Pharmaceutical Research, Vol. 25, No. 3, March 2008 (© 2007)
DOI: 10.1007/s11095-007-9427-9

Research Paper

Predominant Contribution of Rat Organic Anion Transporting Polypeptide-2 (Oatp2) to Hepatic Uptake of β-Lactam Antibiotics

Masanori Nakakariya,[1] Taiki Shimada,[1] Masanori Irokawa,[1] Hiroyuki Koibuchi,[1] Takashi Iwanaga,[1] Hikaru Yabuuchi,[2] Tomoji Maeda,[1] and Ikumi Tamai[1,3]

Received April 12, 2007; accepted July 24, 2007; published online September 22, 2007

Purpose. To identify the rat hepatic basolateral transporters involved in the hepatic uptake of β-lactam antibiotics using nafcillin as a model β-lactam antibiotic that undergoes extensive biliary excretion.
Materials and Methods. Uptake by isolated rat hepatocytes and *Xenopus laevis* oocytes expressing organic anion transporting peptides (Oatp1, 2, and 4) and organic anion transporter (OAT2) was evaluated.
Results. Nafcillin uptake by isolated rat hepatocytes was saturable with the K_m of 210 μM and was significantly inhibited by anionic compounds (estrone-3-sulfate and sulfobromophthalein), but not by cationic compounds (tetraethylammonium and 1-methyl-4-phenylpyridinium). In an *in vitro* uptake study by *Xenopus* oocytes expressing hepatic basolateral membrane transporters, nafcillin was transported by multiple Oatps with K_m values of 4120 μM (Oatp1/Oatp1a1), 198 μM (Oatp2/Oatp1a4), and 1,570 μM (Oatp4/Oatp1b2), though it was not transported by hOAT2. Comparison of affinity and analysis by the relative activity factor method indicated that Oatp2 is the predominant contributor to the hepatic uptake of nafcillin. Cefadroxil, cefazolin, cefmetazole, cefoperazone, cefsulodin, and cephalexin, though not cefotaxime or ceftriaxone, were also substrates of Oatp2.
Conclusion. These findings suggest that Oatp2 plays a key role in the hepatic uptake of nafcillin and most other β-lactam antibiotics in rats.

KEY WORDS: biliary excretion; hepatic uptake; oatp; organic anion; transporter.

INTRODUCTION

It is well known that most β-lactam antibiotics are eliminated from the body through the renal elimination pathway (1,2), while others are eliminated into the bile via the liver (3–5). It has been suggested that molecular weight and lipid solubility are factors determining the biliary excretion of β-lactam antibiotics (6,7), but the underlying mechanisms have not been established.

We have investigated the mechanisms of hepatic uptake of β-lactam antibiotics through the basolateral membrane using freshly isolated rat hepatocytes and a tissue sampling single injection technique (8–12), and we examined the efflux mechanism using isolated rat canalicular membrane vesicles (13). We found that most β-lactam antibiotics were transported by a common organic anion transporter(s), though its molecular identity remains to be identified.

Various transporter molecules have been shown to be involved in hepatic uptake and biliary excretion at the basolateral and the bile canalicular membranes, and their significance in hepatic handling of endogenous and xenobiotic compounds was demonstrated (14). Identification of the transporters involved in the hepatobiliary transport of drugs should enable us to predict potential drug–drug interactions based on competition at transporters, as well as the effects of genetic variants of transporters on the pharmacokinetics of drugs (15–17). Various organic anion- and cation-specific transporters have been identified at the basolateral membrane of hepatocytes, including organic anion transporting polypeptide (Oatp; 18), organic anion transporter (Oat; 19), and organic cation transporter (Oct; 20). OATP/Oatp have many subtypes, and play major roles in the hepatic handling of a variety of anionic xenobiotics, including methotrexate, pravastatin, troglitazone, and irinotecan (15,21–23).

As regards β-lactam antibiotics, rat Mrp2, which is expressed in the canalicular membrane of the liver, was shown to be involved in the biliary excretion of benzylpenicillin (PCG; 24). Further, PCG is transported by OATP1B1 and by Npt1 in basolateral membrane of human and mouse

[1] Department of Membrane Transport and Pharmacokinetics, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamasaki, Noda, Chiba 278-8510, Japan.
[2] GenoMembrane, Inc., Tsurumi-ku, Yokohama 230-0040, Kanagawa, Japan.
[3] To whom correspondence should be addressed. (e-mail: tamai@rs.noda.tus.ac.jp)

ABBREVIATIONS: BSP, sulfobromophthalein; $E_1$3S, estrone-3-sulfate; MPP^+, 1-methyl-4-phenylpyridinium; Mrp, multidrug resistance-associated protein; Ntcp, Na^+-taurocholate cotransporting polypeptide; OAT/Oat, organic anion transporter; OATP/Oatp, organic anion transporting polypeptide; PCG, benzylpenicillin; RAF, relative activity factor; TEA, tetraethylammonium.

hepatocytes, respectively (25,26). However, since several transporters, including their subtypes, are expressed in hepatic basolateral membrane, a more comprehensive study is needed to identify the transporter molecules involved in biliary excretion of β-lactam antibiotics.

