BILIARY EXCRETION OF PRAVASTATIN IN RATS: CONTRIBUTION OF THE EXCRETION PATHWAY MEDIATED BY CANALICULAR MULTISPECIFIC ORGANIC ANION TRANSPORTER (cMOAT)

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ABSTRACT:

The biliary excretion of pravastatin in normal rats and Eisai hyperbiliruminemic rats (EHBRs) was examined in vivo and in vitro using bile canalicular membrane vesicles (CMVs). In vivo, the total body clearances at steady-state (CL\text{tot}) for both rat strains decreased as the infusion rate increased. At the lowest infusion rate, CL\text{tot} for normal rats was 1.6 times higher than that for EHBRs. Under this set of conditions, the biliary excretion clearance (CL\text{bil}) of pravastatin was 3-fold higher than that for EHBRs. The CL\text{bil} fell markedly with increasing C\text{liver}, defined as the biliary excretion rate at steady-state divided by the concentration in the liver (C\text{liver}), for normal rats was 180 μM; in contrast, the degree of saturation was slight if any in EHBRs. In vitro, the uptake of pravastatin by CMVs prepared from normal rats exhibited clear ATP-dependence, whereas only a minimal effect of ATP was observed on the uptake by CMVs from EHBRs. Transport kinetic studies were performed over a wide range of pravastatin concentration (0.2–10,000 μM) with a tracer tritium-labeled pravastatin. Saturation was observed both in the ATP-dependent (K\text{m} \approx 220 μM) and ATP-independent (K\text{m} \approx 480 μM) uptake of CMVs prepared from normal rats. ATP-dependent uptake of 2,4-dinitrophenyl glutathione, a typical substrate for the canalicular multispecific organic anion transporter (cMOAT), was inhibited by pravastatin in a concentration-dependent manner and the resultant inhibitory constant of pravastatin (170 μM) was comparable with the K\text{m} value of ATP-dependent pravastatin uptake itself. In conclusion, biliary excretion of pravastatin is mediated mainly by cMOAT in normal rats. This can explain the decrease in the biliary excretion of pravastatin in EHBRs.

The HMG-CoA \(^1\) reductase inhibitor, pravastatin, as far as its pharmacological effect (decrease in the plasma cholesterol level) is concerned, is relatively liver specific (1). As one of the reasons for this tissue-specific activity, we have previously demonstrated that after iv administration of pravastatin, liver accounted for most of the uptake compared with other tissues (2). Based on the results obtained with in vitro isolated rat hepatocytes, pravastatin was taken up actively by a so-called “multispecific anion transporter (3)” (4). In addition, the permeability surface area products for the influx of unbound pravastatin evaluated in four different experimental systems (in vivo, in vitro liver perfusion, isolated cells, and primary culture cells) were in good agreement (5), indicating that the active transport mechanism on the liver surface for this drug is entirely responsible for its initial distribution into the liver. As described above, regarding the hepatic uptake of pravastatin, we and others (4, 6, 7) have identified a number of characteristics.

After oral administration, pravastatin was well absorbed from the gastrointestinal tract (70% in rats and more than 50% in dogs) (8). Regarding transport across the intestinal brush-border membrane, a proton-gradient dependent carrier-mediated mechanism is involved (9). Pravastatin was taken up efficiently by the liver as described above, and enterohepatic circulation has been suggested to take place (8). These carrier-mediated transport mechanisms contribute to the efficient exposure of the target enzyme in the liver to the drug administered orally; distribution to other tissues is restricted because of its high hydrophilicity. Consequently, these features of pravastatin, in the light of its disposition in the body, lead to liver-selective inhibition of cholesterol biosynthesis.

