Carrier-Mediated Mechanism for the Biliary Excretion of the Quinolone Antibiotic Grepafloxacin and Its Glucuronide in Rats

HIROYUKI SASABE, AKIRA TSUJI and YUICHI SUGIYAMA
Faculty of Pharmaceutical Sciences (H.S. and Y.S.), University of Tokyo, Tokyo, Japan and Faculty of Pharmaceutical Sciences (A.T.), University of Kanazawa, Kanazawa, Ishikawa, Japan
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ABSTRACT
Grepafloxacin (GPFX) has a comparatively greater hepatobiliary transport than other quinolone antibiotics. The biliary excretion mechanism of GPFX was investigated in a series of in vivo and in vitro studies with Sprague-Dawley rats and the mutant strain Eisai-hyperbilirubinemia rats (EHBR), which have a hereditary defect in their biliary canalicular multispecific organic anion transport system (cMOAT). The biliary excretion of the parent drug in EHBR was 38% of that in normal rats, whereas the 3-glucuronide, a main metabolite of GPFX, was scarcely excreted into the bile in EHBR. To clarify the biliary excretion mechanism of GPFX, studies of uptake by bile canalicular membrane vesicle (CMV) were performed. ATP dependence was observed in the uptake of GPFX by CMV, although the extent was not very marked, whereas no ATP-dependent uptake was observed by CMV prepared from EHBR. An inhibition study of the ATP-dependent uptake of the glutathione conjugate, 2,4-dinitrophenyl-S-glutathione (DNP-SG), a typical substrate for cMOAT, was performed in order to differentiate among the affinities of six quinolone antibiotics for this transporter. All quinolone antibiotics inhibited the ATP-dependent uptake of DNP-SG with different half-inhibition concentrations (IC50), and GPFX had the lowest IC50 value. The uptake of GPFX-glucuronide by CMV from normal rats showed a marked ATP dependence, whereas there was little ATP-dependent uptake in EHBR. The Km value (7.2 μM) for the higher-affinity component of the glucuronide uptake was comparable to the Ki value (9.2 μM) of the glucuronide in terms of inhibition of the ATP-dependent uptake of DNP-SG, which indicates that DNP-SG and the glucuronide may share the same transporter, cMOAT. The Ki value of the glucuronide observed in this inhibition was less than 1/200 that of the parent, which suggests that the glucuronide had a much higher affinity than the parent drug. These results lead us to conclude that at least a part of the GPFX transport and a major part of its glucuronide transport across the bile canalicular membrane are by a primary active transport mechanism mediated by cMOAT.

The total body clearance of many NQ occurs mainly via metabolic elimination and urinary excretion, whereas the biliary clearance of newly developed quinolones such as GPFX and SPFX is greater than for other NQ (Matsunaga et al., 1991; Akiyama et al., 1995). As a first step in clarifying the mechanism that determines the degree of biliary clearance of NQ, we previously studied the hepatic uptake using isolated rat hepatocytes and found that NQ are taken up by the liver via a carrier-mediated active transport system (Sasabe et al., 1997). However, the biliary clearance was governed not only by the degree of hepatic uptake but also by the intrinsic ability of excretion into bile across the bile canalicular membrane. Therefore, in order to understand the mechanism for the effective hepatobiliary transport of GPFX, it is necessary to assess the transport system involved in the excretion process.

Some primary active transport systems driven by cellular ATP hydrolysis are known to mediate the biliary excretion of a number of compounds. Of these, 1) the first transporter is for bile acids (Inoue et al., 1984; Adachi et al., 1991; Nishida et al., 1991) such as TCA; 2) the second is for amphipathic organic cations and neutral compounds, including anticancer drugs (Kamimoto et al., 1989), called P-glycoprotein; 3) the third (cMOAT) is for organic anions, including a number of conjugates such as LTC4 (Ishikawa et al., 1990; Sathirakul et al., 1994), an endogenous compound, and DNP-SG (Kobayashi et al., 1990), a glutathione conjugate, and some glucuronide conjugates of exogenous compounds (Shimamura et al., 1994; Takenaka et al., 1995a, 1995b). Multiplicity in transporters for organic anions has been recently investigated (Takenaka et al., 1995a; Yamazaki et al., 1996).