Nafcillin is excreted into bile to the extent of 97 and 90% in dogs and humans, respectively, and is classified as a biliary excretion type of β-lactam antibiotic (3,27). Since protein binding of nafcillin is relatively high (>90%), the contribution of glomerular filtration to the excretion of nafcillin may be small. Although other β-lactam antibiotics, such as cefazolin and cefmetazole, also exhibit high protein binding (>80%), they are mainly excreted into urine. Since these three β-lactam antibiotics all have similar lipid solubility and molecular weight, other factors must account for the biliary excretion of nafcillin. Nafcillin is known to interact with bilirubin and sulfobromophthalein (BSP), which are substrates of OATPs (28), and it inhibits the uptake of PCG by isolated rat hepatocytes in a competitive manner (8). Therefore, it is possible that nafcillin is taken up by rat hepatocytes via a common transporter for β-lactam antibiotics.

The purpose of the present study is to molecularly identify the transporter(s) responsible for the hepatic uptake of nafcillin and other β-lactam antibiotics, in order to throw light on the underlying mechanisms that determine the biliary excretion of these drugs and other anionic xenobiotics. In addition, since organic anion transporting polypeptides (Oatps) appear to be involved, at least in part, in nafcillin transport, and plural subtypes of Oatps are expressed at the basolateral membranes in rat hepatocytes, we compared the relative importance of each Oatp subtype in the hepatic handling of anionic compounds.

MATERIALS AND METHODS

Chemicals

[^{3}H]Digoxin (0.87 TBq/mmol), [^{3}H]estrone-3-sulfate, ammonium salt (1.7 TBq/mmol), and [^{3}H]prostaglandin E_2 (7.4 TBq/mmol) were purchased from Perkin Elmer Life Sciences, Inc. (Boston, MA). [^{14}C]Inulin carboxyl (92.5 MBq/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Nafcillin, ceftriaxone, cefoperazone, cefotaxime, cafazolin, cefadroxil, digoxin, estrone-3-sulfate ($E_1$3S), BSP, and 1-methyl-4-phenylpyridinium (MPP^+) were purchased from Sigma-Aldrich (St. Louis, MO). Cephalexin and tetraethylammonium (TEA) were purchased from Wako Pure Chemicals (Osaka, Japan). Cefmetazole and cefsulodin were from USP Convention Inc. (Rockville, MD) and MP Biomedicals L.L.C. (Aurora, OH), respectively. All other reagents were purchased from Sigma-Aldrich (St Louis, MO), Wako Pure Chemicals (Osaka, Japan), Kanto Chemicals (Tokyo, Japan), or Nacalai tesque (Kyoto, Japan).

Animals

Male Wistar rats (7–8 weeks old) were purchased from Sankyo Labo Service, Inc. (Hamamatsu, Japan). Rats were housed three per cage with free access to commercial chow and tap water, maintained on a 12-h dark/light cycle (8:00 A.M.–8:00 P.M. light) in an air-controlled room (temperature, 24.5 ± 1°C; humidity, 55 ± 5%). *Xenopus laevis* were purchased from Hamamatsu Biological Research Service, Inc. (Hamamatsu, Japan). Animal care and experimentation were conducted according to the guidelines of the Tokyo University of Science.

cDNA Cloning of Oatp1 (Oatp1a1), Oatp2 (Oatp1a4), Oatp4 (Oatp1b2), moat1 (Oatp2b1, Oatp9), and OAT2

The human OAT2 (*SLC22A7*) gene was PCR-amplified using human liver-derived HepG2 cell cDNA as a template, with upstream primer 5′-GGATCCGAGCTGGCTGGA TACTAGAGG-3′ and downstream primer 5′-TCTAGAT AGGCAGAGGGTTCGATACTC-3′ (both synthesized by Hokkaido System Science, Hokkaido, Japan), and Ex taq DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan), based on the reported human OAT2 gene sequence (GeneBank accession number NM 006672). A major 1.8-kbase polymerase chain reaction product was ligated into the TA cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and then human OAT2 cDNA was digested with *Bam*HI and *Xba*I, and ligated to pcDNA3.1 (Invitrogen). The obtained cDNA sequences were analyzed and confirmed to be the same as the reported one.

