occur in certain clinical situations. In actual fact, an interaction between digoxin and quinine, quindine, or verapamil has been demonstrated to involve biliary excretion in humans (Angelin et al., 1987; Hedman et al., 1990, 1991). Coadministration of bilirubin inhibits the systemic elimination of indocyanine green, which is eliminated mainly in the bile (Kanai, 1972).

To predict biliary excretion, at least three membrane transport processes, uptake and efflux at the sinusoidal membrane and excretion at the canalicular membrane, need to be considered. The degree of the inhibition of each process has to be separately examined and the obtained information should be combined, based on a mathematical model, to predict the “net” excretion from circulating plasma into bile. Both isolated hepatocytes and canalicular membrane vesicles (CMVs) have been widely used to analyze the uptake and excretion processes, whereas little information is available on an in vitro system for assessing sinusoidal efflux.

ABBREVIATIONS: CMV, canalicular membrane vesicle; HPLC, high-performance liquid chromatography; $f_p$, free fraction in plasma; $f_u$, free fraction in liver; $Cl_{total}$, total body clearance; $Cl_{bile,p}$, biliary clearance with respect to circulating plasma; $Cl_{bile,h}$, biliary clearance with respect to the liver concentration; $C_{ss,p}$, steady-state plasma concentration; $C_{ss,h}$, steady-state liver concentration; $V_{bile}$, biliary excretion rate; $Cl_{int,bile}$, intrinsic clearance for net biliary excretion; $P_{in}$, intrinsic clearance for hepatic uptake; $P_{ex}$, intrinsic clearance for the sinusoidal efflux; $P_3$, intrinsic clearance for biliary excretion across canalicular membrane; $Q_p$, hepatic plasma flow rate; $X_{liver}$, the amount of drug in the liver; AUC, area under the curve; $f_u, (plasma)$, unbound inhibitor concentration in plasma; $f_u, (liver)$, unbound inhibitor concentration in liver; $R_b$, blood-to-plasma concentration ratio; cMOAT/MRP2, canalicular multispecific organic anion transporter/multiresistance protein 2.
The purpose of the present study is to establish a rational methodology for predicting drug interactions involving biliary excretion from in vitro transport studies. Both isolated hepatocytes and CMVs were used to determine the intrinsic potential for an interaction involving hepatic uptake and biliary excretion at the sinusoidal and canalicular membranes, respectively. For the analysis of net excretion, we have proposed methods combining both types of potential inhibition. To demonstrate the validity of such in vitro/in vivo extrapolation, the drug interaction between methotrexate and probenecid, which has been reported in clinical situations (Aherne et al., 1978), was experimentally established in rats. Probenecid administration increased the plasma methotrexate concentration 2- to 3-fold (Aherne et al., 1978). Considering that the amount of methotrexate excreted into the bile was, at most, 30% of the intravenous dose in humans (Nuernberg et al., 1990), the interaction involving biliary excretion may not have a major impact on such an increase in systemic exposure to methotrexate. In fact, the urinary clearance of methotrexate, which accounts for approximately 70 to 90% of the total body clearance, fell when probenecid was coadministered (Aherne et al., 1978). However, these results cannot completely rule out the possibility of an interaction involving biliary excretion. In addition, in rats, biliary excretion is the major elimination pathway for methotrexate, and 72% of an intravenous dose was recovered in the bile (Masuda et al., 1997). Therefore, this interaction may be a useful model in rats. Both the hepatic uptake and biliary excretion clearance of methotrexate at the sinusoidal and canalicular sides, respectively, were directly analyzed in rats in vivo in the presence of probenecid to allow a comparison with the predicted values based on the in vitro data.

Experimental Procedures

Animals and Materials. Male Sprague-Dawley rats weighing 250 to 300 g (Nihon-ikagaku, Tokyo, Japan) were used throughout the experiments. All animals were treated humanely. The studies reported in this manuscript have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. [3',5',7',9'-H]Methotrexate, sodium salt ([3H]methotrexate, 6.50 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Methotrexate (Ametopterin), ATP, AMP, and probenecid were from Sigma Chemical Co. (St. Louis, MO).

