Comparative Inhibitory Effects of Different Compounds on Rat Oatp1 (*Slc21a1*)- and Oatp2 (*Slc21a5*)-Mediated Transport

Yoshihisa Shitara,[1] Daisuke Sugiyama,[2] Hiroyuki Kusuhara,[2,3] Yukio Kato,[2,3] Takaaki Abe,[4] Peter J. Meier,[5] Tomoo Itoh,[1] and Yuichi Sugiyama[2,3,6]

Received September 12, 2001; accepted October 26, 2001

Purpose. The purpose of the present study is to examine the selectivity of various inhibitors towards the rat organic anion transporting polypeptides 1 (Oatp1: gene symbol *Slc21a1*) and 2 (Oatp2: *Slc21a5*).
Methods. The inhibitory effects of 20 compounds on the Oatp1-mediated transport of estradiol 17β-D-glucuronide and on the Oatp2-mediated transport of digoxin were examined in cDNA-transfected LLC-PK_1 cells.
Results. Among the compounds examined in this study, nonsteroidal anti-inflammatory drugs, deoxycorticosterone, and quinidine preferentially inhibited Oatp1, whereas digoxin, quinine, and rifampicin preferentially inhibited Oatp2 at low concentrations. On the other hand, propionic acid, α-ketoglutarate and *p*-aminohippurate showed no inhibitory effects on either transporter up to a concentration of 1000 μM. The K_i values of ibuprofen and quinidine were estimated to be 19 and 13 times lower for Oatp1 compared with Oatp2, whereas the values for rifampicin, quinine, and digoxin were 13, 20, and 100< times lower for Oatp2 compared with Oatp1.
Conclusions. At low concentrations, some of the tested inhibitors exert selective inhibition of either Oatp1- or Oatp2-mediated substrate transport. These selective inhibitors may be used at appropriate concentrations to estimate the maximum contribution of Oatp1 or Oatp2 to the total substrate uptake into rat hepatocytes.

KEY WORDS: Oatp; organic anion transporter; hepatic transport.

INTRODUCTION

The liver plays an important role in the detoxification and elimination of many drugs and other xenobiotic substances. Drug detoxification in the liver is initiated by hepatic uptake across the sinusoidal membrane, followed by metabolism and biliary excretion so that the hepatic uptake rate is one of the determinants of drug disposition (1).

Previously, drug uptake studies were performed in sinusoidal membrane vesicles and in isolated and cultured hepatocytes to characterize the transport properties of hepatocellular drug uptake (2). In isolated and primary cultured hepatocytes, it is also possible to predict the *in vivo* elimination rate of drugs from the initial uptake rate in isolated or primary cultured hepatocytes by considering the number of hepatocytes per gram of liver (1,3). More recent studies to investigate the molecular mechanism of hepatic drug uptake used cloning to identify the drug transporters expressed at the sinusoidal membrane of rat hepatocytes. Transporters involved in the hepatic uptake of anionic compounds can be categorized into two families: the organic anion transporting polypeptide (Oatp) family and the organic anion transporter (Oat) family. It has been established that Oatp1 (gene symbol: *Slc21a1*), Oatp2 (*Slc21a5*), Oatp4 (*Slc21a10*) and Oat2 (*Slc22a7*) and Oat3 (*Slc22a8*) are expressed in rat liver (4–9). Functional analyses have revealed that many therapeutic drugs are also substrates for members of these organic anion transporter families (10,11).

Studies with cRNA-injected *Xenopus laevis* oocytes and cDNA-transfected mammalian cells have shown that many kinds of substrates, including organic anions (4,12,13), steroids (5,14), and bulky organic cations (15), are transported by members of the Oatp family. Other studies show that taurocholate, triiodothyronine, and thyroxine are common substrates of Oatp1, Oatp2, and Oatp4 (6,16,17), whereas bile acids, including cholate and glycocholate, organic anions, including estradiol 17β-D-glucuronide ($E_2$17βG) and estrone 3-sulfate, ouabain, and type II organic cations, including N-(4,4-azo-n-pentyl)-21-deoxyajmaline and rocuronium, are substrates of both Oatp1 and Oatp2 (15,17). In addition, digoxin is a specific substrate of Oatp2 (5,6) and leucotriene C_4 is recognized as a substrate by Oatp1 and Oatp4, but not by Oatp2 (6,17,18). These findings suggest that the hepatic transporters have a partially overlapping substrate specificity, *i.e.,* some substrates are transported by more than one transporter.