Mutant rats such as TR(−) (Jansen et al., 1985; Nishida et al., 1992a) and EHBR (Mikami et al., 1986), which have an

ABBREVIATIONS: NQ, quinolone antibiotics; GPFX, grepafloxacin; SPFX, sparflloxacin; LFLX, lomefloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; ENX, enoxacin; TCA, taurocholic acid; cMOAT, bile canalicular multispecific organic anion transport system; LTC4, leukotriene C4; EHBR, Eisai-hyperbilirubinemia rats; CMV, bile canalicular membrane vesicle; DNP-SG, 2,4-dinitrophenyl-S-glutathione; Km, Michaelis constant; Vmax, maximum uptake rate; Fsn, nonspecific uptake clearance; AUC, area under the curve; TLC, thin-layer chromatography.
inherited deficiency in their cMOAT, are a useful tool for investigating the transport mechanism involved in biliary excretion. Recently, using molecular biological techniques, the organic anion transporters from livers of Wistar and Sprague-Dawley strains of rat have been successfully cloned (Paulusma et al., 1996; Ito et al., 1997), and it has become clear that the transporter has two different ATP-binding regions. The origin of the molecular mutation in TR(−) and EHBR has also been identified (Mayer et al., 1995; Paulusma et al., 1996; Ito et al., 1997).

The biliary excretion of the glutathione conjugate LTC4 and those of organic anions such as dibromosulfophthalein (DBSP) and the β-lactam antibiotic cefodizime are markedly reduced in EHBR (Huber et al., 1987; Sathirakul et al., 1993). The studies using CMVs have directly demonstrated the ATP-dependent uptake of LTC4 (Ishikawa et al., 1990; Fernandez-Checa et al., 1992), whereas there was no uptake of these compounds by CMV prepared from mutant rats, TR(−) and EHBR, although the ATP-dependent uptake of TCA remained normal (Takenaka et al., 1995a). These results suggest that biliary excretion of LTC4 is mediated by cMOAT. Moreover, it has been reported that the glucuronides of some drugs are actively excreted into bile by cMOAT, although this transporter does not recognize the sulfate conjugates of these same drugs (Kobayashi et al., 1991; Shimamura et al., 1994; Takenaka et al., 1995a, 1995b).

The membrane vesicle experiments are very useful for transporter studies. For example, the vesicle studies of kidney and intestine indicated the possible presence of transporters that mediate the excretion of ofloxacin into urine and the absorption of enoxacin via the brush-border membrane of each organ (Okano et al., 1990; Iseki et al., 1992; Hirano et al., 1994). However, the excretion mechanism by which NQ are transported across the bile canalicular membrane has never been investigated until now.

In order to understand how the active transport system for organic anions mediates excretion of NQ from liver cells to bile, we selected GPFX, which has a greater hepatobiliary organic anions mediates excretion of NQ from liver cells to bile, we selected GPFX, which has a greater hepatobiliary flux. In this study, we investigated the transport mechanism of the 3-glucuronide, the main metabolite of GPFX, as well as that of GPFX itself.

### Methods and Materials

**Chemicals.** [14C]GPFX (1.17 MBq/μmol, radiochemical purity 97.1%) was obtained from Amersham International (Buckinghamshire, UK). [3H]5-DNP-SG was synthesized from 1-chloro-2,4-dinitrobenzene and radiolabeled glutathione (GSH) in the presence of glutathione S-transferase by the method described by Kobayashi et al. (1990). [3H]GSH (1.62 MBq/μmol, 99.9%) and [3H]TCA, (128 MBq/μmol, 98.5%) were purchased from New England Nuclear Corp (NEN, Boston, MA). The [3H]-glucuronide of GPFX was isolated from the bile of rats given i.v. [3H]GPFX at a dose of 3.7 MBq/3.2 μmol/rat (1.7 mg/rat). Unlabelled 3-glucuronide was obtained from the bile of rats receiving a constant infusion (57 μg/69.6 nmol/min/kg). Bile (1 ml) was applied to a pretreatment column (Bond Elut C18, 3 ml/500 mg, Varian, CA), eluted with methanol-water (15:85, v/v) and the eluate injected into an HPLC column to purify the 3-glucuronide. Unlabeled GPFX, SPFX, LFLX, CPFX, ENX and OFLX were synthesized or purified by Otsuka Pharmaceutical company (Tokyo, Japan). ATP, creatine phosphate and creatine phosphokinase were purchased from Sigma Chemical Co. (St. Louis, MO).