As for the biliary excretion of pravastatin, studies have been directed at investigating whether it is a substrate for a primary active transport system, termed the “canalicular multispecific organic anion transporter” (cMOAT), cloned recently (10–12). Interest has arisen in this topic as it has become clear that most classes of organic anionic compounds and their conjugates are excreted in bile via this transport system (13, 14). For this reason, studies with mutant rats, such as TR\(^-\) and Eisai hyperbilirubinemic rats (EHBRs), in which this transport system is defective, are extremely useful (13–16). We demonstrated that the steady-state biliary excretion of pravastatin fell to 40% in EHBRs compared with that in normal rats in an in vitro single-pass liver perfusion study (17). The bile to liver cytosol unbound drug concentration ratio, however, was approximately 90 for both rat strains. Furthermore, ATP-dependent uptake by bile canalicular membrane vesicles (CMVs) was observed not only for normal rats but even...

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1 Abbreviations used are: HMG-CoA; hydroxymethylglutaryl CoA, EHBRs; Eisai hyperbiliruminemic rats, cMOAT; canalicular multispecific organic anion transporter, CMVs; bile canalicular membrane vesicles, CL\text{tot}; total body clearance, t; infusion rate, C\text{liver}; plasma concentration of pravastatin at steady-state, V\text{max}; biliary excretion rate, K\text{m}; Michaelis constant, V\text{max}; maximum transport velocity, P\text{app}; nonspecific diffusion clearance, AIC; Akaike’s information criterion, K\text{i}; inhibitory constant, CL\text{ass}; biliary excretion clearance, C\text{m}; maximal secretory rate.

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for EHBRs, although the degree of stimulation by ATP was slight in EHBRs. Adachi et al. (18) have demonstrated that uptake of pravastatin by CMVs from normal rats and EHBRs was dependent on both ATP and the pH gradient. They also showed that there was a reduction in the biliary excretion of pravastatin after iv administration to EHBRs compared with that in normal rats. The experimental evidence shown above suggested the possibility of the presence of multiple transport systems for pravastatin. There still remains a possibility, however, that both transport mechanisms, that is cMOAT-mediated (deficient in EHBRs) and a novel ATP-dependent transporter-mediated (maintained in EHBRs), are involved in biliary excretion of pravastatin. So far, only [14C] labeled pravastatin has been available with a relatively low specific activity; thus it was impossible to examine the transport kinetics of this compound at low concentrations, e.g. to clarify the contribution of cMOAT-mediated transport across the bile canalicular membrane quantitatively.

The purpose of this study is to gain a better understanding of the mechanism of pravastatin excretion into bile and the defect in pravastatin excretion in EHBRs. We carried out a series of in vivo studies involving continuous infusion at different infusion rates over a wide range and compared the biliary excretion at steady state in normal rats and EHBRs. Furthermore, we performed a transport study by CMVs to see if pravastatin is a substrate for cMOAT using newly synthesized [3H]-labeled pravastatin with a specific activity three orders of magnitude higher than that of the [14C] labeled material. For this purpose, we performed an uptake study with [3H] labeled pravastatin using CMVs from both normal and EHBR livers, and an inhibition study of pravastatin on ATP-dependent uptake of [3H] 2,4-dinitrophenyl glutathione (DNP-SG), a typical substrate for cMOAT, by CMVs from normal rat liver.

Materials and Methods

Chemicals. [14C] Pravastatin, [3H] pravastatin, and unlabeled pravastatin were kindly donated by Sankyo Co. Ltd. (Tokyo, Japan). Glutathione, [glycine-2-3H] (44.8 Ci/mmol) purchased from New England Nuclear Corp. (Boston, MA). 1-Chloro-2,4-dinitrobenzen, glutathione S-transferase (from rat liver), ATP (disodium salt), creatine phosphate, and creatine phosphokinase (Type I: from rabbit muscle) were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

The radiochemical purity of [14C] pravastatin synthesized as described previously (8), determined by HPLC, was 98.8%, and the specific activity was 11.3 mCi/mmol. [14C] labeled positions were shown in (8). [3H] labeled pravastatin with a specific activity three orders of magnitude higher than that of the [14C] labeled material. For this purpose, we performed an uptake study with [3H] labeled pravastatin using CMVs from both normal and EHBR livers, and an inhibition study of pravastatin on ATP-dependent uptake of [3H] 2,4-dinitrophenyl glutathione (DNP-SG), a typical substrate for cMOAT, by CMVs from normal rat liver.