The rat Oatp1 (slco1a1), Oatp2 (slco1a4), and Oatp4 (slco1b2) genes were PCR-amplified using rat liver cDNA as a template, with upstream primer 5′- CAATCAGAA GAACACCATGGAAGAAACAGA-3′ and downstream primer 5′- CCTTGAACAGGGCAGTAGAAAACTC -3′, upstream primer 5′- ATGGGAAAATCTGAGAA AAGGGTTGCAAC-3′ and downstream primer 5′- GTA GAAAACTCATTACAGCTTCGTTTTCAG-3′, and upstream primer 5′- TCCCATCACAACCACTGTTCAG TC-3′ and downstream primer 5′- GGGTATTTGAAAA CACAGCAACAGATGTAT-3′, respectively (all synthesized by Invitrogen and KOD-Plus-Ver.2 polymerase [TOYOBO]), based on the reported Oatp1, Oatp2, and Oatp4 gene sequences (GeneBank accession numbers NM_017111, NM_131906, and NM_031650, respectively). Major polymerase chain reaction products were ligated into the cloning vector pCR-Blunt II-TOPO (Invitrogen) and then these cDNAs were digested with *Xho*I and *Asp718I*, and ligated to pcDNA3.1 (Invitrogen). The obtained cDNA sequences were analyzed and confirmed to be the same as the reported ones.

Rat moat1 (slco2b1) was PCR-amplified using rat small-intestinal cDNA as a template, with upstream primer 5′-CTTTGGGAAGAGCAGGTGAG-3′ and downstream primer 5′-GTCCAGTGACCTATTGTCGG-3′ (both synthesized by Hokkaido System Science) and Ex taq DNA polymerase (Takara Shuzo Co. Ltd.), based on the reported moat1 gene sequence (GeneBank accession number NM_08 0786). A major 2.0-kbase polymerase chain reaction product was ligated into the TA cloning vector pCR2.1-TOPO (Invitrogen) and then moat1 cDNA was digested with *Bam*HI and *Xba*I, and ligated to pcDNA3 (Invitrogen). The obtained cDNA sequences were analyzed and confirmed to be the same as the reported one.

Uptake Study by Isolated Rat Hepatocytes

Hepatocytes were isolated from rats by means of the procedure described previously (29). After isolation, the hepato-

cytes were suspended in Hank's buffer (1.26 mM $CaCl_2$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.4 mM $MgSO_4$, 136.9 mM NaCl, 4.2 mM $NaHCO_3$, 0.38 mM Na_2HPO_4, 5 mM D-glucose, pH 7.4). Immediately after preparation of the isolated hepatocytes, suspended hepatocytes were used for uptake experiments within four hours after preparation. To examine the sodium dependence of nafcillin uptake by rat hepatocytes, experiments were performed in Hank's buffer in which sodium was replaced with potassium. The hepatocyte suspension and Hank's buffer containing a test compound were separately incubated at 37°C for 5 min and then transport was initiated by mixing them. At appropriate times, 100 μl aliquots of the mixture were withdrawn and the hepatocytes were separated from the transport medium by centrifugal filtration through a layer of a mixture of silicon oil ($d = 1.07$; SH550; Dow Corning Toray Silicone Co. Ltd., Tokyo, Japan) and liquid paraffin ($d = 0.86$; Wako Pure Chemicals) with a density of 1.03. The uptake rate of nafcillin was corrected for the adherent volume calculated from the [^{14}C]inulin uptake. Digoxin was dissolved in dimethylsulfoxide to give a final concentration 1%.

Uptake Experiments Using *Xenopus laevis* Oocytes Expressing Transporter Genes

Complementary RNAs (cRNAs) of Oatp1, Oatp2, Oatp4, moat1, hOAT2 were prepared by *in vitro* transcription with T7 RNA polymerase in the presence of ribonuclease inhibitor and an RNA cap analog using a mMESSAGE mMACHINE kit (Ambion, Austin, TX). Uptake experiments were conducted as described previously (30). Briefly, for standard experiments, defolliculated oocytes were injected with 50 nl of water containing 25 ng of cRNA, cultured for 3 days at 18°C in modified Barth's solution (96 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ and 10 mM HEPES, pH 7.4) and used for uptake experiments. Uptake was initiated by incubating the oocytes at room temperature in modified Barth's solution containing a test compound. At appropriate times, the oocytes were washed with ice-cold modified Barth's solution to terminate the uptake. Transporter activity was routinely checked using [^{3}H]estrone-3-sulfate (Oatp1, Oatp2, and Oatp4) and [^{3}H]prostaglandin E_2 (moat1 and hOAT2) in each assay, because they are well-established substrates of respective transporters. For quantitation of test compounds, the oocytes were solubilized in 5% sodium dodecyl sulfate solution in the case of radiolabeled compounds ([^{3}H]digoxin, [^{3}H]estrone-3-sulfate, and [^{3}H]prostaglandin E_2). The uptake of radiolabeled compounds was quantified with a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan) after adding 1.2 ml of scintillation fluid (Clear-sol, Nacalai tesque, Kyoto, Japan).