Uptake of [3H]Methotrexate by Isolated Rat Hepatocytes. Hepatocytes were isolated from rats by the procedure described previously (Yamazaki et al., 1992). After isolation, the hepatocytes were suspended at 4°C in albumin-free Krebs-Henseleit buffer [5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 7H₂O, 12 mM HEPES, 5 mM glucose, 2 mM CaCl₂, 118 mM NaCl, 24 mM NaHCO₃, pH 7.3] to give a final concentration of 2 × 10⁶ cells/ml. Cell viability was routinely checked by the trypan blue (0.4% w/v) exclusion test (Yamazaki et al., 1992). Isolated hepatocytes with a viability of more than 90% were routinely used. Cellular protein was determined with Bio-Rad protein assay kit using bovine serum albumin as a standard (Yamazaki et al., 1992). The uptake of [3H]methotrexate was initiated by adding [3H]methotrexate and probenecid to the preincubated (3 min at 37°C) cell suspension. At designated times, the reaction was terminated by separating the cells from the medium by using a centrifugal filtration technique described previously (Yamazaki et al., 1992). The cells were dissolved into the alkaline solution, followed by neutralization, and the radioactivity was determined in scintillation cocktail (Clearsol I; Nacalai Tesque Inc., Kyoto, Japan).

Prediction of Drug Interaction at Biliary Excretion

The inhibition constant (Kᵢ) was obtained by fitting the following equation to the data:

\[ \frac{V_{\text{uptake}}}{V_{\text{uptake}}(\text{inhibitor})} = \frac{1}{1 + I/Kᵢ} \]  

where \( V_{\text{uptake}}(\text{inhibitor}) \) and \( V_{\text{uptake}}(\text{inhibitor}) \) represent the initial uptake rate of [3H]methotrexate in the presence and absence of inhibitor at a concentration of \( I \). This equation was derived based on the assumption of competitive or noncompetitive inhibition and the fact that the [3H]methotrexate concentration is much lower than the \( Kᵢ \) value of methotrexate uptake (under Results).

Uptake of [3H]Methotrexate by CMVs. CMVs were prepared as described previously (Masuda et al., 1997) and suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and were frozen in liquid N₂ and stored at −100°C until used. The uptake of [3H]methotrexate was measured by a rapid filtration technique as described previously (Masuda et al., 1997). The transport medium (250 mM sucrose and 10 mM MgCl₂ in 10 mM Tris-HCl buffer, pH 7.4) containing [3H]methotrexate and probenecid was preincubated for 3 min at 37°C in presence of 5 mM ATP or AMP and an ATP-generating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). The reaction was started by adding the vesicle preparation (10 μg) to the preincubated transport medium and further incubating it at 37°C. The uptake reaction was stopped by the addition of 1 ml of ice-cold stop buffer that contained 100 mM NaCl, 250 mM sucrose, and 10 mM Tris-HCl (pH 7.4). ATP-dependent uptake was determined by subtracting the uptake in the absence of ATP from that in the presence of AMP. The \( Kᵢ \) was obtained based on eq. 1.

Drug Interaction Studies in Vivo. For the simultaneous analysis of 1) the uptake and efflux at sinusoidal membrane, 2) excretion at the canalicular membrane, and 3) net biliary excretion from plasma into the bile, steady-state intravenous co-infusion of methotrexate and probenecid, and the subsequent bolus injection of [3H]methothrexate were performed in rats. Under the ether anesthesia, left and right femoral vein and left femoral artery were cannulated with polyethylene tube (PE-50; Clay Adams, Parsippany, NJ). Bile duct was also cannulated with PE-10. After an intravenous bolus injection (22 μmol/kg methotrexate; 0, 23, 45, 60, 80, or 90 μmol/kg probenecid; and 10 mg/kg inulin), methotrexate (0.164 μmol/min/kg), probenecid (0, 1.1, 2.2, 3.3, or 4.4 μmol/min/kg), and inulin (0.33 mg/min/kg) were simultaneously infused via the femoral vein. Blood was sampled via femoral artery at 60, 120, and 180 min and centrifuged (Microfuge E; Beckman Instruments, Fullerton, CA) to obtain plasma. Bile was sampled at 0 to 30, 30 to 90, 90 to 150, and 150 to 210 min. Both methotrexate and probenecid concentration was determined by HPLC. At 210 min after the start of infusion, [3H]methotrexate (22 μmol/kg) was administered as a bolus via the opposite side of femoral vein, and plasma was obtained at 15, 40, 60, and 80 s after the bolus injection. Approximately a 100-ng piece of the liver was obtained at 30 and 60 s by biopsy technique. At 90 s rats were sacrificed and the liver was resected for the determination of hepatic concentration of methotrexate, probenecid, and [3H]methotrexate. Bile was obtained at 0 to 30, 30 to 60, and 60 to 90 s. The radioactivity in plasma, bile, and the liver were determined by the liquid scintillation counter (LS 6000SE; Beckman Instruments).