In vivo constant-infusion study. Rats were lightly anesthetized with ether and the left and femoral artery and vein were cannulated with heparinized polyethylene tubing (PE-50); the bile duct was cannulated with polyethylene tubing (PE-10). [14C] Pravastatin dissolved in physiological saline was infused through the femoral vein cannula at a flow rate of 15 μl/min/rat for 65 min. The infusion rates (μmol/min/kg) were 0.021, 0.50, 2.5, 4.9, 9.8, and 22 for normal rats and 0.024, 2.5, 4, 6, 10, and 24 for EHBRs. At intervals, arterial blood (200 μl) and bile samples were collected in polyethylene tubes. To obtain plasma, blood was centrifuged at 10,000 g for 2 min in a table-top microcentrifuge (Microfuge E, Beckman Instruments, Inc., Fullerton, CA). Rats were killed at 65 min, and immediately the liver was excised, rinsed with saline, and weighed. A portion (1 g) of the liver was homogenized (33% w/v) in 250 mM sucrose buffer containing 50 mM Tris-HCl (pH 7.4). The total radioactivity in plasma, bile, and homogenate samples was determined using a liquid scintillation spectrophotometer (LC6000SE, Beckman Instruments, Inc., Fullerton, CA).

Thin-layer chromatographic analysis. Five-fold volume of acetonitrile was added to each specimen of both plasma and liver homogenate for deproteinization. After centrifugation of the mixture, the recovery of the radioactivity in the supernatant was greater than 98%. The supernatant was applied to a silica gel TLC plate (Kiesel gel 60 F254 (E. Merck, Darmstadt, Germany)) and developed with CHCl3:MeOH:CH3COOH = 9:1:1 (v/v) as the solvent system (21). The concentration of [14C] pravastatin was determined with a Bio-Image Analyzer (Bas 2000, Fuji Film Co., Ltd. Tokyo, Japan). The bile specimens were diluted with acetonitrile (5-fold dilution (v/v), and the concentration of parent compound was determined in the same way as those in the plasma and liver homogenate.

Transport study in canalicular membrane vesicles (CMVs). CMV were prepared from male SD rats and EHBRs liver as described before (22). According to the method of Kobayashi et al. (19) and were suspended in 50 mM Tris/HCl buffer (pH 7.4) containing 250 mM sucrose. Protein was determined using the method of Bradford (23), using the Bio-Rad protein assay kit with bovine serum albumin as a standard. The transport study of [3H] pravastatin and [3H] DNP-SG was performed by the method reported previously (22, 24). Radioactivity retained on the filter (0.45 μm GVWP, Millipore Corp., Bedford, MA) was determined and the uptake of ligands was normalized with respect to the amount of membrane protein and the concentration of ligand in the medium.

Calculation of pharmacokinetic parameters. The total body clearance (Cltot) was calculated according to the following equation:

\[
Cl_{tot} = \frac{I}{C_{p,ss}}
\]

where \( I \) represents the infusion rate of pravastatin and \( C_{p,ss} \) is the arterial plasma concentration of parent drug 60 min after the beginning of the infusion.

Determination of Kinetic Parameters for the Biliary Excretion of Pravastatin. All the fittings described below were performed by an iterative nonlinear least squares method using a MULTI program (25).

