Species Differences in the Transport Activity for Organic Anions across the Bile Canalicular Membrane

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ABSTRACT
Species differences in the transport activity mediated by canalicul multispecific organic anion transporter (cMOAT) were examined using temocaprilat, an angiotensin-converting enzyme inhibitor whose biliary excretion is mediated predominantly by cMOAT, and 2,4-dinitrophenyl-S-glutathione, a typical substrate for cMOAT, in a series of in vivo and in vitro experiments. Temocaprilat was infused to examine the biliary excretion rate at steady-state. The in vivo transport clearance values across the bile canalicular membrane, defined as the biliary excretion rate divided by the hepatic unbound concentrations, were 9.8, 39.2, 9.2, 1.1, and 0.8 ml/min/kg for mouse, rat, guinea pig, rabbit, and dog, respectively. The \( K_m \) and \( V_{\text{max}} \) values for ATP-dependent uptake of 2,4-dinitrophenyl-S-glutathione into canalicular membrane vesicles were 15.0, 29.6, 16.1, 55.8, and 30.0 \( \mu \)M and 0.38, 1.90, 0.15, 0.47, and 0.23 nmol/min/mg protein, yielding the in vitro transport clearance across the bile canalicular membrane (\( V_{\text{max}}/K_m \)) of 25.5, 64.2, 9.4, 8.4, and 7.7 for mouse, rat, guinea pig, rabbit, and dog, respectively. A close in vivo and in vitro correlation was observed among animal species for the transport clearance across the bile canalicular membrane. These results suggest that the uptake experiments with canalicular membrane vesicles can be used to quantitatively predict in vivo excretion across the bile canalicular membrane.

Early knowledge of the human pharmacokinetics of new drug candidates is of major importance at several stages of the development process, for the selection of compounds, as well as for the design of the initial clinical protocol. To extrapolate pharmacokinetic parameters from animal to human, interspecies scaling based on allometric procedures and on physiologically based models has been used (Dedrick et al., 1970; Iwatsubo et al., 1997; Ito et al., 1998b). Such an approach has been successfully applied, in particular to the scaling of hepatic metabolism and urinary excretion. Recently, by comparing the metabolic activity of the cDNA product of P-450 isozymes cloned from animal species, the molecular basis for the presence of interspecies differences in the metabolic activity has been established (Ito et al., 1998b).

In contrast to these elimination processes, only a few reports are available that describe species differences in the biliary excretion of xenobiotics; Chatfield and Green (1978) examined the biliary excretion of benoxaprofen in five animal species and concluded that there is a marked species difference in its route of excretion. In addition, Duggan et al. (1975) reported that the biliary excreted amount of indomethacin and its metabolites also varied among animal species, whereas there is a good correlation among them between total biliary excretion of indomethacin and intestinal lesions, which is induced by indomethacin and/or its metabolites. Using the isolated canalicular membrane vesicles (CMVs), it has been recently shown that excretion across the bile canalicular membrane is mediated by several kinds of ATP-binding cassette transmembrane transporters (Suzuki and Sugiyama, 1998; Müller and Jansen, 1997; Keppler and König, 1997). These include multidrug resistance 1 (MDR1) and MDR2, which are predominantly responsible for the excretion of amphipathic cationic and neutral compounds (such as vinca alkaloids) and phospholipids (such as phosphatidyl choline), respectively. Bile salt export pump (BSEP)