Although some substrates are known to be taken up into hepatocytes by multiple cloned transporters, investigations of the relative contribution of individual transporters to the overall hepatic uptake have been rather limited until now. One of the methods to estimate their contribution is to inhibit a single or multiple transporter(s) by using a specific inhibitor. Although the contribution of drug-metabolizing enzymes (the isoforms of cytochrome P450) has been examined in detail with use of this method (19), the contribution of transporters remains to be investigated.

In the present study, we focused particularly on the function of Oatp1 and Oatp2 and analyzed the comparative effect of inhibitors on these two transporters using Oatp1 and Oatp2 stably expressing LLC-PK_1 cells.

MATERIALS AND METHODS

Materials

[^{3}H]$E_2$17βG (1628 GBq/mmol) and [^{3}H]digoxin (703 GBq/mmol) were purchased from New England Nuclear

[1] School of Pharmaceutical Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108-8641, Japan.
[2] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
[3] CREST, Japan Science and Technology Corporation, Japan.
[4] Department of Neurophysiology, Tohoku University School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan.
[5] Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital Zurich, Rämistrasse 100, CH-8091 Zurich, Switzerland.
[6] To whom correspondence should be addressed. (e-mail: sugiyama@mol.f.u-tokyo.ac.jp)

ABBREVIATIONS: Oatp, organic anion transporting polypeptide; oat, organic anion transporter; $E_2$17βG, estradiol-17β-D-glucuronide; CL_{uptake}, uptake clearance; K_i, inhibition constant; P_{dif}, nonsaturable uptake clearance; NSAIDs, nonsteroidal anti-inflammatory drugs.

0724-8741/02/0200-0147/0

(Boston, MA). Unlabeled $E_2$17βG, digoxin, quinidine, and quinine were purchased from Sigma-Aldrich (St. Louis, MO). Ibuprofen and indomethacin were purchased from Wako Pure Chemicals (Tokyo, Japan). All other chemicals were commercially available and of analytical grade.

Construction of Oatp1- and Oatp2-Expressing LLC-PK_1 Cells

The construction of the Oatp1-expression vector is described by Kouzuki *et al* (20). Full-length cDNA for Oatp2 was initially cloned in the plasmid pBluescript SK(-) (Stratagene, La Jolla, CA) (6). Oatp2 cDNA was excised with *EcoR*V and *Hinc*II (Takara, Shiga, Japan) and subcloned into the *Xho*I site in the $pCXN_2$ vector (21) after converting to blunt ends. For the control study, $pCXN_2$ alone was used (vector transfected). For transfection, LLC-PK_1 cells were cultured in six-well culture plates in medium 199 (Sigma-Aldrich) supplemented with 10% fetal bovine serum and an antibiotic-antimycotic agent (Life Technologies, Grand Island, NY). At 30% confluence, cells were exposed to serum-free medium 199 containing plasmid (1 μg/mL) and lipofectAMINE (10 μg/mL) (Life Technologies). At 8 h after transfection, the medium containing plasmid and lipofectAMINE was replaced with culture medium containing 10% fetal bovine serum. Then, 2 days after transfection, 800 μg/mL G418 sulfate (Promega, Madison, WI) was added to the medium followed by culturing for a further 2 weeks to allow the selection of transfected cells. Expression levels of transporters were determined by Northern blot analysis, and cells with the highest expression levels were selected and used for all further studies.