In vivo. Three types (models 1–3) of equations (eqs. 2–4) described below were fit to the data obtained at the different infusion rates.

\[
V_{bile} = V_{max, in vivo} C_{liver} (K_{M, in vivo} C_{liver} + C_{liver}) + P_{diff, in vivo} C_{liver}
\]

(2)

\[
V_{bile} = V_{max, in vivo} C_{liver} (K_{M, in vivo} C_{liver} + C_{liver})
\]

(3)

\[
V_{bile} = P_{diff, in vivo} C_{liver}
\]

(4)

Where \( V_{bile} \) is the biliary excretion rate of pravastatin (nmol/min/kg) from 55–65 min, \( C_{liver} \) is the pravastatin concentration in the liver (μM), estimated
as the amount of pravastatin in the liver (nmol/g liver) assuming that 1 g liver = 1 ml, and K_{M, in vivo} (µM), V_{max, in vivo} (nmol/min/kg) and P_{dif, in vivo} (ml/min/kg) represent the Michaelis constant as for C_{liver, max} maximum excretory velocity and nonspecific diffusion clearance, respectively. The input data were weighted as the reciprocal of the observed values, and the algorithm used for the fitting was the Damping Gauss Newton Method (25). AIC values were used to assess the suitability of the models (26).

In vitro. The kinetic parameters for pravastatin uptake by CMVs were estimated according to the following equation:

\[ v = \frac{V_{max, in vivo} S}{(K_M, in vivo + S)} + P_{dif, in vivo} S \]  

(5)

where \( v \) is the uptake rate of the ligand by CMVs, assessed by the uptake amount at 2 min (nmol/min/mg protein), \( S \) is pravastatin concentration (µM), \( V_{max, in vivo} \) is the maximum uptake rate by CMVs (nmol/min/mg protein), \( K_M, in vivo \) is the Michaelis constant (µM) and \( P_{dif, in vivo} \) is the nonspecific uptake clearance (µl/min/mg protein).

The inhibitory constant (K_i) value of pravastatin on the ATP-dependent uptake (obtained by subtracting the uptake in the absence of ATP from that in the presence of ATP) of [3H] DNP-SG (1 µM) was calculated according to the following equation, from the inhibition data obtained by varying the pravastatin concentration (1 - 100,000 µM):

\[ v = \frac{V_{max}}{S(K_i + S)} + P_{dif} S \]  

(6)

where \( K_i \) is the inhibitory constant (µM) of pravastatin, \( v \) is the pravastatin concentration (1 - 100,000 µM), \( S \) is the DNP-SG concentration (1 µM), the \( K_M (15.8 \mu M) \) and \( P_{dif} (1.30 \mu l/min/mg) \) values obtained by a different experiment were used;\(^2\) and the parameters \( K_i \) and \( V_{max} \) were obtained by the fitting.

Results

Disposition of Pravastatin in Vivo. The plasma concentrations of pravastatin for both rat strains reached a plateau approximately 40 min after the beginning of the iv infusion, 0.02 - 24.0 µmol/min/kg, i.e. a steady-state had been attained (data not shown). Fig. 2(a) shows the relationship between the infusion rate (I) and the total body clearance (CL_{tot}), defined as the infusion rate divided by the plasma concentration of pravastatin at steady-state (60 min after the beginning of infusion; C_{p,ss}, eq. 1). The values of CL_{tot} for each rat declined with increasing infusion rate. At the lowest infusion rate, the values of C_{p,ss} were less than 1 µM for each rat (0.44 ± 0.06 µM for normal rats and 0.81 ± 0.09 µM for EHBRs, respectively), and the CL_{tot} (ml/min/kg) for normal rats (48.0 ± 5.5) was 1.6 times higher than that for EHBRs (29.8 ± 3.1) (fig. 2(b)).

The plasma concentrations of pravastatin in normal rats and EHBRs. Each symbol and vertical and horizontal bars represent the mean ± SE of three rats.