**ABBREVIATIONS:** CMV, canalicular membrane vesicles; cMOAT, canalicular multispecific organic anion transporter; SD, Sprague-Dawley; EHBR, Eisai hyperbilirubinemic rat; DNP-SG, 2,4-dinitrophenyl-S-glutathione; ALP, alkaline phosphatase; LAP, leucine aminopeptidase; γ-GT-Pase, γ-glutamyl transpeptidase; GST, glutathione S-transferase; CL\(_{\text{Lipid,plasmal}}\), biliary excretion clearance defined by lipid concentration; CL\(_{\text{Unbound,plasmal}}\), biliary excretion clearance defined by lipid unbound concentration; CL\(_{\text{Lipid,plasmal}}\), biliary excretion clearance defined by liver concentration; CL\(_{\text{Unbound,Liver,p}}\), biliary excretion clearance defined by liver unbound concentration; \( C_{\text{plasmal}} \), plasma concentration; \( C_{\text{Lipid,Liver,p}} \), liver concentration; \( C_{\text{Unbound,Liver,p}} \), liver unbound concentration; \( f_{\text{plasmal}} \), the plasma unbound fraction; \( f_{\text{Liver,p}} \), the liver unbound fraction; MRP, multidrug resistance-associated protein; BSEP, bile salt export pump.
mediates the biliary excretion of bile acids (such as taurocholate) (Gerloff et al., 1998), and canalicular multispecific organic anion transporter (cMOAT) is responsible for the biliary excretion of many organic anions (Oude Elferink et al., 1995, 1997; Yamazaki et al., 1996; Keppler and Köng, 1997; Kusuhara et al., 1998). By comparing the transport activity across the bile canalicular membrane between normal and mutant rats whose cMOAT expression is hereditarily defective [such as TR− and Eisai hyperbilirubinemic rats (EHBR)], the substrate specificity of cMOAT has been determined (Oude Elferink et al., 1995; Keppler and Köng, 1997; Kusuhara et al., 1998; Suzuki and Sugiyama, 1998). These include clinically important drugs such as temocaprilat (an angiotensin-converting enzyme inhibitor), pravastatin (a 3-hydroxy methylglutaryl-CoA reductase inhibitor), methotrexate and irinotecan, along with conjugated metabolites (such as glucuronide conjugates (e.g., bilirubin glucuronides) and glutathione conjugates (e.g., leukotriene C4 and glutathione disulfide) (Suzuki and Sugiyama, 1998). Recently, cDNA cloning and functional analysis of the cloned cDNA product have been performed on cMOAT (Büchler et al., 1996; Paulusma et al., 1996; Ito et al., 1996, 1997, 1998a; Madon et al., 1997; Evers et al., 1998; van Aube et al., 1998). Interspecies differences in the activity of this transporter are not fully understood.

The purpose of the present study is to investigate interspecies differences in the transport of anionic drugs across the bile canalicular membrane. Temocaprilat was used as a model compound, because its biliary excretion is predominantly mediated by cMOAT (Ishizuka et al., 1997). The efficient biliary excretion of temocaprilat provides pharmacokinetic advantage, particularly in the treatment of patients with renal failure (Suzuki et al., 1993). We have previously suggested that the efficient biliary excretion of temocaprilat, compared with other angiotensin-converting enzyme inhibitors, is due to its much higher affinity for cMOAT rather than a difference in the ability to be taken up by hepatocytes across the sinusoidal membrane (Ishizuka et al., 1997, 1998). In the present study, we examined the interspecies differences in the transport activity of organic anions across the bile canalicular membrane in a series of in vivo and in vitro experiments in mouse, rat, guinea pig, rabbit, and dog.

**Experimental Procedures**

**Materials.** [3H]Temocaprilat (7.7 Ci/mmol) was synthesized by Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan), whereas unlabeled temocaprilat was synthesized by Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan), whereas unlabeled temocaprilat was synthesized in our laboratory. [3H]2,4-Dinitrophenyl-S-glutathione ([3H]DNPS-G) was synthesized enzymatically using [glycine-2-3H]glutathione (DuPont-New England Nuclear Corp., Boston, MA), 1-chloro-2,4-dinitrobenzene, and glutathione S-transferase (GST) according to the method described previously (Kobayashi et al., 1990). Unlabeled DNPS-G was synthesized chemically by a procedure based on a method previously reported (Saxena and Henderson, 1995), and its purity was checked by HPLC (more than 99%). [3H]Taurocholate was purchased from DuPont-New England Nuclear Corp. ATP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals used were commercially available and reagent grade products. Male ddY mouse, Sprague-Dawley (SD) rats and Hartley guinea pigs were purchased from SLC Co., Ltd. (Shizuoka, Japan). Japanese white rabbits and beagle dogs were purchased from Shiraishi Animal Laboratories (Tokyo, Japan) and Nihon Nosan K.K. (Kanagawa, Japan), respectively. Animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co., Ltd. (Tokyo, Japan).