Uptake Study

For the uptake study, cells were seeded on 12-well culture plates at 1.2×10^5 cells/well. After 2 days, culture medium was replaced with the same medium containing 4 mM sodium butyrate (Sigma-Aldrich) and cultured overnight to induce the transporters (22). Before initiation of the uptake study, cells were washed twice with Krebs-Henseleit buffer and preincubated in 0.5 mL of the same buffer at 37°C. The uptake study was initiated by replacing the Krebs-Henseleit buffer with 0.5 mL of the same buffer containing radiolabeled substrates and incubated at 37°C. In all uptake studies, [^{3}H]-$E_2$17βG and [^{3}H]-digoxin were used as radiolabeled substrates for Oatp1- and Oatp2-expressing cells, respectively, because their uptakes were sufficiently high to detect the effects of inhibitors. For the inhibition study, inhibitors and radiolabeled substrates were added simultaneously. The incubation period was 2 min and 5 min for the uptake of $E_2$17βG in Oatp1-expressing cells and digoxin in Oatp2-expressing cells, respectively, because preliminary experiments had shown that the transport rate was linear over these time periods (data not shown). At designated times, buffer was removed to terminate the reaction, and the cells were washed four times with ice-cold Krebs-Henseleit buffer. Then, the cells were dissolved in 0.5 mL 0.1 N NaOH overnight, followed by neutralization with an equal volume of 0.1 N HCl. Then, 0.8-mL aliquots were transferred to scintillation vials, and the radioactivities associated with cells and medium were determined in a liquid scintillation counter (LS6000SE; Beckman Coulter, Fullerton, CA). The remaining 0.1-mL aliquots of cell lysate were used to assay protein by using the Lowry method (23) with bovine serum albumin as a standard.

Data Analysis

The uptake of $E_2$17βG and digoxin was expressed as the uptake volume [μL/mg protein], defined as the amount of isotopes taken up into cells [dpm/mg protein] divided by their concentration in the incubation medium [dpm/μL]. The initial uptake rate of substrates was expressed as an uptake clearance (CL_{uptake}) [μL/min/mg protein], defined as the initial velocity of uptake divided by the incubation time.

The kinetic parameters for the uptake of $E_2$17βG and digoxin in the transporter-expressing cells were obtained by using the following equation:

$$v_0 = \frac{V_{max} \cdot S}{K_m + S} + P_{dif} \cdot S \quad (1)$$

where v_0 is initial uptake rate, S is the substrate concentration, K_m is the Michaelis constant, V_{max} is the maximal uptake rate, and P_{dif} is the nonsaturable uptake clearance.

The inhibition constant (K_i) was calculated by using the following equation:

$$CL_{uptake} = \frac{CL_{uptake}(0) - P_{dif}}{1 + I/K_i} + P_{dif} \quad (2)$$

where CL_{uptake} (0) is the uptake clearance in the absence of inhibitors, P_{dif} is the nonsaturable uptake clearance, and I is the inhibitor concentration. The above equations were fitted to the data from the uptake or inhibition study by using the nonlinear least-squares method by means of the computer program, MULTI (24), to obtain the required parameters. The input data were weighed as the reciprocal of the observed values, and the Damping Gauss Newton method was used as the fitting algorithm.

All results from the inhibition experiments are given as a percent of the control. As the number of cell passages increased, the contribution of transporter-mediated uptake to the total uptake into the transfected cells fell from 87.4 to 51.9% for Oatp1 and from 79.7 to 59.3% for Oatp2, presumably because of a reduction in the level of expression. However, we found that none of the inhibitors affected the uptake into vector-transfected LLC-PK_1 cells.