**In vivo study.** Male Sprague-Dawley rats (Nihon Ikaagaku, Tokyo, Japan) and EHBR (SLC Japan, Shizuoka) weighing approximately 250 to 300 g were used throughout the experiments. Under light ether anesthesia, the femoral artery and the femoral vein were cannulated with polyethylene catheters (PE-50) for blood sampling and injection of GPFX, respectively. The bile duct was cannulated with a polyethylene catheter (PE-50) for bile collection; both ureters were also cannulated to collect urine. Radiolabeled GPFX was given i.v. at a dose of 5 mg/kg (788 KBq/13.9 μmol/kg). Bile was collected in preweighed test tubes at 10-min intervals until 1 hr after administration and then at 30 min intervals from 1 to 2 hr after administration. Urine was collected in test tubes at 30-min intervals throughout the experiment. Plasma was prepared by centrifugation of blood samples (10,000 × g, Microfuge, Beckman, Fullerton, CA).

**TLC analysis.** The metabolites of GPFX were purified from rat bile and human urine after p.o. administration of GPFX at a dose of 40 mg/kg and 400 mg/body, respectively. Then they were identified using mass spectrometry and 1H-NMR spectrometry as described by Akiyama et al. (1995). The metabolites in rats and humans were the same except for one minor metabolite.

In this study, unchanged GPFX and its metabolites in bile, urine and plasma were separated by TLC to determine their concentrations. Plasma and bile were mixed with three volumes of acetonitrile and centrifuged to precipitate proteins. Aliquots of these supernatants were applied to TLC plates (Kieselgel 60 F254, Merck, FRG), and developed with chloroform-methanol-28% ammonia (7:3:0.5, v/v/v). In addition, the urine was directly applied to TLC plates. Plasma samples from three rats were combined, because the volumes of plasma were small and the radioactivity in each plasma sample was low.

The radioactive profiles on the TLC plates were analyzed using a Bio-Imaging analyzer (Bas2000, Fuji Film, Tokyo, Japan). In this analysis, the Rf of GPFX and its 3-glucuronide were approximately 0.6 and 0.15, respectively. The radioactivity in the 3-glucuronide fraction of GPFX in bile disappeared after digestion with β-glucuronidase (Sigma, G-0251), whereas that in the parent fraction increased.

It has been reported by Akiyama et al. (1995) that unchanged GPFX is largely present in rat plasma and its 3-glucuronide accounts for approximately 1/10 the GPFX concentration in plasma. Moreover, the 3-glucuronide is the predominant component in rat bile, although the 4'-sulfate, M-1 and M-2 could also be detected. M-1 and M-2 with a cleaved piperadine ring have almost the same Rf (approximately 0.4) on TLC and could not be distinguished from each other. Therefore, their combined excretion was assessed. Akiyama reported that the total amount of M-1 and M-2 in bile was similar to that of the

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**Fig. 1.** Chemical structure of GPFX and its metabolic pathway.
4'-sulfate (Akiyama et al., 1995). We were able to take another fraction in which the radioactivity was similar to that of M-1 and M-2 in this TLC analysis of rat bile. The 4'-sulfate fraction of GPFX was unaffected by treatment with either β-glucuronidase or aryl sulfatase, (Sigma, S-9754) which was reported to be unable to digest sulfate where the sulfur atom was directly bound to a nitrogen atom (Akiyama et al., 1995). The fraction for which the Rf value on TLC was approximately 0.2 was considered the 4'-sulfate on the basis of the afore-mentioned information.

The biliary and urinary clearances of GPFX and its glucuronide were calculated by dividing the amount excreted in bile or urine by the AUC of the corresponding plasma concentration profiles.

Preparation of CMVs. CMV were prepared from male SDR and EHBR using a slight modification of the method of Meier et al. (1984). After suspension of vesicles in 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose, the vesicles were frozen in liquid N2 and stored at −80°C until required. The transport activity of CMV used in this study was also checked by measuring the ATP-dependent uptake of standard substrates, [3H]TCA (1 μM) and [3H]DNP-SG (1 μM), for a 2-min incubation period at 37°C. Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard, using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Uptake of [14C]GPFX and [14C]GPFX-glucuronide by CMV. The uptake of [14C]GPFX or [14C]GPFX-glucuronide was measured by the rapid-filtration technique described by Ishikawa et al. (1990). The transport medium (10 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 10 mM MgCl2 contained 50 μM [14C]GPFX or [14C]GPFX-glucuronide, 5 mM ATP and ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). The transport medium (final 40 μl) was mixed with 10 μl of vesicle suspension (20 μg protein) and incubated at 37°C. The uptake reaction was stopped by addition of 1 ml of ice-cold buffer containing 100 mM NaCl, 250 mM sucrose and 10 mM Tris-HCl (pH 7.4) at designated times. This reaction mixture (900 μl) was then filtered through a 0.45-μm HAWP filter (Millipore Corp., Bedford, MA) and washed twice with 5 ml of ice-cold buffer. In the uptake study of GPFX, the filter was steeped in 1% bovine serum albumin solution for one night before the experiment in order to reduce nonspecific adsorption of GPFX to the filter. Radioactivity retained on the filter was determined by liquid scintillation counting. The uptakes of these compounds by CMV were normalized with respect to both the amount of vesicles and the concentration of ligand in the reaction mixture. In the saturation study of the uptake of GPFX-glucuronide, the transport medium contained 5, 10, 20 and 50 μM [14C]GPFX-glucuronide. For higher concentrations, such as the final 100, 200 and 1000 μM, the ligand solution was prepared with radiolabeled (50 μM) and unlabeled GPFX-glucuronide.