In Vitro Transport Experiment Using CMVs. CMVs, prepared as previously reported (Kobayashi et al., 1990), were suspended in 50 mM Tris buffer (pH 7.4) containing 250 mM sucrose. Enrichment of marker enzymes [alkaline phosphatase (ALP), leucine aminopeptidase (LAP) and γ-glutamyl transpeptidase (γ-GT Pase)] in CMVs compared with the liver homogenate was determined using p-nitrophenylphosphate, L-leucyl-p-diethylaminolinaldil, and L-γ-glutamyl-p-N-ethyl-N-hydroxyethylaminolinaldil as substrates, respectively. In addition, the orientation of the CMVs was determined by examining the nucleotide pyrophosphatase in the absence and presence of 0.2% of Triton X-100 (Böhme et al., 1994).

The transport study was performed using the rapid filtration technique described in a previous report (Ishikawa et al., 1990). Transport medium (10 mM Tris, 250 mM sucrose, 10 mM MgCl2, 6H2O, pH 7.4) containing radiolabeled compound (35 µl), with or without unlabeled substrate, was preincubated at 37°C for 3 min and then rapidly mixed with 5 µl of CMV suspension (10–20 µg protein), with or without 5 mM ATP and ATP-regenerating system (10 mM creatine phosphate, 100 µg/ml creatine phosphokinase). The uptake of glutathione conjugates was determined in CMVs pretreated with acivicin (1 mM), an irreversible inhibitor of γ-GTPase, for 30 min (Ballatori and Dutczak, 1994; Niinuma et al., 1999). The transport reaction was stopped by the addition of 1 ml ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4). The stopped reaction mixture was filtered through a 0.45-µm GVWP filter (Millipore Corp., Bedford, MA) and then washed twice with 5 ml of stop solution. Radioactivity retained on the filter was determined using a liquid scintillation counter (LSC-3500; Aloka Co., Tokyo, Japan). Uptake was normalized with respect to the amount of membrane protein.

**Estimation of Biliary Excretion Clearance by In Vivo Infusion.** The animals were anesthetized with i.p. pentobarbital, and bile specimens were collected via the common bile duct with gallbladder isolated. [3H]Temocaprilat with unlabeled temocaprilat was infused i.v. via the femoral or jugular vein and, at each time point, blood was collected by heparinized syringe and the plasma immediately separated by centrifugation. Bile specimens were collected into preweighed tubes at the specified intervals. The radioactivity of plasma and bile samples was measured by scintillation spectrophotometer (LSC-3500; Aloka Co.).

After the in vivo experiments, liver homogenate (16 or 33% w/v) in PBS (pH 7.4) was prepared to determine the liver binding of temocaprilat. Liver homogenates were centrifuged through an MPS-3 membrane (Amicon Division, W. R. Grace & Co., Beverly, MA) at 3000 rpm for 10 to 20 min. The buffer containing [3H]temocaprilat was also centrifuged to determine the recovery of the ligands through the membrane (−90%). The unbound fraction (fu,homogenate) was calculated as follows:

\[
\text{fu, homogenate} = \frac{C_{\text{filtrate}}}{C_{\text{homogenate}}}R
\]  

(1)

where \(C_{\text{filtrate}}\) and \(C_{\text{homogenate}}\) denote the concentration in the filtrate after ultrafiltration and that in the liver homogenate, respectively. \(R\) denotes the recovery determined in the control experiment (\(R = 0.9\)). The value of the unbound fraction in liver (fu,liver) was obtained by extrapolation from the observed values (fu,homogenate 16 or 33%) as follows:

\[
\text{fu,liver} = \text{fu, homogenate}(16 \text{ or } 33%) \rho (d \cdot (1 - \text{fu, homogenate}(16 \text{ or } 33%))
\]  

(2)

where \(d\) denotes the dilution factor of the homogenate.