RESULTS

Concentration-Dependent Uptake of $E_2$17βG and Digoxin in Oatp1- and Oatp2-Expressing LLC-PK_1 Cells

The concentration-dependent uptake of $E_2$17βG and digoxin was examined in Oatp1- and Oatp2-expressing LLC-PK_1, respectively, to evaluate these experimental systems. Eadie-Hofstee plots of their uptake in transporter-expressing cells and mock-transfected cells are shown in Fig. 1. As shown in Fig. 1, concentration-dependent uptake of the substrates was observed in transporter-expressing cells but not in mock-transfected cells (Fig. 1). The obtained K_m, V_{max}, and P_{dif} values for the uptake of $E_2$17βG in Oatp1-expressing cells were 11.2 ± 4.7 μM, 114 ± 42 pmol/min/mg protein and 0.726 ± 0.479 μL/min/mg protein, respectively. The corresponding values for the uptake of digoxin in Oatp2-expressing

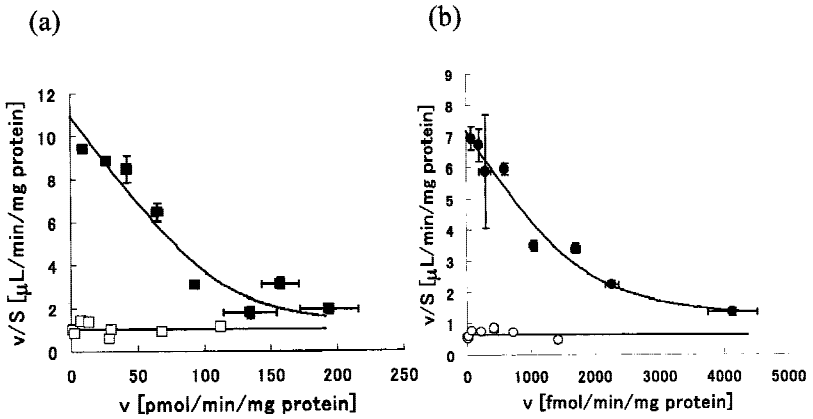

Fig. 1. Eadie-Hofstee plots of the uptake of $E_2$17βG into Oatp1-expressing LLC-PK_1 cells (a) and digoxin into Oatp2-expressing LLC-PK_1 cells (b). Uptake of [^{3}H]-$E_2$17βG (■) or [^{3}H]-digoxin (●) by oatp1- and oatp2-expressing LLC-PK_1 cells, respectively, was examined in the presence of various concentrations of unlabeled substrates. Uptake of $E_2$17βG (□) and digoxin (○) by vector-transfected cells is also shown. Each point and bar represent the mean ± SE (n = 3). The solid lines represent the fitted lines.

cells were 279 ± 57 nM, 1.76 ± 0.33 fmol/min/mg protein, and 0.856 ± 0.171 μL/min/mg protein.

Comparative Effect of Inhibitors on Oatp1- and Oatp2-Mediated Transport

To identify inhibitors that selectively inhibit Oatp1- or Oatp2-mediated transport, the inhibitory effects of a wide range of different compounds on the Oatp1-mediated transport of $E_2$17βG and the Oatp2-mediated transport of digoxin were compared. As shown in Table I, 17 compounds showed a concentration-dependent inhibition of both Oatp1 and Oatp2. In contrast, propionic acid (a monocarboxylate), α-ketoglutarate (a dicarboxylate), and *p*-aminohippurate inhibited neither Oatp1 nor Oatp2, even at concentrations as high as 1000 μM (Table I). Some inhibitors showed a selective inhibition of Oatp1 or Oatp2: NSAIDs (indomethacin, ibuprofen, ketoprofen, and naproxen), deoxycorticosterone, and quinidine preferentially inhibited Oatp1-mediated transport, whereas rifampicin, digoxin, and quinine preferentially inhibited Oatp2-mediated transport (Table I). To investigate their selectivity for these transporters, more detailed studies of their inhibitory effects were performed.

Inhibitory Effects of Ibuprofen

The concentration-dependent inhibitory effect of ibuprofen on the Oatp1-mediated transport of $E_2$17βG and the Oatp2-mediated transport of digoxin are shown in Fig. 2. In this study, ibuprofen concentrations ranged from 0 to 3000 μM. Although ibuprofen inhibited both Oatp1 and Oatp2 in a concentration-dependent manner, it inhibited Oatp1 more potently at low concentrations (Fig. 2). In contrast, ibuprofen had no effect on nonsaturable uptake into vector-transfected cells (Fig. 2). The K_i values for Oatp1 and Oatp2 were 126 ± 77 μM and 2430 ± 1590 μM (mean ± computer-calculated SD), respectively, supporting its selective inhibitory effect on Oatp1.