Uptake of [3H]DNP-SG and [3H]TCA by CMV. The uptake of [3H]DNP-SG (1 μM) and that of [3H]TCA (1 μM) were examined by the same method as for GPFX-glucuronide except for a change in the volume of the incubation mixture (final 20 μl) and amount of vesicles (10 μg protein). The uptake reaction was stopped at 2 min. For the inhibition of the uptake of [3H]DNP-SG, the reaction mixture was incubated in the presence of some NQ at concentrations of 0.1, 0.3, 1, 3 and 10 mM or of GPFX-glucuronide at concentrations of 0.1, 1, 10 and 100 μM and 1, 3 and 10 mM.

Estimation of kinetic parameters. For the ATP-dependent uptake of GPFX-glucuronide by CMV, which was obtained by subtracting the uptake in the absence of ATP from that uptake in its presence, the following equation was used.

\[ V_0 = \frac{V_{\text{max}} \times S}{K_{m1} + S} + \frac{V_{\text{max}} \times S}{K_{m2} + S} \]  

(1)

Where \( V_0 \) is the initial uptake rate of the drug (pmol/min/mg protein), S is the drug concentration in the medium (μM), \( K_{m1} \) and \( K_{m2} \) are the Michaelis constants (μM), and \( V_{\text{max1}} \) and \( V_{\text{max2}} \) are the maximum uptake rates (pmol/min/mg protein). The foregoing equation was fitted to the uptake data set by an iterative nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981) in order to obtain estimates of kinetic parameters. The input data were weighted as the reciprocal of the observed values, and the Damping Gauss Newton Method algorithm was used for fitting.

In the inhibition studies of [3H]DNP-SG ATP-dependent uptake by GPFX-glucuronide at several concentrations, Eq. 2 was fitted to the data on the uptake velocity (\( V_0 \)) and inhibitor concentrations (I) for competitive inhibition in order to obtain the \( K_i \) of the inhibitor.

\[ V_0 = \frac{V_{\text{max}} \times S}{K_m \times \left(1 + \frac{I}{K_i}\right) + S} \]  

(2)

The DNP-SG concentration (S) and \( K_m \) were kept constant (1.0 μM and 15.8 μM, respectively). This \( K_m \) value was obtained from another study in this laboratory (Niinuma et al., 1997).

Results

Biliary excretion profiles of GPFX and GPFX-glucuronide in normal rats and EHBR. After a single i.v. administration of GPFX at a dose of 5 mg/kg to normal rats and EHBR, biliary excretion profiles were compared. The biliary excretion of GPFX in EHBR was 38% of that in normal rats (fig. 2A), whereas 3-glucuronide of GPFX, which is a major metabolite of GPFX, underwent very little excretion into the bile in EHBR (fig. 2B). Combined excretions of M-1 and M-2 with a cleaved piperadine ring exhibited a pattern similar to that of unchanged drug in terms of the comparison between normal rats and EHBR (fig. 2C). There was no difference in the biliary excretion of its 4'-sulfate between normal rats and EHBR (fig. 2D).