Determination of Protein Binding in Plasma and Liver. Plasma (100 μl) obtained at 180 min during intravenous infusion was directly applied to MPS ultrafiltration tube (Millipore Corporation, Bedford, MA). The filter binding of methotrexate and probenecid was 2.59 and 6.24%, respectively. All binding was normalized with respect to the filter blank. The 25% (w/v) homogenate was prepared from the liver sample obtained at the end of in vivo study using a Teflon homogenizer (Iuchi, Tokyo, Japan) in 50 mM Tris-HCl (pH 7.4). This homogenate was then serially diluted by the same buffer to make 16.8 and 8.3% homogenates, and 1.0 ml of each was applied to Centrisart I 10,000D (Sartorius AG, Goettingen, Germany).
The ultrafiltration tube was then centrifuged at 800g for 5 min, and the methotrexate and probenecid concentration in the filtrate was determined by HPLC as unbound concentration. The filter binding of methotrexate and probenecid was 3.07 and 8.08%, respectively. All binding was normalized with respect to the filter blank. The free fraction in plasma (f_S) was determined as the ratio of unbound concentration in the filtrate to the total concentration. For the calculation of free fraction in liver (f_L), the bound concentration in each homogenate was first calculated by subtracting the free concentration from the total concentration, and the ratio of bound to free concentration at 100% homogenate concentration was then extrapolated by the linear regression of the plot of such ratio against the homogenate concentration. The f_L was calculated as the reciprocal of the sum of one plus the ratio of bound to free concentration.

**HPLC Determination.** The 25 μl of plasma or the bile diluted 10 times with H2O was deproteinized with 100 μl of 1 M HClO4 containing the internal standard (2.5 μg/ml aminopterin for methotrexate and 5.0 μg/ml sulfamethazine for probenecid), followed by centrifugation at 4°C and 10,000g for 10 min. One milliliter of 25% (w/v) liver homogenate was added to 1 ml of 1 M HClO4 containing the internal standard, followed by centrifugation at 4°C and 10,000g for 10 min. The supernatant (100 μl) was neutralized with 25 μl of 3 M K2HPO4, and 20 μl of the obtained sample was subjected to HPLC. The HPLC analysis for methotrexate and probenecid was performed according to previous reports (Nurenberg, 1989; Tellingen et al., 1989; Nurenberg et al., 1990; Nakamura et al., 1996) using Inertsil ODS-3 (250 × 4.6 mm, particle size 5 μm) column (Tosoh, Tokyo, Japan). The mobile phase consisted of acetonitrile:0.05 M phosphate buffer (pH 6.2) : 1.9 (v/v) for methotrexate and acetonitrile:0.01 M phosphate buffer (pH 7.4) = 1.5 (v/v) for probenecid with a flow rate of 1.0 ml/min. The UV detector was operated at a wavelength of 303 and 254 nm for methotrexate and probenecid, respectively. The detection limit for methotrexate and probenecid was 35 and 50 ng/ml plasma, 35 and 50 ng/ml bile, and 14 and 20 ng/g liver, respectively.

**Pharmacokinetic Analysis.** Total body clearance (CLtotal), biliary clearance with respect to circulating plasma (CLint,bile), and biliary clearance with respect to the liver concentration (CLint,liver) were calculated by the following equations:

\[
CL_{int,liver} = \frac{I}{C_{pass}} \quad (2)
\]

\[
CL_{int,bile} = \frac{V_{bile}}{C_{pass}} \quad (3)
\]

\[
CL_{int,bile,h} = \frac{V_{bile}}{C_{pass}} \quad (4)
\]