(a) Open symbol represents the data for normal rats. Solid line is the least-squares fit of data to eq. 2. Dotted line represents the nonspecific diffusion calculated with the value of nonspecific uptake clearance of P_{dif, in vivo} for normal rats. Dashed line is the theoretical curve of saturable uptake. Obtained kinetic parameters for normal rats are as follows: K_{M, in vivo} = 181 ± 7.6 µM, V_{max, in vivo} = 3440 ± 1410 nmol/min/kg, and P_{dif, in vivo} = 4.92 ± 1.58 ml/min/kg. (b) Closed symbol represents the data for EHBRs. Solid line is the fitted line by eq. 4, and the resultant P_{dif, in vivo} for EHBRs is 1.45 ± 0.07 ml/min/kg.

were less than 1 µM for each rat (0.44 ± 0.06 µM for normal rats and 0.81 ± 0.09 µM for EHBRs, respectively), and the CL_{tot}(ml/min/kg) for normal rats (48.0 ± 5.5) was 1.6 times higher than that for EHBRs (29.8 ± 3.1) (fig. 2(b)).

Fig. 3 shows the concentration-dependence of the biliary excretion of pravastatin at steady-state. At any infusion rate, the biliary excretion by normal rats was higher than for EHBRs. For normal rats, the biliary excretion clearance (CL_{bilex}) was calculated as V_{liver} divided by C_{liver} decreased with the increase in C_{liver} (from 14.2 ± 3.2 at C_{liver} = 0.73 ± 0.09 µM to 8.70 ± 1.26 at C_{liver} = 793 ± 33 µM). For EHBRs, the values fell from 4.00 ± 0.88 at C_{liver} = 0.91 ± 0.17 µM to 1.44 ± 0.35 at C_{liver} = 2110 ± 250 µM. To obtain the kinetic parameters for the biliary excretion of pravastatin, three different models described in eqs. 2-4 were fit to the data. Model 1 fit the data for normal rats best (the AIC values were as follows: 26.1 for model 1, 71.4 for model 2, and 42.2 for model 3), and the kinetic parameters obtained are listed in table 1. For EHBRs, the AIC values were comparable among the 3 models (23.4 for model 1, 22.5 for model 2, and 22.6 for model 3); however, in practical terms reliable values for parameters were obtained only for model 3 (for model 1, the computer calculated SD values were >10-fold larger than the fit values; for model 2, the K_M value calculated was more than twice the highest value for C_{liver, observed experimentally}). The P_{dif, in vivo} value in model 3 was calculated as 1.45 ± 0.07 ml/min/kg (table 1).

Transport Study in Canaliculic Membrane Vesicles (CMVs). As shown in fig. 4(a), the uptake of [3H] pravastatin by CMVs prepared from normal rats exhibited ATP-dependence at 0.2 µM (tracer only), whereas no stimulatory effect of ATP was observed in the presence of 2 nM of unlabeled pravastatin. For CMVs from EHBRs, a minimal effect of ATP was observed in the uptake of pravastatin (fig. 4(b)); however, concentration-dependence was observed in CMVs from both rat strains. The kinetic parameters (mean ± computer calculated SD) for the uptake of pravastatin by CMVs from normal rats using the data shown in fig. 5 were obtained as listed in table 1.

The inhibitory profiles of pravastatin on the ATP-dependent uptake...
Comparable with the K_M of [3H] DNP-SG (1 mM) and ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml of creatine phosphokinase) in the medium. Circles represent the tracer uptake. Each point and vertical bar represent the mean ± SE of three determinations. * Significantly higher than the control (p<0.05).

CMVs (10 μg protein) were incubated with (closed) or without (open) ATP (5 mM) and ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml of creatine phosphokinase) in the medium. Circles represent the tracer uptake. Each point and vertical bar represent the mean ± SE of three determinations. * Significantly higher than the control (p<0.05).

Table 1: Kinetic parameters for pravastatin transport

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<th>In vivo</th>
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<td>Normal rats*</td>
<td>K_M, in vivo (μM) 181 ± 78</td>
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<td>V_max, in vivo (pmol/min/mg protein) 3440 ± 1410</td>
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<td>P_dif, in vivo (μl/min/mg protein) 4.92 ± 1.58</td>
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<td>P_dif, in vivo (μl/min/mg protein) 1.45 ± 0.07</td>
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* Data shown in Fig. 3(b).