Urinary excretion profiles of GPFX and GPFX-glucuronide in normal rats and EHBR. No difference in the urinary excretion of GPFX was observed between normal rats and EHBR (table 1). 4'-Sulfate, M-1 and M-2 also showed no difference in urinary excretion between normal rats and EHBR, whereas the urinary excretion (0–120 min after administration of GPFX at a dose of 5 mg/kg) including 0.79 μmol/kg. Parent (panel a), 3-glucuronide (panel b), M-1 and M-2 (panel c) and 4'-sulfate (panel d) in bile samples were separated by TLC. Each point represents the mean ± S.E. of three rats. * P < .05, ** P < .01 (significantly different from the excretion over 120 min in normal rats using Student’s t test).
Inhibition of [3H]DNP-SG uptake into CMV (fig. 5). GPFX, which exhibit relatively greater hepatobiliary excretion, had greater inhibitory effects; they inhibited the [3H]DNP-SG uptake to 54.7 ± 1.7% and 60.0 ± 4.6% of the control, respectively, at a concentration of 3 mM.

Uptake profiles of GPFX-glucuronide by CMV prepared from normal rats and EHBR. The uptake of GPFX-glucuronide by CMV prepared from normal rats exhibited a marked ATP dependence and an overshoot phenomenon (fig. 6). The ATP-dependent uptake of GPFX-glucuronide by CMV prepared from EHBR was markedly reduced compared with CMV prepared from normal rats (table 3). The ATP-dependent uptake showed a saturable phenomenon. When the data were converted into Eadie-Hofstee plots, this saturable component included higher- and lower-affinity components (fig. 7). This uptake provided a $K_m$ of 7.2 ± 2.4 μM and a $V_{max}$ of 0.460 ± 0.110 nmol/min/mg protein for the high-affinity component and a $K_m$ of 169 ± 110 μM and a $V_{max}$ of 0.657 ± 0.102 nmol/min/mg protein for the low-affinity component.

Inhibition of [3H]DNP-SG uptake by GPFX and GPFX-glucuronide. The GPFX-glucuronide inhibited the ATP-dependent uptake of [3H]DNP-SG by CMV in a concentration-dependent manner (fig. 8). The glucuronide in this inhibition study has a $K_i$ of 9.2 ± 1.7 μM, which is comparable to its own $K_m$ (7.2 μM) for the higher-affinity component of ATP-dependent uptake. The $K_i$ of the parent drug obtained in the same study was 1.89 ± 0.80 μM, which demonstrated that the affinity of the glucuronide is approximately 200 times higher than that of the parent (fig. 8).

**Discussion**

It has been proved that the carrier-mediated active transporter contributes to the transport of organic anions from liver cells to bile. Detailed studies of the organic anion transporter have been performed using mutant rat strains, TR(−) and EHBR, in which cMOAT is hereditarily defective with respect to biliary excretion (Ishikawa et al., 1993, 1994; Yamazaki et al., 1996). In the present study, the biliary excretion of GPFX in EHBR was reduced to approximately one-third that in normal rats (fig. 2A). A main metabolite of GPFX, the 3-glucuronide, was scarcely excreted into bile in EHBR (fig. 2B), which indicates that cMOAT mediates the major portion of its biliary excretion. Such reduced biliary excretion in EHBR has also been observed for the biliary excretion of nonconjugated organic anion, DBSP, glutathione conjugates, LTC₄, BSP-GSH and glucuronides of...
TABLE 2
Biliary and urinary clearance of GPFX and its glucuronide based on plasma concentrations after a single i.v. dose of \[^{14}C\]GPFX to normal rats and EHBR

<table>
<thead>
<tr>
<th></th>
<th>Biliary Clearance (ml/min/kg)</th>
<th>Urinary Clearance (ml/min/kg)</th>
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<tbody>
<tr>
<td></td>
<td><strong>Parent</strong></td>
<td><strong>3-Glucuronide</strong></td>
</tr>
<tr>
<td>Normal rats</td>
<td>1.79 ± 0.05</td>
<td>15.53 ± 0.90</td>
</tr>
<tr>
<td>EHBR</td>
<td>0.52 ± 0.01**</td>
<td>0.09 ± 0.01**</td>
</tr>
</tbody>
</table>

These clearance values were calculated by dividing the amount excreted over the period up to 120 min after an administration by the AUC_{0–120 min} for the plasma concentrations.

Each point represents the mean ± S.E. of three rats.

**P < .01** (significantly different from the corresponding excretion over 120 hr in normal rats using Student’s t test).

Fig. 4. Uptake profiles of GPFX by canicular membrane vesicles prepared from normal rats (panel a) and EHBR (panel b). Uptake of GPFX was determined by incubating the vesicles (20 μg) at 37°C for 2, 5, 15 and 30 min after the addition of GPFX. The incubation mixture (40 μl) contained 50 μM \[^{14}C\]GPFX with (●) or without (○) 5 mM ATP, 10 mM creatine phosphate and 100 μg/ml creatine phosphokinase. Each column and vertical bar represents the mean ± S.E. of three different experiments for EHBR. *P < .05, **P < .01 (significantly different from the uptake in the corresponding ATP(−) group using Student’s t test).