Inhibitory Effects of Rifampicin

The concentration-dependent inhibitory effect of rifampicin on the Oatp1-mediated transport of $E_2$17βG and the Oatp2-mediated transport of digoxin is shown in Fig. 3. The concentration of rifampicin ranged from 0 to 300 and 0 to 500 μM for the inhibition of Oatp1 and Oatp2, respectively. Rifampicin preferentially inhibited Oatp2 rather than Oatp1 (Fig. 3). Its K_i values for Oatp1 and Oatp2 were 18.2 ± 11.2 μM and 1.46 ± 0.58 μM (mean ± computer-calculated SD), respectively.

Inhibitory Effects of Quinine and Quinidine

We also investigated the inhibitory effects of quinine and its stereoisomer quinidine on Oatp1 and Oatp2. The concentrations of quinine and quinidine ranged from 0 to 1000 μM and from 0 to 400 μM, respectively. As shown in Fig. 3, although quinine inhibited Oatp2 more potently, its stereoisomer, quinidine, preferentially inhibited Oatp1 (Fig. 4). The K_i values of quinine for Oatp1 and Oatp2 were 76.7 ± 15.6 μM and 3.81 ± 1.36 μM (mean ± computer-calculated SD), respectively, and the corresponding values for quinidine were 9.27 ± 4.92 μM and 120 ± 27 μM (mean ± computer-calculated SD) for Oatp1 and Oatp2, respectively.

Inhibitory Effect of Digoxin

Although digoxin inhibited Oatp1-mediated transport only minimally, its inhibitory effect on Oatp2 was potent at 100 μM (Table I). On the basis of these results, we focused on the inhibitory effect of digoxin at lower concentrations to examine its selectivity for Oatp2. Up to 10 μM, digoxin inhibited Oatp2 in a concentration-dependent manner without affecting Oatp1 (Fig. 5). Digoxin (10 μM) almost completely inhibited Oatp2-mediated transport without significantly inhibiting Oatp1-mediated transport, suggesting that digoxin is a selective inhibitor of Oatp2 (Fig. 5). The K_i value for the Oatp2-mediated transport was 0.196 ± 0.037 μM (mean ±

Table I. Oatp1-Mediated Transport of $E_2$17βG and Oatp2-Mediated Transport of Digoxin in the Presence of Inhibitors[a]