Estimation of Uptake Clearance of Nafcillin by Oatp2 in Rat Hepatocytes

To estimate the contribution of Oatp2 to the overall hepatic uptake, we adopted the relative activity factor (RAF) method, which has been applied to examine the relative contributions of hepatic uptake transporters OATP1B1 and OATP1B3 to apparent hepatic uptake (31). Based on this method, we estimated the contribution of Oatp2 to the overall uptake of nafcillin by rat hepatocytes. Since digoxin could be a selective substrate of Oatp2 among the Oatps which are expressed in liver (32,33), it was used as a reference compound for Oatp2-mediated uptake. The ratio of the uptake clearance of the reference compound in hepatocytes ($CL_{hepatocyte,\ digoxin}$) to that in the Oatp2 expression system ($CL_{Oatp2,\ digoxin}$) was calculated and defined as R_{act}. The hepatic uptake clearance of nafcillin by Oatp2 ($CL_{hepatocyte,\ nafcillin,\ Oatp2}$) was calculated by multiplying the uptake clearance of nafcillin in Oatp2-expressing oocytes ($CL_{Oatp2,\ nafcillin}$) by R_{act}, according to the following equation.

$$R_{act} = CL_{hepatocyte,\,digoxin} / CL_{Oatp2,\,digoxin} \quad (1)$$

$$CL_{hepatocyte,\,nafcillin,\,Oatp2} = CL_{Oatp2,\,nafcillin} \times R_{act} \quad (2)$$

The uptake clearance by hepatocytes was determined by calculating the slope of the uptake plot between 30 and 45 s for digoxin and between 15 and 60 s for nafcillin. The saturable component of the uptake clearance by hepatocytes was determined by subtracting clearance in the presence of 250 μM digoxin and 20 mM nafcillin, respectively.

The uptake clearance by *Xenopus* oocytes expressing Oatp2 was obtained from the uptake at 15 min for digoxin and 30 min for nafcillin. Specific uptake by Oatp2 was determined by subtracting the uptakes by water-injected oocytes from those by Oatp2 cRNA-injected oocytes.

HPLC Analysis of β-Lactam Antibiotics

The amounts of nafcillin and other β-lactam antibiotics in oocytes and hepatocytes were determined by high-performance liquid chromatography (HPLC). The oocytes and hepatocytes were collapsed by sonication in 400 μl of a mixture of 75% methanol and 25% water, and centrifuged at 13,000×*g* for 15 min. The resultant supernatant was transferred to a new tube and evaporated to dryness under a vacuum. The residue was dissolved in 150 μl of mobile phase, vortex-mixed, and injected to the HPLC instrument. HPLC analyses were carried out on a Alliance system (Waters, Milford, MA) consisting of the 2690 separation module, and the 2487 dual-wavelength absorbance detector (224 nm for nafcillin, 229 nm for cefadroxil, 275 nm for cefazolin, 250 nm for cefmetazole and cefotaxime, 240 nm for cefoperazone, 280 nm for cefsulodin, and 260 nm for cephalexin, and 274 nm for ceftriaxone). An analytical column, 250 × 4.6-mm i.d. Mightysil RP-18GP Aqua column (Kanto Chemical, Tokyo, Japan), was used with mobile phases of a mixture of 30 mM phosphate buffer (pH 3.0) and methanol in ratios of 40:60% for nafcillin, 90:10% for cefadroxil, 75:25% for cefazolin and cefmetazole, 70:30% for cefoperazone, 95:5% for cefsulodin, and 80:20% for cephalexin, cefotaxime, and ceftriaxone, at a flow rate of 1 ml/min and at 40°C. The Alliance HPLC system was controlled with Empower software (Waters ver. 1, Milford, MA).

Analytical Methods

To estimate the kinetic parameters for saturable transport, the initial uptake rate (v) was fitted to the following

equation by nonlinear least-squares regression analysis using KaleidaGraph (Synergy Software, Reading, PA).

$$v = V_{max} \times s/(K_m + s) + kd \times s$$

where v, s, K_m, V_{max} and kd are the uptake rate, concentration of substrate, the half-saturation concentration (Michaelis constant), the maximum transport rate, and the apparent non-saturable first-order rate constant, respectively. All data were expressed as means ± SEM, and statistical analysis was performed by the use of Student's t test with $p < 0.05$ as the criterion of significance.

RESULTS

Characterization of Nafcillin Uptake by Isolated Rat Hepatocytes

The uptake of nafcillin (100 μM) by rat hepatocytes increased linearly up to 1 min (Fig. 1a). Thus, the initial uptake rate was obtain as the slope of the uptakes at 15 and 30 s and was used in the subsequent studies. The concentration dependence of nafcillin uptake by rat hepatocytes was studied (Fig. 1b). The uptake was saturable, and the K_m, V_{max}, and kd values were 210 ± 112 μM, 58.8 ± 25.0 pmol s^{-1} mg $protein^{-1}$, and 0.0145 ± 0.0113 μl s^{-1} mg $protein^{-1}$, respectively (Table I). In addition, the inhibitory effects of various compounds were examined. Uptake of nafcillin was reduced by anionic compounds (BSP and E_13S), whereas cationic compounds (TEA and MPP^+) had no effect (Fig. 2). Moreover, when extracellular sodium ions were replaced with potassium ions at equimolar concentration, the uptake of nafcillin was not changed (Fig. 2).