The plasma unbound fraction (fu,plasma) was also determined by centrifugation of plasma containing [3H]temocaprilat through an MPS-3 membrane (Amicon Division, W. R. Grace & Co.).
Binding to Liver Cytosolic Protein(s). To determine cytosolic binding protein, the cytosolic fraction (105,000 g) was prepared from liver homogenate (33% w/v) in 250 mM sucrose and 50 mM Tris-HCl buffer (pH 7.4) from SD rats (Takenaka et al., 1995). After adding [3H]temocaprilat (1 μM) and incubating at 37°C for 15 min, the cytosol was analyzed by HPLC using a gel filtration column (TSK-gel G2000SWx, 30 cm × 7.8 mm i.d.; Tosoh Co., Ltd., Tokyo, Japan). The solvent system used was 50 mM sodium phosphate buffer (pH 7.4) at a flow rate of 0.5 ml/min, and fractions (0.25 ml) were collected. The protein concentration was measured spectrophotometrically at 280 nm, and the radioactivity was determined in a scintillation spectrophotometer (LS-5500; Aloka Co.). GST activity in the eluted fractions of liver cytosol was determined by monitoring the formation of DNP-SG (absorbance at 340 nm) from 1-chloro-2,4-dinitrobenzene (Sugiyama et al., 1981). The GST from rat liver and rat albumin (Sigma Chemical Co.) was also applied to HPLC to confirm its retention time.

Data Analysis. All data are represented as their means ± S.E. Biliary excretion clearance defined for the plasma (CLbile(plasma)), the total (CLunbound(liver)), and unbound concentration in the liver (CLunbound(u,liver)) was calculated by dividing the cumulative amount excreted into bile by the plasma concentration (Cplasma), the total (Cunbound) and unbound concentration (Cunbound × Cplasma), respectively. Student’s t test was used to determine the significance of differences. Uptake rates were fitted to the Michaelis-Menten equation using nonlinear least-squares program, Win-Nonlin (version 1.1; Statistical Consultants Inc., Lexington, KY), to calculate the kinetic parameters.

Results

In Vitro Transport Experiment Using CMVs. The enrichment of marker enzymes compared with liver homogenate and the percentage of inside-out membrane vesicles are summarized in Table 1. The activities of ALP, LAP, and γ-GTPase in CMVs were several times higher than those in liver homogenate and, in addition, approximately 35% of CMVs were composed of inside-out membrane vesicles, which is comparable with that reported for CMVs prepared from male Wistar rats (Böhme et al., 1994). The uptake of DNP-SG and taurocholate into CMVs prepared from five different animal species was stimulated in the presence of the ATP and ATP-generating system, although the degree of ATP stimulation varied among species (Fig. 1).

Using these CMVs, we measured the concentration dependence of the initial uptake rate of DNP-SG. The initial uptake rate was calculated 2 min after initiation of the reaction. Although the initial uptake rate in the absence of ATP increased linearly with concentration, the rate in the presence of ATP showed saturation. The Eadie-Hoffstee plot for the ATP-dependent uptake of DNP-SG and the calculated parameters are shown in Fig. 2 and Table 2, respectively. Although the K_m values were comparable among species (15–56 μM), the V_max for rat CMVs was markedly higher than for other species (Table 2).

To examine the affinity of temocaprilat for cMOAT, we estimated the uptake parameters of temocaprilat into mouse CMVs (Fig. 3A) and also examined the effect of temocaprilat on the uptake of [3H]DNP-SG into CMVs prepared from five experimental animals (Fig. 3, B and C). The parameters for the uptake of temocaprilat into mouse CMVs were calculated to give a V_max value of 0.61 ± 0.14 nmol/min/mg protein and a K_m value of 104 ± 36 μM (mean ± computer calculated S.D.). The ATP-dependent uptake of [3H]DNP-SG into CMVs was reduced by temocaprilat with the calculated IC_{50} values of 474 ± 95, 364 ± 43, 437 ± 71, 461 ± 55, and 447 ± 51 μM (mean ± computer calculated S.D.), for mouse, rat, guinea pig, rabbit, and dog, respectively.

Estimation of Biliary Excretion Clearance by In Vivo Infusion. The species differences in the biliary excretion of temocaprilat, a substrate for rat cMOAT, were then measured in vivo. Pharmacokinetic parameters after i.v. infusion of temocaprilat are summarized in Table 3. The biliary excretion clearance (CLunbound(u,liver)), defined for the unbound concentration in the liver, was markedly higher in rats than in other experimental animals. A good correlation (r^2 = 0.945) was observed between this in vivo biliary excretion clearance and the uptake of DNP-SG into CMVs (Fig. 4).