Fig. 6. Uptake profiles of GPFX-glucuronide by CMV prepared from normal rats and EHBR.

TABLE 3
Uptake of GPFX-glucuronide by CMV prepared from normal rats and EHBR

<table>
<thead>
<tr>
<th></th>
<th>Normal Rats</th>
<th>EHBR</th>
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<tr>
<td>Uptake (μl/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ATP(+)</strong></td>
<td>91.1 ± 4.2**</td>
<td>24.2 ± 1.6**</td>
</tr>
<tr>
<td><strong>ATP(−)</strong></td>
<td>5.5 ± 0.2</td>
<td>9.5 ± 0.9</td>
</tr>
</tbody>
</table>

Uptake of GPFX-glucuronide was determined by incubating the vesicles (20 μg) at 37°C for 2 min after the addition of GPFX-glucuronide (10 μM). Each point represents the mean ± S.E. of 5 to 6 determinations.

**P < .01** (significantly different from the corresponding ATP(−) uptake using Student’s t test).

The plasma concentration profile of parent drug in EHBR did not differ substantially from that in normal rats (fig. 3A), whereas GPFX-glucuronide concentration increased with time in EHBR (fig. 3B). This increase is thought to be due to back flux of the glucuronide produced from the liver cells to the plasma compartment, caused by a marked reduction in the biliary excretion of the glucuronide.

The urinary excretion of GPFX-glucuronide increased in EHBR (table 1). Inasmuch as the urinary excretion clearance of GPFX did not change in EHBR (table 2), this increase is thought to reflect the increase in the plasma concentration of GPFX-glucuronide that came from the reduction in its biliary excretion.

It may be that the reduction in the biliary excretion of the glucuronide in mutant rats is caused by a deficiency not only in biliary excretion ability but also in conjugating ability. Moreover, when some endogenous substances such as bilirubin glucuronide cannot be excreted into the bile in the mutant rats, and do accumulate in liver cells, the biliary excre-
Fig. 7. Eadie-Hofstee plot of GPFX-glucuronide uptake by CMV prepared from normal rats. Uptake of GPFX-glucuronide was determined by incubating the vesicles (20 μg) at 37°C for 2 min after the addition of [14C]GPFX-glucuronide. The mixture (40 μl) contained 5, 10, 20, 50, 100, 200 and 1000 μM [14C]GPFX-glucuronide with or without 5 mM ATP, 10 mM creatine phosphate and 100 μg/ml creatine phosphokinase. ATP-dependent uptake (●) was calculated by subtracting ATP(−) uptake from the corresponding ATP(+) uptake. The solid line was obtained as follows. First, ATP-dependent uptake data were fitted to equation 1, and then the fitted line was converted to the V0/S vs. V0 form (Eadie-Hofstee plot). Each plot represents the mean ± S.E. of three determinations.

Fig. 8. Effect of GPFX and GPFX-glucuronide on the ATP-dependent uptake of DNP-SG by CMV prepared from normal rats. Uptake of DNP-SG was determined by incubating the vesicles (10 μg) at 37°C for 2 min after the addition of [3H]DNP-SG. The incubation mixture (20 μl) contained 1 μM [3H]DNP-SG and GPFX-glucuronide (●) 0, 0.0001, 0.001, 0.01, 0.1, 1, 3 and 10 mM or GPFX (○) 0.1, 1, 3 and 10 μM with or without 5 mM ATP, 10 mM creatine phosphate and 100 μg/ml creatine phosphokinase. ATP-dependent uptake was calculated by subtracting ATP(−) uptake from the corresponding ATP(+) uptake. The solid line was obtained as follows. First data were fitted to equation 2, and then the fitted line was converted to the V0/S vs. I form. Each plot and vertical bar represent the mean ± S.E. of three determinations.