Inhibitors	Oatp1-mediated transport[b] [% of control]	Oatp2-mediated transport[b] [% of control]	
Indomethacin			
1000 μM	1.16 ± 3.87	14.3 ± 2.6	
100 μM	13.6 ± 5.0	92.5 ± 10.1	**[c]
10 μM	78.7 ± 6.0	106 ± 15.6	
Ibuprofen			
1000 μM	10.0 ± 3.5	57.0 ± 3.6	**
100 μM	69.7 ± 13.3	93.9 ± 2.1	
10 μM	116 ± 8	74.7 ± 4.9	
Ketoprofen			
1000 μM	6.89 ± 2.94	30.2 ± 1.7	**
100 μM	64.8 ± 2.4	96.7 ± 7.0	*
10 μM	79.1 ± 1.2	75.2 ± 11.6	
Naproxen			
1000 μM	19.9 ± 1.8	52.2 ± 1.1	**
100 μM	87.4 ± 2.2	93.9 ± 2.0	
10 μM	87.9 ± 5.2	72.9 ± 10.2	
Corticosterone			
100 μM	14.3 ± 2.5	32.9 ± 4.8	
10 μM	43.4 ± 2.9	65.7 ± 10.9	
Deoxycorticosterone			
100 μM	1.34 ± 2.26	13.6 ± 7.1	
10 μM	20.6 ± 5.5	75.3 ± 12.7	*
Digoxin			
100 μM	71.6 ± 12.7	8.57 ± 4.77	*
10 μM	105 ± 6	13.3 ± 0.97	**
Methotrexate			
1000 μM	82.9 ± 3.0	77.7 ± 7.5	
100 μM	111 ± 8	97.6 ± 8.2	
10 μM	135 ± 5	124 ± 15	
Penicillin G			
1000 μM	55.3 ± 6.2	81.0 ± 4.3	
100 μM	110 ± 1	109 ± 12	
10 μM	115 ± 9	90 ± 9.1	
Verapamil			
1000 μM	3.93 ± 3.89	4.12 ± 1.56	
100 μM	7.04 ± 8.28	14.0 ± 3.81	
10 μM	99.6 ± 11.0	87.5 ± 16.3	
Cyclosporin A			
30 μM	27.6 ± 3.4	27.4 ± 13.8	
3 μM	88.1 ± 2.9	60.1 ± 5.9	*
0.3 μM	87.0 ± 4.6	91.6 ± 12.0	
Tolbutamide			
1000 μM	32.9 ± 3.9	47.3 ± 10.7	
100 μM	101 ± 3	99.2 ± 4.9	
10 μM	94.4 ± 3.6	108 ± 6	
Glibenclamide			
1000 μM	2.33 ± 2.80	1.61 ± 1.20	
100 μM	5.55 ± 6.37	22.2 ± 5.8	
10 μM	27.1 ± 6.5	64.7 ± 9.4	
Rifampicin			
1000 μM	10.9 ± 3.3	5.64 ± 9.73	
100 μM	46.6 ± 3.4	17.9 ± 9.7	
10 μM	106 ± 10	32.0 ± 6.8	*
Cimetidine			
1000 μM	79.4 ± 4.4	54.0 ± 5.8	
100 μM	90.9 ± 13.6	94.9 ± 6.2	
Quinidine			
400 μM	20.6 ± 9.3	26.4 ± 4.1	
100 μM	8.36 ± 23.76	90.6 ± 10.4	
25 μM	47.3 ± 7.5	129 ± 11	*
Quinine			
400 μM	1.13 ± 1.83	2.14 ± 13.64	
100 μM	40.4 ± 3.4	11.2 ± 14.4	
25 μM	97.9 ± 5.1	28.4 ± 11.2	**
α-Ketoglutarate			
1000 μM	100 ± 5	84.6 ± 3.1	
Propionic acid			
1000 μM	89.4 ± 1.8	121 ± 6	
p-Aminohipprate			
1000 μM	144 ± 9	124 ± 17	
100 μM	118 ± 6	139 ± 21	

[a] Substrate uptake was measured by incubating cells with 0.1 μM $E_2$17βG or 50 nM digoxin in the presence or absence of inhibitor.
[b] Values are CL_{uptake} in cDNA-transfected cells minus that in mock-transfected cells, normalized by their control estimated in the absence of inhibitors and expressed as mean ± SE of three separate studies.
[c] Statistically significant difference between Oatp1- and Oatp2-mediated transport by Dunnet's test.
* $P < 0.05$; ** $P < 0.01$.

computer-calculated SD), which is much smaller than that for Oatp1 (>100 μM).

DISCUSSION

In the present study, we have investigated the effect of 20 compounds on Oatp1- and Oatp2-mediated transport to find a selective inhibitor for each of these transporters. As candidates for such selective inhibition, we chose compounds that (i) are actively taken up into rat hepatocytes, (ii) are inhibitors of transporter-mediated uptake in rat hepatocytes, and/or (iii) are substrates or inhibitors of Oatp family transporters. Although many of them inhibited both Oatp1 and 2 to comparable degrees, some of them preferentially inhibited one transporter: NSAIDs, deoxycorticosterone, and quinidine preferentially inhibited Oatp1, whereas digoxin, rifampicin, and quinine preferentially inhibited Oatp2. These compounds may act as selective inhibitors at appropriate concentrations. Comparison of the K_i values of digoxin for Oatp1 and Oatp2 revealed that this cardiac glycoside acts as a selective inhibitor of Oatp2 over a wide range of concentrations (from 3.7 to ~ 10 < μM). In contrast, the selectivity of the other inhibitors described above was not high; indeed there was only a 13- ~20-fold difference in K_i values between Oatp1 and Oatp2.