Binding to Liver Cytosolic Protein(s). To determine the cytosolic binding protein for temocaprilat, cytosol prepared from SD rat liver was analyzed by HPLC using a gel filtration column and the GST activity in the eluted fractions was measured (Fig. 5). Three major peaks associated with [3H]temocaprilat were obtained from the elution pattern, the retention times of which were 11.5, 14.0, and 17.5 min. The GST activity was found at around 17.5 min in each HPLC fraction, and authentic GST from rat liver had almost the same retention time.

Discussion

In the present study, we examined the biliary excretion of temocaprilat among several animal species. In rats, approximately 80% of the infused dose was excreted into the bile, consistent with our previous in vivo finding after i.v. bolus administration (Ishizuka et al., 1997). The biliary excreted amount of temocaprilat differed among animal species; 81, 77, 28, 3, and 24% of the dose was excreted into bile in mouse, rat, guinea pig, rabbit, and dog, respectively. Moreover, allometric relationship with body weight did not hold for the intrinsic clearance for the biliary excretion of temocaprilat defined by liver concentration. These results suggest the importance of characterizing the transport properties across the hepatocellular plasma membrane in these animal species.

We determined the ATP-dependent transport using CMVs in vitro and found that the clearance for transport across the bile canalicular membrane in vivo and that for ATP-depen-

<table>
<thead>
<tr>
<th>Characterization of CMVs</th>
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<tr>
<td>Enrichment of the marker enzymes in CMVs compared with liver homogenate as well as the fraction of inside-out CMVs were measured. Results are mean ± S.E. of three independent preparations.</td>
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<tr>
<td></td>
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<tr>
<td>Mouse</td>
</tr>
<tr>
<td>ALP</td>
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<tr>
<td>γ-GTPase</td>
</tr>
<tr>
<td>LAP</td>
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<tr>
<td>Inside-out ratio (%)</td>
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TABLE 1
dent uptake into CMVs in vitro correlates well among several animal species (Fig. 4). As a model compound to determine the transport activity in vitro, we used DNP-SG, a typical substrate for cMOAT, because the ATP-dependent uptake of this compound into CMVs is much higher than that of temocaprilat. Therefore, any species difference in vitro should be much more easily detected with this ligand. In our preliminary experiments, although the ATP-dependent uptake of temocaprilat into CMVs was observed in all species, it was too small to allow estimation of the associated kinetic parameters for animal species other than mice and rats (data not shown). We also examined the effect of temocaprilat on the uptake of [3H]DNP-SG into CMVs prepared from five experimental animals (Fig. 3). The ATP-dependent uptake of [3H]DNP-SG into CMVs was reduced in a concentration-dependent manner by unlabeled temocaprilat and calculated IC50 values for the inhibitory effect of temocaprilat on the ATP-dependent uptake of [3H]DNP-SG into CMVs were almost comparable among species. Collectively, these results suggest that the affinity for cMOAT might be also comparable among species. As reported previously (Ishizuka et al., 1997), the IC50 value in rat CMVs (364 ± 0.03 µM) was approximately four times higher than the Km of temocaprilat for rat cMOAT (92.5 ± 0.09 µM; Ishizuka et al., 1997). This discrepancy was also found in mouse CMVs (IC50 = 474 ± 0.09 µM and Km = 104 ± 0.09 µM). One of the possible explanations for the discrepancy for the IC50 and Km of temocaprilat was the existence of multiplicity for the transport systems (Ishizuka et al., 1997). The similarity in the affinity of temocaprilat for cMOAT among animal species, however, was further confirmed by the comparable Km values between mice (104 ± 0.09 µM; Fig. 3) and rats (92.5 ± 0.09 µM; Ishizuka et al., 1997).