![Fig. 4. Time profiles for the uptake of [1H] pravastatin by the CMVs prepared from normal rats (a) and EHBR (b).](Image)

Discussion

We have previously suggested that a primary active transport mechanism which is maintained in EHBRs contributes at least partly to the biliary excretion of pravastatin. However, the decrease in the biliary excretion of pravastatin in EHBRs suggested that this drug is also a substrate for cMOAT (17). So far, only the [14C] labeled radioisotope of this compound with a relatively low specific activity has been available, and this has prevented us carrying out a detailed transport study, especially the uptake by CMVs. For example, we could not reduce the radiolabeled pravastatin concentration below 200 μM to obtain reliable counts in the CMV uptake study (17). Also, the lowest concentration in the study of Adachi et al. with CMVs was 100 μM (18). With [14C] pravastatin, we were unable to examine the possibility that other transport system(s) with higher affinity might be involved. In general, transport studies using CMVs were performed at a ligand concentration around 10^-8 ~ 10^-5 M range (19, 22, 24).
addition, we observed a clear decrease in the biliary excretion of pravastatin in EHBRs in perfused liver at 10⁻⁶ M for C_{liver} with the infusate concentration of 1 μM (17), which was slightly higher than the therapeutic concentration of this drug (≤ 0.3 μM) (1). In the present study, we have been able to examine the biliary excretion of pravastatin in normal rats and EHBRs in *in vitro* CMV studies at much lower concentrations using newly synthesized [³H] labeled pravastatin with a specific activity three orders of magnitude higher than that of the [¹⁴C] labeled material.

Furthermore, although membrane vesicles are an artificial experimental system, they are a useful tool for investigating the precise mechanism of transport. However, a quantitative assessment based on such results alone may not reflect accurately what is happening *in vivo*. Consequently, to examine the contribution of any transport mechanism to the biliary excretion of pravastatin, studies should be performed with other experimental systems that are more physiological than membrane vesicles. Therefore, we also examined the biliary excretion of pravastatin in an *in vivo* constant infusion study.

We found that the biliary excretion of pravastatin is actually reduced in EHBRs compared with that in normal rats *in vivo* under a wide range of plasma and/or liver concentrations (fig. 3). These results suggest that cMOAT-mediated transport is involved in the biliary excretion of pravastatin. *In vitro*, the uptake of pravastatin by CMVs prepared from normal rats exhibited clear ATP-dependence, whereas a minimal ATP-dependence was observed in the uptake by CMVs from EHBRs (fig. 4), which is consistent with our previous study (17). Saturation was observed both in ATP-dependent (K_M, 220 μM) and ATP-independent (K_M, 480 μM) uptake by CMVs prepared from normal rats (fig. 5). ATP-dependent uptake of DNP-SG was inhibited by pravastatin in a concentration-dependent manner and the resultant K_M of pravastatin (170 μM) was comparable to the K_M value of ATP-dependent pravastatin uptake itself (fig. 6). Furthermore, those values (K_M and K_J) obtained with *in vitro* CMVs were comparable with that (K_M) *in vivo* (table 1). These results indicate that pravastatin shares the same transporter on the bile canalicular membrane as DNP-SG, a typical substrate for cMOAT, and this causes the decrease in biliary excretion in EHBRs *in vivo*.

To demonstrate directly that cMOAT-mediated transport is actually involved in the biliary excretion of pravastatin in EHBRs *in vivo* is quite difficult without using cMOAT knock-out animals or specific inhibitors of cMOAT that are effective under *in vivo* conditions. So far, a cMOAT knock-out mouse has not been developed, and a cMOAT-specific inhibitor has yet to be discovered. We consider that our approach in this study, i.e. quantitative comparison of the kinetic parameters obtained for both *in vivo* and *in vitro* experimental systems, is presently the most reliable and practical way to evaluate the contribution of the cMOAT-mediated transport to biliary excretion under *in vivo* conditions.