Differences in the contribution of biliary clearance to the total clearance of NQ may be due to variations in the affinity for the transport systems involved. Therefore, in order to differentiate the affinity of NQ for cMOAT, we tested the inhibitory effects of NQ on the CMV uptake of [3H]DNP-SG; this is a glutathione conjugate reported to be transported exclusively by cMOAT (Ninuma et al., 1997). GPFX with a Kᵢ (9.2 μM) of 1.89 mM had the highest affinity for cMOAT among the NQ tested (fig. 5). Although the CMV uptake of NQ apart from GPFX has not been examined, these results may suggest that the other NQ could be substrates for cMOAT. GPFX and SPFX have been classified as to the biliary excretion type of NQ (Sekine, 1991; Akiyama et al., 1995). GPFX and SPFX had greater inhibitory effects than the urinary excretion type, LFLX, which suggests that the affinity of NQ for cMOAT may be one of the factors that determines the degree of biliary clearance. On the other hand, GPFX and SPFX also have a higher affinity for the transporter that mediates uptake into liver cells, whereas LFLX and GPFX have a lower affinity (Sasabe et al., 1997). The difference in the biliary clearance of NQ might stem from differences in affinity for both uptake and excretion transporters among NQ.

GPFX is conjugated to glucuronide and sulfate at the 3-carboxyl group and 4- amino group in the piperazine ring, respectively (fig. 1). These conjugations are known as major metabolites of GPFX (Akiyama et al., 1995). Urinary excretion of the GPFX-glucuronide was much smaller than its biliary excretion (table 1), which suggests that the glucuronide produced is excreted mainly into bile. Biliary excretion of this 3-glucuronide of GPFX was scarcely observed in EHBR, whereas that of the 4'-sulfate was similar to that in normal rats (fig. 2). These phenomena were also observed in earlier results from our laboratory involving the biliary excretion of E-3040 conjugates (Takenaka et al., 1995a,b). The glucuronide was excreted by cMOAT, whereas the sulfate was excreted by another transporter (Takenaka et al., 1995a,b).

Moreover, the uptake of the GPFX-glucuronide by CMV consisted predominantly of ATP-dependent transport (fig. 6), which was not observed to any great extent in CMV from EHBR (table 3), which indicates that a large fraction of the biliary excretion was mediated by cMOAT. The Kᵢ value (9.2 μM, fig. 8) of the glucuronide for the uptake of [3H]DNP-SG by CMV was comparable with the Kᵢ value (7.2 μM) of the high-affinity component in its own ATP-dependent uptake (fig. 7). Therefore, GPFX-glucuronide and DNP-SG may share the same transporter, cMOAT. The Kᵢ value (9.2 μM) of the glucuronide produced was less than 1/200 of that of the parent (1.89 mM, fig. 8), which suggests that the glucuronide had a much higher affinity for cMOAT. Moreover, these results from the in vitro study correlated with those from the in vivo study in which 29% of the uptake of exogenously administered anionic compounds may also be reduced by secondary effects, the endogenous substances inhibiting the transport. Jansen et al. (1987) and Elfferink et al. (1989) have investigated this using a double-mutant rat derived from TR(−) and Gunn rats that has an inherited deficiency in its conjugation with glucuronic acid. Although the double-mutant rat did not exhibit cholestasis of conjugated bilirubin, the biliary excretion of DNP-SG and BSP was reduced in this rat, as in the TR(−) strain. These results rule out the possibility of secondary effects and suggest that the reduction in biliary excretion of these organic anionic compounds is directly caused by the deficiency in cMOAT in the mutant rat. Furthermore, ATP dependence was observed in the uptake of these compounds by CMV (Nishida et al., 1992a,b), and this ATP-dependent uptake was reduced markedly in CMV prepared from EHBR, which indicates that the biliary excretion is by primary active trans-

port. Therefore, the reduction in the biliary excretion of GPFX and its glucuronide in EHBR strongly suggests that their excretion is mediated by cMOAT (fig. 2A,B).

To further clarify the mechanism for the biliary excretion of GPFX, we examined the uptake by CMV prepared from normal rats and EHBR. We observed ATP-dependent uptake only by CMV from normal rats and not from EHBR (fig. 4), which indicates that the biliary excretion of GPFX may be mediated at least partly by cMOAT if we consider the in vitro results (fig. 2A) together. However, this small ATP-dependent uptake indicates that GPFX may have a relatively weak affinity for cMOAT.

Although the double-mutant rat did not exhibit cholestasis of conjugated bilirubin, the biliary excretion of DNP-SG and BSP was reduced in this rat, as in the TR(−) strain. These results rule out the possibility of secondary effects and suggest that the reduction in biliary excretion of these organic anionic compounds is directly caused by the deficiency in cMOAT in the mutant rat. Furthermore, ATP dependence was observed in the uptake of these compounds by CMV (Nishida et al., 1992a,b), and this ATP-dependent uptake was reduced markedly in CMV prepared from EHBR, which indicates that the biliary excretion is by primary active trans-

port. Therefore, the reduction in the biliary excretion of GPFX and its glucuronide in EHBR strongly suggests that their excretion is mediated by cMOAT (fig. 2A,B).