where I, Cpass, Vbile, and Cpass represent infusion rate (nmol/min/kg), plasma concentrations at steady state (μM), biliary excretion rate at steady state (nmol/min/kg), and hepatic concentration at steady state (μM), respectively. Cpass was determined as the mean values of the plasma methotrexate concentrations at 60, 120, and 180 min. Vbile was determined as the mean value of biliary excretion rate of methotrexate from 90 to 150 min and that from 150 to 210 min. Cpass was determined as the hepatic methotrexate concentration at the end of in vivo experiment. To calculate Cpass, the specific gravity of the liver was assumed to be unity. Thus, the amount in the liver (nmol/g liver) can be regarded as the hepatic concentration (μM), and the units of CLint,bile,h should be milliliters per minute per kilogram. For calculation of the hepatic uptake clearance (CLuptake), the biexponential equation was fitted to the plasma concentration-time profile for [3H]methotrexate (Cp) by means of a nonlinear iterative least-squares method using a MULTIT program (Yamaoka et al., 1981) and the area under the plasma concentration-time curve (AUC) was calculated from the integration of the exponential equation. The integration plot was obtained by plotting Xliver/Cp against AUC/Cp, the initial slope of this plot representing the CLuptake, where Xliver represents the amount of [3H]methotrexate once taken up by hepatocytes and calculated as the amount of [3H]methotrexate in the liver plus that recovered in bile. The input data for all the fitting were weighted as the reciprocal of the square of the observed values, and the algorithm used for the fitting was the Damping Gauss Newton method.

The intrinsic clearances for the net biliary excretion (CLint,bile,h), hepatic uptake across sinusoidal membrane (P1), and excretion across canalicular membrane (P3) was calculated based on the following equations using well stirred model:

\[
CL_{int,bile} = \frac{Q_f f_p CL_{int,bile,h}}{Q_f + f_p CL_{int,bile,h}(1 - \text{hematocrit})/R_b} \quad (5)
\]

\[
CL_{uptake} = \frac{Q_f f_p P_1}{Q_f + f_p}(1 - \text{hematocrit})/R_b \quad (6)
\]

\[
P_3 = CL_{int,bile,h}/f_L \quad (7)
\]

where Qf is the hepatic plasma flow rate and was set to be 34.8 ml/min/kg according to our previous data (Kato et al., 1999). The hematocrit is the hematocrit and was set to be 0.45. The Rb is the blood-to-plasma concentration ratio and was measured to be 0.83. The CLint,bile can be expressed as (Miyauchi et al., 1993):

\[
CL_{int,bile} = P_f P_3/(P_f + P_3) \quad (8)
\]

where P2 represents the intrinsic clearance of the efflux across the sinusoidal membrane from the liver.

**In Vitro/in Vivo Extrapolation.** The decrease in P1 by probenecid was predicted based on the following equation:

\[
R_{uptake} = P_1/(\text{inhibitor})/P_1 = 1/(1 + I_{u,plasma}/K_i) \quad (9)
\]

where Iu,plasma was the unbound concentration in the extracellular space and Ki was obtained using isolated hepatocytes. Since probenecid is a low-clearance drug, the probenecid concentration in the extracellular space was assumed to be equal to its concentration in circulating plasma. The decrease in P3 by probenecid was predicted by the following equation:

\[
R_{excretion} = P_3/(\text{inhibitor})/P_3 = 1/(1 + I_{u,liver}/K_i) \quad (10)
\]

In this case Iu,liver was the unbound probenecid concentration in the liver (f_LCpass) and Ki was obtained using CMVs.

For the analysis of the interaction involving net biliary excretion, the decrease in CLint,bile was estimated based on the following equation (see details under Discussion):

\[
CL_{int,bile} - \text{inhibitor}/CL_{int,bile} - \text{inhibitor} = R_{uptake} \times R_{excretion} \quad (11)
\]

As an alternative method, the decrease in CLint,bile was estimated from eq. 11 where Rexcretion was obtained using Iu,plasma instead of Iu,liver:

\[
R_{excretion} = P_3/(\text{inhibitor})/P_3 = 1/(1 + I_{u,plasma}/K_i) \quad (12)
\]

**Results**

**Drug Interaction Study in Vivo.** To demonstrate the in vitro/in vivo extrapolation, we first attempted to establish a model of the interaction in rats since in experimental animals we can directly determine the intrinsic clearance for the hepatic uptake and biliary excretion of methotrexate in both in vivo and in vitro systems, and compare the degree of the inhibition between the two systems. During co-infusion of methotrexate and probenecid, the plasma concentration profile and biliary excretion rate of methotrexate attained a steady state within 180 min (Fig. 1). Co-infusion of probenecid increased the plasma methotrexate concentration while reducing its biliary excretion rate (Fig. 1).