Kinetic analysis revealed that the K_m values for the uptake of $E_2$17βG and digoxin in Oatp1- and Oatp2-expressing LLC-PK_1 cells, respectively, were within the same range as the reported values (5,20), and the P_{dif} for both substrates in transporter-expressing cells were close to their uptake clearance in mock-transfected cells (Fig. 1). These results suggest that the saturable transport of each substrate is mediated mainly by Oatp1 or Oatp2, and the nonsaturable transport is due to passive diffusion, similarly to the passive diffusion in mock-transfected cells. The uptake in mock-transfected cells did not show any concentration dependence, suggesting that LLC-PK_1 cells do not have any endogenous transporters that

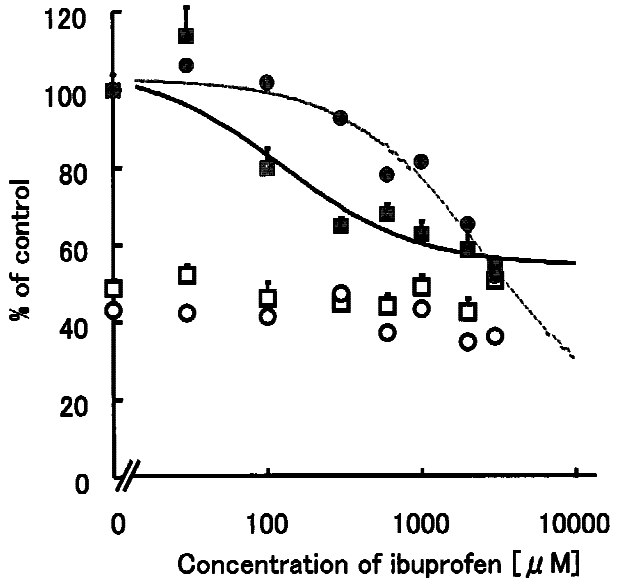

Fig. 2. Concentration-dependent effect of ibuprofen on the function of Oatp1 and Oatp2. Uptake of 0.1 μM [^{3}H]-$E_2$17βG (■) or 50 nM [^{3}H]-digoxin (●) by Oatp1- and Oatp2-expressing LLC-PK_1 cells, respectively, was examined in the presence or absence of ibuprofen. Uptake of $E_2$17βG (□) and digoxin (○) in vector-transfected cells is also shown. Results are given as a percent of the control. Each point and bar represent the mean ± SE (n = 9 from three independent cell preparations). The solid and dotted lines represent the fitted line for the Oatp1-mediated uptake of $E_2$17βG and Oatp2-mediated uptake of digoxin, respectively.

make a significant contribution to any uptake. Therefore, most of the saturable transport seen in transporter-expressing cells can be regarded as being mediated by only a single transporter: Oatp1 or Oatp2.

The results of the present study should be discussed in relation to the previous findings in isolated hepatocytes. In our previous reports, the transport properties of ibuprofen and indomethacin in primary cultured rat hepatocytes and COS-7 cells transiently expressing Oatp1, along with the inhibitory effect of indomethacin on the function of Oatp1, were investigated (20,24,25). Although both compounds were actively taken up into rat hepatocytes, this uptake was not mediated by Oatp1 (20,25). Indomethacin inhibited the function of Oatp1 in a concentration-dependent manner, with IC_{50} values of 10–100 μM (Table I), which is within the same range as the estimated K_i value in an earlier report (26). In addition, we have shown that both NSAIDs are able to inhibit the function of Oatp2, with higher K_i values than those for Oatp1 (Table I and Fig. 2).