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Differences in the contribution of biliary clearance to the total clearance of NQ may be due to variations in the affinity for the transport systems involved. Therefore, in order to differentiate the affinity of NQ for cMOAT, we tested the inhibitory effects of NQ on the CMV uptake of [3H]DNP-SG; this is a glutathione conjugate reported to be transported exclusively by cMOAT (Ninuma et al., 1997). GPFX with a Kᵢ (9.2 μM) of 1.89 mM had the highest affinity for cMOAT among the NQ tested (fig. 5). Although the CMV uptake of NQ apart from GPFX has not been examined, these results may suggest that the other NQ could be substrates for cMOAT. GPFX and SPFX have been classified as to the biliary excretion type of NQ (Sekine, 1991; Akiyama et al., 1995). GPFX and SPFX had greater inhibitory effects than the urinary excretion type, LFLX, which suggests that the affinity of NQ for cMOAT may be one of the factors that determines the degree of biliary clearance. On the other hand, GPFX and SPFX also have a higher affinity for the transporter that mediates uptake into liver cells, whereas LFLX and GPFX have a lower affinity (Sasabe et al., 1997). The difference in the biliary clearance of NQ might stem from differences in affinity for both uptake and excretion transporters among NQ.

GPFX is conjugated to glucuronide and sulfate at the 3-carboxyl group and 4- amino group in the piperazine ring, respectively (fig. 1). These conjugations are known as major metabolites of GPFX (Akiyama et al., 1995). Urinary excretion of the GPFX-glucuronide was much smaller than its biliary excretion (table 1), which suggests that the glucuronide produced is excreted mainly into bile. Biliary excretion of this 3-glucuronide of GPFX was scarcely observed in EHBR, whereas that of the 4'-sulfate was similar to that in normal rats (fig. 2). These phenomena were also observed in earlier results from our laboratory involving the biliary excretion of E-3040 conjugates (Takenaka et al., 1995a,b). The glucuronide was excreted by cMOAT, whereas the sulfate was excreted by another transporter (Takenaka et al., 1995a,b).

Moreover, the uptake of the GPFX-glucuronide by CMV consisted predominantly of ATP-dependent transport (fig. 6), which was not observed to any great extent in CMV from EHBR (table 3), which indicates that a large fraction of the biliary excretion was mediated by cMOAT. The Kᵢ value (9.2 μM, fig. 8) of the glucuronide for the uptake of [3H]DNP-SG by CMV was comparable with the Kᵢ value (7.2 μM) of the high-affinity component in its own ATP-dependent uptake (fig. 7). Therefore, GPFX-glucuronide and DNP-SG may share the same transporter, cMOAT. The Kᵢ value (9.2 μM) of the glucuronide produced was less than 1/200 of that of the parent (1.89 mM, fig. 8), which suggests that the glucuronide had a much higher affinity for cMOAT. Moreover, these results from the in vitro study correlated with those from the in vivo study in which 29% of the
biliary excretion of the parent remained and minimal glucuronide was excreted into bile in EHBR (table 2). However, small but significant ATP-dependent uptake of GPFX-glucuronide was observed in EHBR CMV (approximately one-fifth that in normal rats, table 3). We have already suggested that the glucuronide of E-3040 was taken up into CMV not only by cMOAT but also by the other transporter that exists in EHBR, an interpretation based on the results of mutual inhibition studies of DNP-SG and E-3040 glucuronide (Niinuma et al., 1997). Thus we propose that multiplicity exists in the biliary transporter for organic anions. The!multiplicity may also be evident in this study using CMV in that the biliary excretion of GPFX-glucuronide may also be partially mediated by a transporter that was present in EHBR. However, this assumption seems to be inconsistent with the in vivo result that GPFX-glucuronide was scarcely excreted into bile in EHBR (table 2). This apparent discrepancy, however, may be resolved by assuming that some endogenous substance, such as bilirubin glucuronide, which accumulates in liver cells of EHBR, may inhibit excretion under in vivo conditions.

At least part of the biliary excretion of GPFX and a large portion of the main metabolite 3-glucuronide were shown to occur by active primary transport mediated by cMOAT with respect to hepatobiliary transport. Moreover, the affinity of NQ for cMOAT may be one of the factors that determines the degree of biliary clearance.

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References