The pharmacokinetic parameters for methotrexate were calculated and shown in Table 1. The CLint,bile was 64% of CLtotal in the absence of probenecid (Table 1). Both CLtotal and CLint,bile were reduced by probenecid (Table 1). To directly
estimate the effect of probenecid on the excretion of methotrexate across the bile canalicular membrane, the $CL_{bile,h}$ was calculated and was also found to be reduced by probenecid in a dose-dependent manner (Table 1). Neither the $f_p$ nor $f_h$ of methotrexate was much affected by probenecid (Table 1). In the present study we also determined the urinary excretion rate of methotrexate. The sum of its biliary and urinary excretion rates accounted for approximately $102 \pm 3\%$ of the infusion rate in the absence of probenecid. Thus, the metabolism of methotrexate under these conditions should be minor and any potential effect of an interaction involving metabolism should be minimal as far as the biliary excretion of methotrexate is concerned.

To directly analyze the hepatic uptake of methotrexate, a tracer amount of $[3H]$methotrexate was administered when the plasma methotrexate concentration has reached steady state, followed by integration plot analysis for the hepatic uptake of $[3H]$methotrexate (Fig. 2). The initial slope of this plot was reduced by probenecid (Fig. 2). The obtained $CL_{uptake}$ was reduced in a probenecid dose-dependent manner. The $CL_{uptake}$ was almost comparable with $CL_{bile,p}$ in the absence of probenecid (Table 1).

Inhibition of $[3H]$Methotrexate Transport by Probenecid Both in Isolated Hepatocytes and CMVs. It has been reported that methotrexate is actively taken up by isolated rat hepatocytes (Horne et al., 1976; Gewirtz et al., 1984). In the present study, the uptake of $[3H]$methotrexate by isolated rat hepatocytes was found to be linear up to 3 min during the incubation (data not shown) and, therefore, the initial velocity of its uptake was assessed as the uptake at 2 min. Probenecid inhibits $[3H]$methotrexate uptake in a concentration-dependent manner. Considering that the $[3H]$methotrexate concentration in the medium (0.5 $\mu$M) was chosen so that it was much lower than its reported $K_m$ value (5.9 $\mu$M, Gewirtz et al., 1980; 23 $\mu$M, Honscha and Petzinger, 1999), the $K_i$ was estimated based on eq. 1 and found to be 180 $\mu$M (Fig. 3A). Such inhibition by probenecid was compatible with previous findings ($K_i = 100–200 \mu$M; Gewirtz et al., 1984).

It has also been reported (Masuda et al., 1997) that methotrexate excretion across the bile canalicular membrane is mainly mediated by a primary active transport system called the canalicular multispecific organic anion transporter (cMOAT/MRP2). The ATP-dependent uptake of $[3H]$methotrexate by rat CMVs was linear up to 3 min during incubation (data not shown) and, therefore, the initial velocity of uptake was assessed as the uptake at 2 min at a substrate concentration of 1.0 $\mu$M, which was much lower than the reported $K_m$ value (300 $\mu$M; Masuda et al., 1997). Probenecid inhibits the ATP-dependent $[3H]$methotrexate uptake in a concentration-dependent manner with a $K_i$ of 51.6 $\mu$M (Fig. 3B).

**Determination of Unbound Probenecid Concentration Both in Plasma and Liver.** For the extrapolation from in vitro to in vivo, we directly determined the unbound inhibitor (probenecid) concentration both in plasma and liver. The plasma probenecid concentration at 180 min after the start of the infusion increased in parallel with its infusion rate (Table 2). Neither the $f_p$ nor the $f_h$ of probenecid exhibited any clear dose-dependent change (Table 2), suggesting that its protein binding both in plasma and liver is almost linear. The unbound concentration of probenecid in the liver ($f_hC_{hiss}$) was lower than that in plasma ($f_pC_{pis}$) (Table 2).