Because the present in vivo and in vitro studies were performed under linear conditions, the results suggest that
Correlation between the uptake of DNP-SG into CMVs in vitro with CMVs (A) and the effect of temocapril on the uptake of [3H]DNP-SG into CMVs prepared from five experimental animal livers (B and C; mouse (O), rat (●), guinea pig (▲), rabbit (■) and dog (○)). Uptake of [3H]temocapril and [3H]DNP-SG containing different concentrations of temocapril into CMVs was measured in the presence of ATP. The solid line represents the fitted line obtained from nonlinear least-square method. Each point represents the mean ± S.E. (n = 3–4).

Fig. 3. Eadie-Hofstee plot for the uptake of temocapril into mouse (□) and rat (○) (taken from our previous report, Ishizuka et al., 1997) CMVs (A) and the effect of temocapril on the uptake of [3H]DNP-SG into CMVs prepared from five experimental animal livers [B and C: mouse (O), rat (●), guinea pig (▲), rabbit (■) and dog (○)]. Uptake of [3H]temocapril and [3H]DNP-SG containing different concentrations of temocapril into CMVs was measured in the presence of ATP. The solid line represents the fitted line obtained from nonlinear least-square method. Each point represents the mean ± S.E. (n = 3–4).

Table 3
Pharmacokinetic parameters after i.v. infusion of temocapril
Temocapril was i.v. infused to experimental animals. The plasma concentration and the biliary excretion rate were determined under the steady-state condition. At the end of the experiments, the liver was removed to determine the concentration of temocapril. Results are mean ± S.E. of three independent experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit of Measure</th>
<th>Mouse</th>
<th>Rat</th>
<th>Guinea Pig</th>
<th>Rabbit</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>kg</td>
<td>0.044 ± 0.002</td>
<td>0.26 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>3.47 ± 0.03</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td>Bile flow</td>
<td>µl/min/kg</td>
<td>32.3 ± 5.6</td>
<td>35.4 ± 2.9</td>
<td>158.1 ± 11.4</td>
<td>48.5 ± 0.9</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Infusion rate</td>
<td>nmol/min/kg</td>
<td>0.97</td>
<td>0.60</td>
<td>0.74</td>
<td>0.62</td>
<td>0.41</td>
</tr>
<tr>
<td>Km,plasma</td>
<td>nM</td>
<td>0.15 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Km,liver</td>
<td>nM</td>
<td>149 ± 18</td>
<td>46 ± 6</td>
<td>193 ± 25</td>
<td>80 ± 5</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Cmax</td>
<td>pmol/g</td>
<td>562 ± 129</td>
<td>267 ± 34</td>
<td>121 ± 7</td>
<td>73 ± 2</td>
<td>456 ± 90</td>
</tr>
<tr>
<td>Cmax,liver</td>
<td>pmol/g</td>
<td>94 ± 19</td>
<td>12 ± 1</td>
<td>23 ± 1</td>
<td>18 ± 1</td>
<td>129 ± 27</td>
</tr>
<tr>
<td>CLtot</td>
<td>ml/min/kg</td>
<td>7.5 ± 0.6</td>
<td>12.5 ± 1.6</td>
<td>3.9 ± 0.5</td>
<td>7.9 ± 0.5</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>CLplasma</td>
<td>ml/min/kg</td>
<td>5.9 ± 0.8</td>
<td>9.7 ± 1.2</td>
<td>1.1 ± 0.2</td>
<td>0.24 ± 0.02</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>CLliver</td>
<td>ml/min/kg</td>
<td>1.6 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>0.27 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>CLliver,plasma</td>
<td>ml/min/kg</td>
<td>9.8 ± 1.9</td>
<td>39.2 ± 3.3</td>
<td>9.2 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>0.80 ± 0.13</td>
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</tbody>
</table>

The Vmax/Km for transport across the bile canalicular membrane in vivo can be quantitatively predicted from the same value determined in vitro with CMVs (Fig. 4). An in vivo-in vitro correlation for the Km, value of another cMOAT substrate, pravastatin, has been demonstrated previously in rats (Yamazaki et al., 1997). The biliary excretion clearance of pravastatin under steady-state conditions in vivo fell markedly with an increase in the liver concentration with an in vivo Km value of 180 µM, which was comparable with the Km (220 µM) for the ATP-dependent uptake of pravastatin into CMVs (Yamazaki et al., 1997). Collectively, both the Vmax and Km values for the biliary excretion in vivo can be predicted from in vitro transport studies.