Uptake of Nafcillin by *Xenopus* Oocytes Expressing Rat Oatps and Human OAT2

To examine whether nafcillin could be transported by hepatic transporters, the uptakes of nafcillin by oocytes expressing rat Oatp1, Oatp2, Oatp4, moat1, and human OAT2 were measured. The time courses of nafcillin uptake by Oatp1, Oatp2, and Oatp4 were examined using oocytes expressing these transporters and are shown in Fig. 3. The uptakes of nafcillin by oocytes expressing Oatps increased linearly up to 90 min for Oatp1 and Oatp2, and up to 180 min for Oatp4, and were significantly higher than those by water-injected oocytes ($p < 0.05$; Fig. 3). Moreover, uptakes of nafcillin at 120 min by *Xenopus* oocytes expressing moat1 (20.3 nl/oocyte) was significantly higher than that by oocytes injected with water (control, 5.96 nl/oocyte; data not shown). Since Oatp1, Oatp2, and Oatp4 are expressed on the basolateral membranes of rat hepatocytes, we focused on these three transporters in the following study. Accordingly, the uptakes at 60 min for Oatp1 and Oatp2 and at 150 min for Oatp4 were routinely used for measurement of the initial uptake rate in subsequent studies. The oocytes expressing OAT2 showed no significant nafcillin uptake, while the uptake of [^{3}H]prostagrandin E_2 as a positive control of OAT2 activity was clearly observed by OAT2 (data not shown).

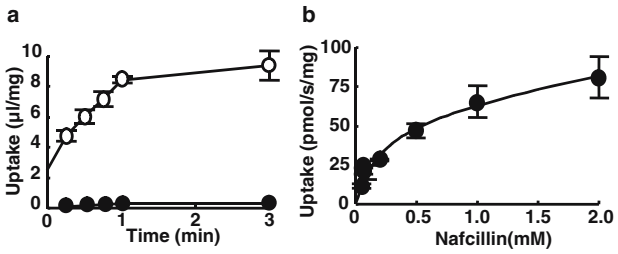

Fig. 1. Time course and concentration dependence of nafcillin by isolated rat hepatocytes. **a** Uptake of nafcillin (100 μM) by the isolated rat hepatocytes was measured in the presence (*closed circles*) or absence (*open circles*) of 20 mM nafcillin over 3 min at 37°C and pH 7.4. **b** Nafcillin uptake by isolated rat hepatocytes was measured over the concentration range of 50–2,000 μM at 15 and 30 s, 37°C and pH 7.4. Each result represents the mean ± SEM ($n = 4$).

The concentration dependence of nafcillin uptake was next examined (Fig. 4). The uptake was saturable, and the K_m values of nafcillin uptake were 4,120 ± 962 μM (Oatp1, Fig. 4a), 198 ± 45.7 μM (Oatp2, Fig. 4b), and 1,570 ± 459 μM (Oatp4, Fig. 4c). The V_{max} values of nafcillin transport were 241 ± 33.3 pmol 60 min^{-1} $oocyte^{-1}$ (Oatp1), 16.3 ± 0.900 pmol 60 min^{-1} $oocyte^{-1}$ (Oatp2), and 11.9 ± 2.10 pmol 150 min^{-1} $oocyte^{-1}$ (Oatp4; Table I).

Estimation of Contribution of Oatp2 to Nafcillin Uptake with the RAF Method

To estimate the contribution of Oatp2 to naficillin uptake by rat hepatocytes, the uptake clearance of the reference compound (digoxin for Oatp2) was estimated in *Xenopus* oocytes and rat hepatocytes. The measured values of uptake clearance of digoxin in isolated hepatocytes and Oatp2-expressing oocytes were 117.0 μl min^{-1} mg $protein^{-1}$ and 26.4 nl min^{-1} $oocyte^{-1}$, respectively (Fig. 5a and b). Consequently, R_{act} was calculated to be 4,431 oocyte/mg protein. The measured values of uptake clearance of nafcillin by rat hepatocytes and Oatp2-expressing oocytes were 4.86 μl min^{-1} mg $protein^{-1}$, and 0.993 nl min^{-1} $oocyte^{-1}$, respectively (Figs. 1a and 5c). From these data, the estimated uptake clearance of nafcillin mediated by Oatp2 in rat hepatocytes was calculated to be 4.40 μl min^{-1} mg $protein^{-1}$. Thus, the contribution of Oatp2 to the overall uptake of nafcillin in rat hepatocytes was determined to be 90.6%. In other words, Oatp2 is the predominant contributor to the apparent uptake of nafcillin by hepatocytes.

Uptake of β-Lactam Antibiotics by *Xenopus* Oocytes Expressing Oatp2

Since Oatp2 was found to be the key Oatp subtype in the hepatic basolateral uptake of nafcillin, the uptake of other β-lactam antibiotics via Oatp2 was examined. As shown in Table II, the uptakes of cefadroxil, cefazolin, cefmetazole, cefoperazone, cefsulodin, and cephalexin by oocytes expressing Oatp2 were higher than those by water-injected oocytes, although cefotaxime and ceftriaxone did not appear to be transported by Oatp2. Accordingly, it was demonstrated that Oatp2 can transport most β-lactam antibiotics.