<table>
<thead>
<tr>
<th>Probenecid $\mu$mol/min/kg</th>
<th>$CL_{total}$ $\text{ml/min/kg}$</th>
<th>$CL_{bile,p}$ $\text{ml/min/kg}$</th>
<th>$CL_{bile,h}$ $\text{ml/min/kg}$</th>
<th>$CL_{uptake}$ $\text{ml/min/kg}$</th>
<th>$f_p$</th>
<th>$f_h$</th>
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<tbody>
<tr>
<td>0</td>
<td>14.7 ± 1.0</td>
<td>9.43 ± 0.33</td>
<td>13.1 ± 0.3</td>
<td>11.9 ± 0.8</td>
<td>0.642 ± 0.026</td>
<td>0.217 ± 0.010</td>
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<tr>
<td>1.1</td>
<td>9.63 ± 0.19</td>
<td>5.43 ± 0.13</td>
<td>8.65 ± 0.64</td>
<td>8.75 ± 0.35</td>
<td>0.574 ± 0.005</td>
<td>0.287 ± 0.023</td>
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<td>2.2</td>
<td>8.18 ± 0.93</td>
<td>4.59 ± 0.10</td>
<td>6.98 ± 0.19</td>
<td>8.12 ± 0.41</td>
<td>0.590 ± 0.005</td>
<td>0.237 ± 0.019</td>
</tr>
<tr>
<td>3.3</td>
<td>6.94 ± 0.15</td>
<td>2.72 ± 0.11</td>
<td>6.29 ± 0.49</td>
<td>4.82 ± 0.23</td>
<td>0.539 ± 0.013</td>
<td>0.333 ± 0.022</td>
</tr>
<tr>
<td>4.4</td>
<td>6.37 ± 0.81</td>
<td>2.27 ± 0.13</td>
<td>6.14 ± 0.29</td>
<td>5.10 ± 0.35</td>
<td>0.582 ± 0.007</td>
<td>0.250 ± 0.024</td>
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</table>
uptake of $[3H]$methotrexate was determined for a 2-min incubation in the hepatocytes (A) and CMVs (B). In each experimental system, the initial $[3H]$methotrexate was plotted. The straight lines indicate the fitted lines in the presence or absence of probenecid. In B, the ATP-dependent uptake of $[3H]$methotrexate plotted. The initial slope in this plot represents the CL$_{uptake}$ of $[3H]$methotrexate. The values are expressed as means ± S.E. of four rats.

Extrapolation for Inhibition of Methotrexate Transport by Probenecid in Vivo from In Vitro Data. To assess methotrexate transport activity across sinusoidal and canalicular membranes, avoiding the effect of protein binding and plasma flow rate, the intrinsic clearances, $P_i$ and $P_3$, respectively, were calculated from the CL$_{uptake}$ and CL$_{bile,h}$ (Fig. 4). Based on eqs. 9 and 10, the reduction in $P_1$ and $P_3$, respectively, was predicted from the $K_i$ values assessed in vitro and the unbound probenecid concentration measured in vivo (Fig. 4). In each case, the predicted line was comparable with the actual data for $P_1$ and $P_3$ (Fig. 4).

Next, to assess the net biliary excretion activity of methotrexate from the extraacellular space to the bile, the intrinsic clearance (CL$_{int,bile}$) was calculated from CL$_{bile,p}$ (Fig. 5A). The reduction in CL$_{int,bile}$ by probenecid was predicted based on eq. 11, the predicted points being slightly lower than the actual data for CL$_{int,bile}$ (Fig. 5A). When the unbound probenecid concentration in plasma rather than in liver was used for the prediction, the predicted line was also slightly lower than the actual CL$_{int,bile}$ data (Fig. 5A). Similar results were obtained for the prediction of the organ clearance (CL$_{bile,p}$) as shown in Fig. 5B.