The K_M, *in vivo* defined as a total concentration in the liver, for normal rats was around 180 μM (table 1). It may be appropriate to compare the K_M, *in vivo* value corrected for intracellular binding (K_M) for unbound drug concentration in the liver; K_M, *u. in vivo*, with K_M obtained by *in vitro* CMV study, since it is a general concept in pharmacokinetics that only the unbound drug can penetrate the membrane. Previously we evaluated the unbound fraction of pravastatin in liver cytosol at steady-state in the single-pass liver perfusion study (17). The resultant unbound fraction (the unbound drug concentration in the cytosol vs. total drug concentration in liver homogenate) was 0.54 in rats at C_{liver} (μM); around 5 μM. According to the previous result, the K_M, *u. in vivo*, was calculated to be around 100 μM, assuming that the intracellular binding was linear within the concentration range in the present study. The likelihood that a remarkable nonlinear tissue binding did occur is low since previously we did not observe any significant difference in plasma unbound fraction (fu = 0.67) for normal rats over a wide range of concentration (0.5–1000 μM) (27). Even if saturable binding was involved, the K_M for the unbound drug was estimated within the range of 100–200 μM, a consideration that would lead one to conclude the K_M values for biliary excretion of pravastatin obtained for *in vivo* and *in vitro* experimental systems are comparable regardless of the correction of intracellular binding.

The exact reason for the three times difference in P_{diff, in vivo} values between the two-rat strains are not clear. It is probable, however, that the nonspecific diffusion can be dependent on the bile flow rate. It is well known that bile flow is two to three times lower in EHBRs by nature, mainly because of its deficiency in the excretion of organic anions and their conjugates (28). In addition to this fact, we found infusion rate-dependent (i.e. C_{liver}-dependent) increase in bile flow rate at steady-state for both rat-strains (for normal rats; from 44 to 145 μl/min/kg, for EHBRs; from 27 to 54 μl/min/kg, respectively). It is difficult to argue in a precise manner about the difference in P_{diff, in vivo} obtained under such different values in terms of bile flow rate between two-rat strains. To clarify whether the P_{diff, in vivo} is essentially different, it might be useful to compare the P_{diff, in vitro} values determined *in vitro* using CMVs. However, the absolute values for the uptake of pravastatin by CMVs from EHBRs were minute (fig. 4(b)). Furthermore, a direct comparison of the P_{diff, in vitro} values between the two-rat strains is inappropriate in this case since there may be nonspecific adsorption of the ligand to the outer/inner membrane of the leaky vesicles. Further study is required to investigate the reason for the difference in P_{diff, in vivo} values between the two-rat strains.

We cannot rule out the possibility that the putative primary active transport system we suggested previously is also involved in the biliary excretion of pravastatin. This is because a small, but significant, stimulation of uptake by ATP is exhibited in CMVs from normal rats (fig. 4) (17). Considering the results obtained in the present study, it seems likely that biliary excretion of pravastatin via bile canalicular membrane is mediated mainly by cMOAT and the contribution from other primary active transport system is rather small. Saturation in ATP-independent uptake by CMVs was clearly observed in normal rats (fig. 5(a)), indicating that a certain carrier-mediated ATP-independent transport system may be involved in pravastatin excretion. A small, but distinct, saturable contribution in the biliary excretion in EHBRs was also detected (fig. 3(b)). There has also been a report of clear saturation of uptake by CMVs in the absence of ATP for glutathione (GSH) (29). Uptake of GSH by CMVs from both normal rats and EHBRs does not show any ATP-dependence although the biliary excretion decreased clearly in EHBRs. These authors hypothesized that, for the biliary excretion of GSH, there was almost no GSH in the bile of mutant rats and this could be best explained as a secondary defect owing to *cis*-inhibition by retained endogenous organic anions for the defective carrier and/or loss of *trans*-stimulation by these same substrates which are normally concentratively transported into bile (29). Furthermore, studies using CMVs have demonstrated that both ATP- and membrane potential-dependent transport mechanisms are responsible for the biliary excretion of bilirubin glucoronide (30) and DNP-SG (31), and that a membrane potential-dependent transport system is maintained in mutant rats (30). Tamai et al. (32) also demonstrated that a positive inner membrane potential stimulates the uptake of the cephalosporin antibiotic, cephradime (monovalent anion), by CMVs. We have already demonstrated the multiplicity in the transport systems for organic anions which are deficient in mutant rats (14, 22, 24, 33). At present, we...
cannot argue for a saturable transport of pravastatin which we detected in our *in vitro* (fig. 4) and/or *in vivo* studies in EHBRs (fig. 3 (b)). A number of questions need to be answered, e.g. are these two saturable systems identical or not and what is the driving force(s)? Further studies are required to clarify the mechanism in detail using CMVs as well as molecular biological techniques.