DISCUSSION

Elucidation of the molecular mechanisms of the biliary excretion of drugs is important for efficient drug discovery

Table I. K_m and V_{max} Values of Nafcillin Uptake by *Xenopus* Oocytes Expressing Oatps and by Isolated Hepatocytes in Rats

	Oatp1	Oatp2	Oatp4	Isolated Hepatocytes
K_m (μM)	4,120 ± 962	198 ± 45.7	1,570 ± 459	212 ± 112
V_{max}	241 ± 33.3 pmol 60 min^{-1} $oocyte^{-1}$	16.3 ± 0.900 pmol 60 min^{-1} $oocyte^{-1}$	11.9 ± 2.10 pmol 150 min^{-1} $oocyte^{-1}$	58.8 ± 25.0 pmol s^{-1} $mg\ protein^{-1}$

Each value represents the mean ± SEM (oocytes: $n = 8 - 10$, hepatocytes : $n = 4$).

and development, and also for ensuring appropriate clinical pharmacotherapy, since hepatic handling often significantly affects the effectiveness and toxicity of drugs. There are several factors that influence the biliary excretion of drugs, and in the present study we focused on the hepatic basolateral membrane transporters, since multiple transporter molecules are expressed at the membrane and they mediate the first step of hepatic handling. As model compounds, we used β-lactam antibiotics, focusing mainly on nafcillin, since β-lactam antibiotics include derivatives that exhibit a variety of biliary excretion characteristics. In particular, nafcillin is exclusively excreted into bile (3,27), and we have previously suggested that nafcillin is taken up by rat hepatocytes via organic anion transporters in common with other β-lactam antibiotics (8). Accordingly, in the present study, we evaluated the transport of nafcillin and other several β-lactam antibiotics to identify the transporter molecule(s) responsible for the hepatic uptake in rats.

The uptake of nafcillin by freshly isolated rat hepatocytes was saturable, with a K_m of 210 μM, and was inhibited by anionic compounds (BSP and $E_1$3S), but not by cationic compounds (TEA and MPP^+; Figs. 1b and 2). Moreover, sodium-independent transport of nafcillin was observed in isolated rat hepatocytes (Fig. 2). Therefore, it appears that nafcillin is taken up by rat hepatocytes via a sodium-independent organic anion transporter(s).

By measuring the nafcillin uptake into *Xenopus* oocytes expressing known hepatic organic anion transporters, it was demonstrated that Oatp1, Oatp2, Oatp4 and moat1, but not hOAT2, have the potential to transport nafcillin (Fig. 3). Accordingly, it is possible that multiple rat Oatps are involved in the hepatic uptake of nafcillin. We focused on the uptake of nafcillin by Oatp1, Oatp2 and Oatp4, since they are known to be expressed at the basolateral membrane of rat hepatocytes. From the initial uptakes of nafcillin mediated by Oatp1, Oatp2, and Oatp4, we obtained K_m values of 4,120, 198, and 1,570 μM, respectively (Table I). Oatp2 exhibited highest affinity for nafcillin, and the K_m value of Oatp2 was close to that observed in isolated rat hepatocytes (210 μM). Accordingly, we considered that Oatp2 is likely to play a major role in hepatic uptake of nafcillin.

Then, we evaluated the relative contribution of Oatp2 to the apparent uptake into rat hepatocytes by using the relative activity factor (RAF) method. The RAF method has been applied to evaluate the relative importance of hepatic uptake transporters (31). Since digoxin is a selective substrate of Oatp2 (32,33), it was used as a reference compound for Oatp2-mediated uptake in the present study. The ratio of the uptake clearance of digoxin in rat hepatocytes to that in the Oatp2-expressing system was calculated and defined as R_{act}. The uptake clearance by Oatp2 in hepatocytes was obtained by multiplying the uptake clearance of nafcillin in the Oatp2-expressing system by R_{act}. This study revealed that the contribution of Oatp2 to hepatic uptake of nafcillin is about 91%. The observed affinity of Oatp2 for nafcillin and the contribution to uptake obtained by the RAF method provide strong evidence that Oatp2 is the predominant contributor to the hepatic uptake of nafcillin in rats. This is the first study to elucidate the relative contribution of rat Oatp subtypes to the apparent hepatic uptake of drugs.