**TABLE 2**

Pharmacokinetic parameters of probenecid

<table>
<thead>
<tr>
<th>Probenecid</th>
<th>CL$_{total}$</th>
<th>$C_{\text{in}}$</th>
<th>$f_\phi$</th>
<th>C$_{\text{bile}}$</th>
<th>$f_\phi$</th>
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<tbody>
<tr>
<td>$\mu$mol/min/kg</td>
<td>ml/min/kg</td>
<td>$\mu$M</td>
<td></td>
<td>$\mu$M</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>4.11 ± 0.07</td>
<td>243 ± 4</td>
<td>0.243 ± 0.005</td>
<td>205 ± 12</td>
<td>0.204 ± 0.011</td>
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<tr>
<td>2.2</td>
<td>3.48 ± 0.04</td>
<td>633 ± 19</td>
<td>0.214 ± 0.008</td>
<td>420 ± 40</td>
<td>0.185 ± 0.040</td>
</tr>
<tr>
<td>3.3</td>
<td>4.69 ± 0.08</td>
<td>704 ± 12</td>
<td>0.258 ± 0.005</td>
<td>541 ± 23</td>
<td>0.230 ± 0.009</td>
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<tr>
<td>4.4</td>
<td>4.86 ± 0.15</td>
<td>905 ± 12</td>
<td>0.286 ± 0.005</td>
<td>831 ± 169</td>
<td>0.159 ± 0.035</td>
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</table>

**Discussion**

Compared with the prediction of drug interactions involving hepatic metabolism, the prediction of biliary excretion is a more complicated procedure because at least three membrane transport processes have to be considered to successfully predict the excretion. Therefore, we first attempted to demonstrate the prediction of a drug interaction involving each type of membrane transport based on the Michaelis-Menten equation (eqs. 9 and 10). These equations need both the $K_i$ for the inhibition of the transport system and unbound concentration of the inhibitor directly exposed to the transport system. To demonstrate the validity of the prediction method based on these equations, we obtained the former set of information in vitro (Fig. 3), whereas the latter information was obtained from in vivo experiments (Table 2). The predicted lines based on eqs. 9 and 10 were almost identical to the data obtained in vivo (Fig. 4), suggesting that drug interactions associated with membrane transport via sinusoidal and canalicular membranes can be quantitatively predicted based on the $K_i$ values obtained in isolated hepatocytes and CMVs, respectively, when the unbound concentration of the inhibitor is directly estimated both in plasma and liver in vivo.

It should be noted that there is still limited information about the in vitro system that can assess efflux transport on the sinusoidal membrane. Considering such difficulty, we have proposed the method to avoid false negative prediction, by using eq. 11, of the interaction involving net biliary excretion. This is because the most critical factor that should be avoided in such an approach is a false negative prediction. A false negative prediction is one that does not predict a positive interaction that actually exists in a clinical situation. The intrinsic clearance for the net biliary excretion (CL$_{int,bile}$) is a hybrid of the intrinsic clearances for each membrane penetration ($P_1$, $P_2$, and $P_3$) as shown in eq. 8. Therefore, the CL$_{int,bile}$ can be divided into two extreme cases: the case when $P_2 \ll P_3$, the CL$_{int,bile}$ should be equal to $P_3$, whereas in the case when $P_2 \gg P_3$, the CL$_{int,bile}$ should be $P_1 \times P_3/P_2$. To avoid any false negative predictions, we should only consider the latter case because, in the former case, the inhibition of only $P_1$ has to be considered, whereas in the latter case such inhibition has to be considered for both $P_1$ and $P_3$. If the inhibitor drug also reduces $P_2$, the CL$_{int,bile}$ should be in-
creased and, therefore, $P_2$ does not need to be considered if we want to avoid any false negative predictions. Thus, in all cases, the reduction in $CL_{int,bile}$ should be, at most, the reduction in $P_1$ (sinusoidal uptake) multiplied by that in $P_3$ (canalicular efflux) as expressed in eq. 11. In actual fact, by performing such a multiplication, the predicted values in $CL_{int,bile}$ were only slightly less than the actual values (Fig. 5A), suggesting that this method is suitable for avoiding any false negative predictions. Note that the predicted value based on this method was always lower than the actual $CL_{int,bile}$ value (Fig. 5A). This is reasonable when we consider that the $CL_{uptake}$ was almost comparable with the $CL_{bile,p}$ in the control (Table 1), which means that the rate-limiting step in the net biliary excretion of methotrexate is its uptake ($P_2 \ll P_3$), and therefore, the $CL_{int,bile}$ can be approximated as $P_1$.