In vitro studies with CMVs indicated that the transport activity for DNP-SG was in the order, rat > mouse > guinea pig, rabbit, dog and that this difference may be accounted for largely by the difference in Vmax rather than in Km (Table 2). It was in marked contrast to our previous finding that the low molecular biological studies on this particular protein will reveal species differences in BSEP activity (Gerloff et al., 1998). However, we must be cautious in the interpretation of the in vitro data because the enrichment of these marker enzymes (ALP, LAP, and γ-GTPase) in CMVs compared with liver homogenate differed significantly among animal species (Table 1). Although the exact reason for this discrepancy is unknown, one possible explanation is to assume that these enzymes may not be exclusively located on the bile canalicular membrane. In fact, some reports have been published which suggest that LAP and γ-GTPase are also present in microsomal fractions (Kanno et al. 1984; Goldberg, 1980).
clear answer to the species difference in transport activity could be obtained by using cDNA products cloned from these experimental animals. The species differences in the transport activity of DNP-SG, however, may not necessarily be accounted for only by the difference in cMOAT activity per se. We cannot exclude the possibility that other, as yet unidentified, transporters capable of transporting DNP-SG may also be expressed on CMVs in certain animal species. Previously, we found that MRP6 (Kool et al., 1997) (initially referred to as MLP-1) is also expressed in the liver of both SD rats and EHBR (Hirohashi et al., 1998). In addition, it has been shown that the hepatic expression of MRP3 (Kool et al., 1997) (initially referred to as MLP-2) is observed in EHBR, but not in SD rats (Hirohashi et al., 1998; Kiuchi et al., 1998). Moreover, we showed that the hepatic expression of MRP3 is induced in SD rats by phenobarbital treatment or any other treatment that increases the plasma concentration of bilirubin and/or its glucuronide (Hirohashi et al., 1998). More importantly, we found expression of MRP3 in normal human subjects and, in addition, phenobarbital induced the expression of MRP3 in HepG2 cells in vitro (Kiuchi et al., 1998). Although the uptake of DNP-SG into CMVs can be accounted for by cMOAT in rats because the uptake was almost completely abolished in CMVs from EHBR (Yamazaki et al., 1996; Suzuki and Sugiyama, 1998), it is possible that some other unidentified MRP/cMOAT homologues are involved in the transport of this ligand in CMVs from other animal species.

Finally, it is important to consider the intracellular binding of temocaprilat. A good in vivo and in vitro correlation was observed if the in vivo excretion clearance was defined in terms of the hepatic unbound concentration (CL\text{h,liver}/f_{u,plasma}). This finding confirmed the pharmacokinetic theory which assumes that most pharmacokinetic events, metabolism and membrane transport involve only for the unbound form of drugs. To identify the cytosolic binding protein for temocaprilat, we subjected a mixture of temocaprilat and liver cytosol prepared from SD rats to HPLC using a gel filtration column (Fig. 5). We found three major radioactive peaks, the retention times of which were 11.5, 14.0, and 17.5 min, respectively. The finding that the retention time of the second peak was almost identical with that of rat albumin (13.9 min) and the fact that temocaprilat was highly bound to rat plasma ($f_{u,plasma} = 0.06$, Table 3) suggested that the second chromatographic peak reflected binding to albumin. The fact that the third radioactive peak was almost identical with that of GST activity (Fig. 5) suggests that one of the proteins responsible for temocaprilat binding in the liver cytosol may be ligandin(s) (GST). We could not clearly demonstrate the protein for the first chromatographic peak, but X-fraction, following the nomenclature of Levi et al. (1969), may be a candidate for binding to temocaprilat. Collectively, these proteins may be the main factor determining the free fraction of temocaprilat in liver.

In conclusion, the species differences in the transport of organic anions across the bile canalicular membrane was characterized in vivo and in vitro. The differences in the transport activity of DNP-SG in CMVs among mouse, rat, guinea pig, rabbit, and dog were accounted for largely by the difference in $V_{\text{max}}$ values. In addition, the in vivo excretion activity can be quantitatively predicted from in vitro experiments with CMVs.

References


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