We next examined the contribution of Oatp2 to the transport of other β-lactam antibiotics. Six of eight β-lactam

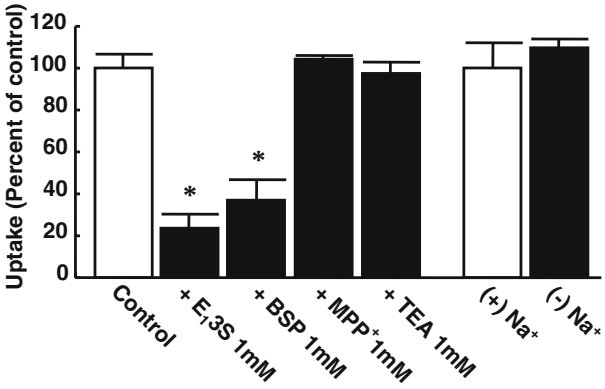

Fig. 2. Effect of various compounds and sodium ions on uptake of nafcillin by isolated rat hepatocytes. Uptake of nafcillin (100 μM) was measured at 15 and 30 s. The results are shown as a percentage of control uptake measured in the absence of inhibitor and in the presence of sodium ions. The concentration of each compound was 1 mM. Each column represents the mean ± SEM ($n = 4$). *Asterisk*, significant difference from the uptake by the control ($p < 0.05$).

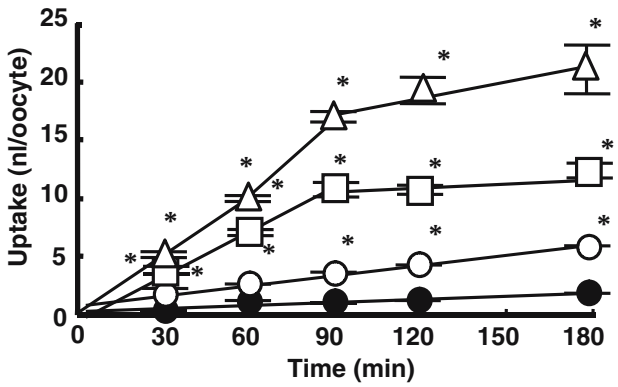

Fig. 3. Time course of nafcillin uptake by *Xenopus* oocytes expressing Oatp1, Oatp2, and Oatp4. Uptake of nafcillin (1 mM) by oocytes injected with cRNA of Oatp1 (*square*), Oatp2 (*triangle*), Oatp4 (*open circles*) or with water (*closed circles*) was measured over 180 min at room temperature and pH 7.4. Each result represents the mean ± SEM ($n = 8 - 10$). *Asterisk*, significant difference from the uptake by water-injected oocytes ($p < 0.05$).

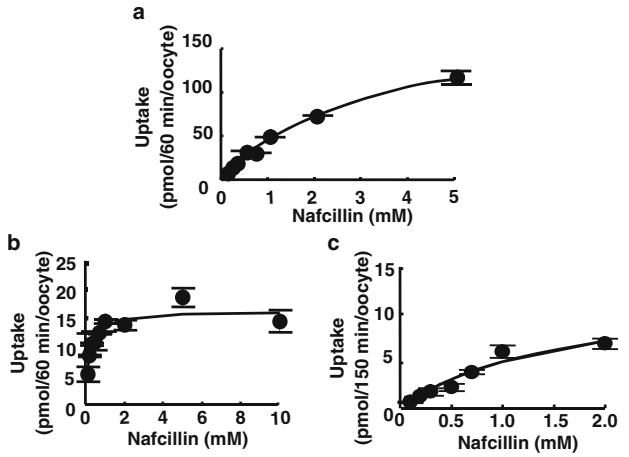

Fig. 4. Concentration dependence of nafcillin uptake by *Xenopus* oocytes expressing **a** Oatp1, **b** Oatp2, and **c** Oatp4. Oatp-mediated uptake of nafcillin by *Xenopus* oocytes was determined by subtracting the uptake into water-injected oocytes from that into Oatp-cRNA-injected oocytes at 60 (Oatp1 and Oatp2) or 150 min (Oatp4) at room temperature and pH 7.4. Each result represents the mean ± SEM (oocytes : $n = 8 - 10$).

antibiotics examined were transported via Oatp2, suggesting that Oatp2 commonly contributes to the hepatic uptake of most β-lactam antibiotics. We previously reported that a common organic anion transporter(s) is involved in hepatic uptake of these drugs (8, 10–12). Furthermore, the inhibition constant K_i of nafcillin for PCG uptake by isolated rat hepatocytes (1,120 μM; 8) is close to the K_m (210 μM) of nafcillin obtained in the present study. Accordingly, Oatp2 may be the key transporter responsible for the hepatic uptake of β-lactam antibiotics.