In this method, however, the critical point in humans is the estimation of $I_u,liver$ by sampling the liver and measuring the unbound inhibitor concentration. In addition, it is quite difficult to demonstrate that the $f_u$ estimated using liver homogenate as in the present study is really the same as the unbound fraction in the liver in vivo although no really suitable methods for the determination of the unbound fraction in organs/tissues have been reported to date. Therefore,
we used $I_{u,\text{plasma}}$ instead of $I_{u,\text{liver}}$ to predict the $R_{\text{excretion}}$ value (eq. 12). Also, in such a case, the predicted line was not very different from the actual values (Fig. 5A), supporting the validity of this method. For inhibitors other than probenecid, however, $I_{u,\text{liver}}$ may be much higher than the $I_{u,\text{plasma}}$ due to its concentrative uptake by hepatocytes. Kanamitsu et al. (2000) proposed a method to determine such differences between $I_{u,\text{plasma}}$ and $I_{u,\text{liver}}$ by using isolated hepatocytes treated with ATP depletors or left untreated.

If the inhibitor drug is administered orally, its concentration in the extracellular space may be higher than that in the circulation because of the hepatic first-pass effect. To estimate the extracellular inhibitor concentration, Ito et al. (1998a,b) proposed using the maximum value for the unbound inhibitor concentration in the portal vein by considering the supply from the circulating plasma as well as the drug absorbed from gastrointestinal lumen following oral administration (Ito et al., 1998a,b).

Unbound inhibitor concentration in the portal vein ≤

\[
\frac{(\text{Maximum inhibitor concentration in circulating blood})}{(\text{Hepatic blood flow rate})} \times (\text{Fraction of the dose absorbed}) / (\text{Absorption rate constant} \times \text{Dose})
\]

To evaluate the suitability of this equation for predicting transporter-mediated interactions, we applied it to the well known digoxin-quinidine interaction (Hedman et al., 1990) where quinidine reduced the biliary clearance of digoxin to approximately half the control value in humans. Since, in that study, quinidine (400 mg) was administered orally and the maximum inhibitor concentration in circulating plasma was reported to be 4.5 μM (Hedman et al., 1990), the estimated maximum value for the unbound inhibitor concentration in the portal vein was 5 μM where the unbound fraction in plasma, the absorption rate constant, the fraction of the dose absorbed, the hepatic blood flow rate and $R_b$ were assumed to be 0.08, 0.1 min⁻¹, 1, 1600 ml/min, and 1. Since the $K_i$ for the inhibition of digoxin uptake by quinidine in isolated human hepatocytes is over 50 μM (Olinga et al., 1998), whereas the $K_m$ of quinidine for P-glycoprotein is 5 μM (Muller et al., 1994), the predicted biliary clearance based on eq. 11 using the maximum value for the unbound inhibitor concentration in the portal vein was 50% of the control in the presence of quinidine. If the unbound inhibitor concentration in circulating blood was used instead of the maximum value for the unbound inhibitor concentration in the portal vein, the predicted value was 93% of the control. Thus, this calculation supports the validity of the prediction based on eq. 11 using the maximum value for the unbound inhibitor concentration in the portal vein, although further examples demonstrating such validity are needed before being able to draw a final conclusion.

Finally, to confirm the validity of this prediction approach, identification of transporters responsible for methotrexate excretion should also be important. Methotrexate excretion across the canicular membrane is reported to be mainly mediated by cMOAT/MRP2 (Masuda et al., 1997) since its excretion is greatly reduced in cMOAT/MRP2-deficient rats. On the other hand, its uptake mechanism by isolated hepatocytes is still controversial. Methotrexate is a folate analog and potently inhibits the uptake of folates by isolated rat hepatocytes (Horne et al., 1978), whereas the inhibition of methotrexate uptake by folates was only minimal at folate concentrations greater than the $K_m$ for folate uptake (Gewirtz et al., 1980). A pH dependence was clearly evident in the uptake of folates, whereas this was not as marked for methotrexate uptake (Horne, 1990). Thus, the uptake mechanism of methotrexate would appear to differ to some extent from that of folates. Honscha et al. (2000) recently cloned a transporter, RL-MTX-1, which may be important as a Na⁺-dependent transporter for methotrexate.

In summary, our data in rats suggest that drug interactions associated with membrane transport via the sinusoidal and canalicular membranes can be quantitatively predicted based on the $K_i$ values obtained in vitro and the unbound concentration of the inhibitor drug in the circulating plasma and the liver, respectively. To predict the degree of inhibition of net biliary clearance, the inhibition of each membrane transport process needs to be combined to avoid false negative predictions.

References


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