Measurement of biliary output at various plasma concentrations has been used frequently, although it is a classical approach, to characterize the biliary excretion of ligands *in vivo*. This was particularly the case before the development of the *in vitro* techniques (34). One criteria, although an indirect one, suggesting that a carrier-mediated excretion mechanism actually exists involving the bile canicular membranes *in vivo* is to demonstrate a definable maximum secretory rate (*T_m*). As far as organic anions are concerned, *T_m* values for several ligands such as bilirubin, indocyanine green, bromosulfophthalein, and dibromosulfophthalein etc. have been reported (24, 34). For pravastatin, no similar study has been conducted. We also measured the pravastatin concentration in the liver and assessed *CL_{liver*} as it may provide additional insight even though it represents a relatively crude approach to intracellular localization and quantitation. Regarding *T_m*, pravastatin exhibits a substantially higher value (6800 nmol/min/kg; fig. 3) than other ligands which are organic anions such as bilirubin, indocyanine green, bromosulfophthalein and dibromosulfophthalein (24, 34). This might be explained not only by the intrinsic transport mechanism in the liver, *e.g.* the hepatic transport capacity for pravastatin is high, but also by the physicochemical characteristics of the ligand itself, *e.g.* pravastatin is very hydrophilic compared with the other amphipathic organic anions described above. Thus, *T_m* may reflect an ephemeral balance between the intrinsic excretory capacity of the liver and the toxicity of the ligand (34).

The therapeutic plasma concentration of pravastatin is less than 0.3 μM (1), which is close to the *C_{p,h}*(0.44 ± 0.06 μM) obtained at the lowest infusion rate for normal rats in the present study (fig. 2(a)). Assuming that there is no significant species difference in the disposition of pravastatin in the body between rat and human, we can roughly estimate the contribution of the carrier-mediated transport in the biliary excretion under clinical conditions. The resultant *K_{M}* in *vivo* (181 μM) is considerably higher than *C_{liver}*(0.73 ± 0.09 μM) at the lowest infusion rate. Therefore, we can evaluate the contribution of the saturable transport to total biliary excretion as follows: *(V_{max, in vivo} * K_{M, in vivo})/(V_{max, in vivo} * K_{M, in vivo} + P_{diff, in vivo} ) × 100 ≈ 80%*. This calculation, combined with the result that indicates that the *K_{M}* and *K* values obtained in *vitro* with CMVs are comparable with that of *K_{M}* in *vivo*, allows us to state that the biliary excretion of pravastatin is governed mainly by a cMOAT-mediated transport mechanism at therapeutic doses. Previously, we clarified that the hepatic uptake of pravastatin occurs >90% by a carrier-mediated transport mechanism that we identified using isolated hepatocytes (4). This is the first case in which both the hepatic uptake and the biliary excretion of one drug can be explained mainly by carrier-mediated transport mechanisms at therapeutic concentrations.

In conclusion, biliary excretion of pravastatin was mediated mainly by cMOAT in normal rats. This can explain the decrease in the biliary excretion of pravastatin in EHBRs *in vivo*. Further studies are required to clarify the nature of the transport mechanism exhibiting saturation without ATP-dependence.

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**References**