We found no significant correlation between uptake clearance via Oatp2 and other pharmacokinetic properties, such as biliary excretion, for the β-lactam antibiotics examined in the present study (Table II). This may be explained in two ways. One possibility is that hepatic uptake is not directly related to biliary excretion because bile canalicular membrane transport is rate-determining for excretion into bile. We have already shown that cefpiramide, a β-lactam antibiotic, is excreted into bile via organic anion transporters across the bile canalicular membrane (13), and multidrug resistance associated protein 2 (Mrp2), which is expressed at the canalicular membrane, plays a dominant role in the biliary excretion of β-lactam antibiotics (24). Therefore, we have to take the canalicular membrane transporters into consideration, as well as the hepatic basolateral membrane transporters, to understand the biliary excretion. The other possible reason for the lack of correlation may be the technical problem of the detection limit of the HPLC analysis. Transport of β-lactam antibiotics other than nafcillin was examined at 5 mM in the present study, since we could detect the uptakes of all the derivatives reliably at this concentration (Table II). It is possible that the hepatic uptake is saturated at 5 mM, since the K_m values of the drugs obtained in isolated hepatocytes were comparable with or less than 5 mM in our previous studies (8,10,11). If transport is saturated, this would account for the lack of correlation between the Oatp2-mediated transport and the pharmacokinetic properties related to biliary excretion. Further studies will be required to evaluate the relationship between the biliary excretion and hepatic uptake via Oatp2.

To identify the factors determining the extent of biliary excretion, a knowledge of the transporters involved in urinary excretion will also be essential, since the extent of biliary excretion represents the balance of urinary and biliary clearances. We previously found that tubular secretion of β-lactam antibiotics was mediated by organic anion transporter (12,34), and it has been reported that OATs localized at the basolateral membrane and Npt1 expressed at the apical membrane in kidney are involved in the excretion of β-

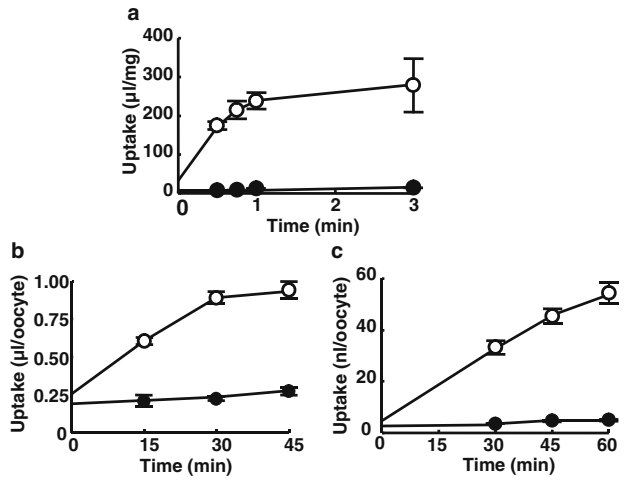

Fig. 5. Uptake of digoxin (**a**, **b**) and nafcillin (**c**) by isolated rat hepatocytes (**a**) and by *Xenopus* oocytes expressing Oatp2 (**b**, **c**). **a** Uptake of [^{3}H]digoxin (100 nM) by rat hepatocytes was measured in the presence (*closed circles*) or absence (*open circles*) of 250 μM unlabeled digoxin over 3 min at 37°C and pH 7.4. **b** Uptake of [^{3}H]digoxin (100 nM) by oocytes injected with cRNA of Oatp2 (*open circles*) or with water (*closed circles*) was measured over 45 min at room temperature and pH 7.4. **c** Uptake of nafcillin (100 μM) by oocytes injected with cRNA of Oatp2 (*open circles*) or with water (*closed circles*) was measured over 60 min at room temperature and pH 7.4. Each result represents the mean ± SEM (oocytes : $n = 8 - 10$, hepatocytes : $n = 4$).

Table II. Uptake of Various **β**-Lactam Antibiotics by *Xenopus* Oocytes Expressing Oatp2

Compound	Uptake (nl/oocyte)	
	Water	Oatp2
Cefoperazone	0.27 ± 0.05	2.60 ± 0.18⋆
Cefsulodin	0.64 ± 0.10	1.36 ± 0.10⋆
Cefmetazole	0.38 ± 0.08	15.9 ± 0.44⋆
Ceftriaxone	0.24 ± 0.05	0.32 ± 0.01
Cephalexin	0.68 ± 0.17	2.70 ± 0.18⋆
Cefazolin	0.62 ± 0.36	8.52 ± 2.01⋆
Cefadroxil	0.82 ± 0.18	2.34 ± 0.60⋆
Cefotaxime	0.20 ± 0.02	0.28 ± 0.07

The uptake of β-lactam antibiotics at the concentration of 5 mM by *Xenopus* oocytes injected with water (control) or with Oatp2-cRNA was measured at 120 min at room temperature and pH 7.4. Each result represents the mean ± SEM ($n = 6 - 7$).
⋆Significant difference from the uptake by water-injected oocytes ($p < 0.05$)

lactam antibiotics, especially the urinary excretion types (35,36).

In conclusion, in the present study we demonstrated that multiple Oatp transporters (Oatp1, Oatp2, Oatp4, and moat1) accept nafcillin and β-lactam antibiotics as substrates, and among them, Oatp2 is the predominant contributor to the hepatic uptake of these drugs. This is the first study to compare the relative contributions of rat Oatp subtypes to the hepatic uptake process. The relationship between Oatp2-mediated uptake and biliary excretion of the β-lactam antibiotics and the functional extrapolation of the present observations in rats to human OATPs will be the next targets of investigation.

ACKNOWLEDGEMENT

A part of this study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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