We also found that rifampicin preferentially inhibited Oatp2-mediated transport (Table I and Fig. 3), which is consistent with the report by Fattinger *et al* (27). However, although these authors reported that 100 μM of rifampicin minimally inhibited Oatp1-mediated transport (reduced to 94% of the control), our results suggest that this concentration of rifampicin reduced the Oatp1-mediated transport of $E_2$17βG to 47% of the control (Table I and Fig. 3). This discrepancy may be due to the difference in the host cells: Fattinger *et al* used cRNA-injected *Xenopus laevis* oocytes, whereas we used cDNA transfected LLC-PK_1 cells.

Our finding that quinine, but not quinidine, is a potent inhibitor of the Oatp2-mediated transport of digoxin supports the previous findings by Hedman and Meijer (28) that the uptake of digoxin into isolated rat hepatocytes is potently inhibited by quinine, but not by quinidine. However, Hedman and Meijer did not determine the K_i values of quinidine and quinine for the uptake of digoxin in isolated rat hepatocytes, although 25 and 50 μM quinine almost completely inhibited the saturable portion, whereas 50 μM quinidine allowed more than half the activity to be retained (28). This result suggests that the K_i values for quinine and quinidine may be $\ll$25μM and >50μM, respectively. These values are consistent with the K_i values determined in the present study (Fig. 4).

We also found that cimetidine, a type I organic cation, did not inhibit the function of either Oatp1 or Oatp2 at low concentrations, which is consistent with the previous report by van Montfoort *et al* (15) who showed that type I organic cations did not significantly interact with Oatp family proteins. In contrast, we found that quinidine and quinine, type II organic cations, inhibited Oatp1 and Oatp2 function. Although we do not yet know if these two compounds are transported by Oatp family proteins, van Montfoort et al showed that N-methyl-quinine is transported by Oatp1, but not by Oatp2, and that N-methyl-quinidine is not transported by either of these transporters (15).

By using a selective inhibitor, the contributions of transporter(s) to the hepatic uptake of drugs of interest can be estimated. Among the selective inhibitors examined in the present study, digoxin is the most useful for estimating the contribution of Oatp2 *in vitro* because of the large difference in K_i values between Oatp1 and Oatp2. This method should be discussed in relation to the method previously proposed.

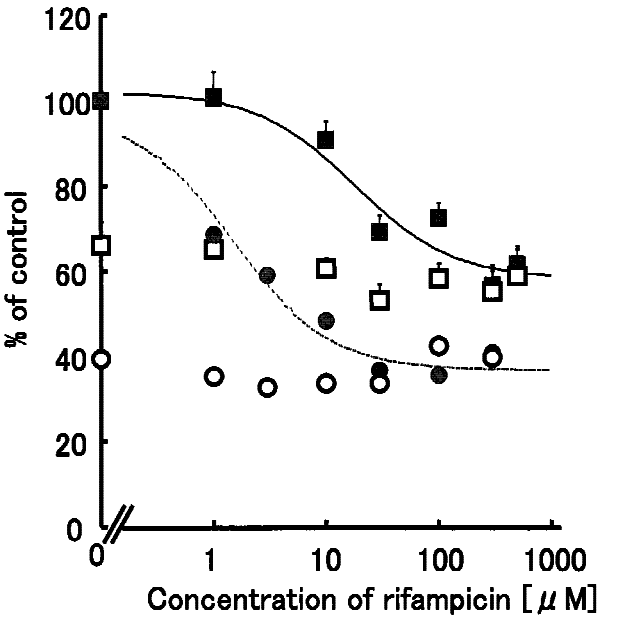

Fig. 3. Concentration-dependent effect of rifampicin on the function of Oatp1 and Oatp2. Uptake of 0.1 μM [^{3}H]-$E_2$17βG (■) or 50 nM [^{3}H]-digoxin (●) by Oatp1- and Oatp2-expressing LLC-PK_1 cells, respectively, was examined in the presence or absence of rifampicin. Uptake of $E_2$17βG (□) and digoxin (○) in vector-transfected cells is also shown. Results are given as a percent of the control. Each point and bar represent the mean ± SE (n = 9 from three independent cell preparations). The solid and dotted lines represent the fitted line for the Oatp1-mediated uptake of $E_2$17βG and Oatp2-mediated uptake of digoxin, respectively.

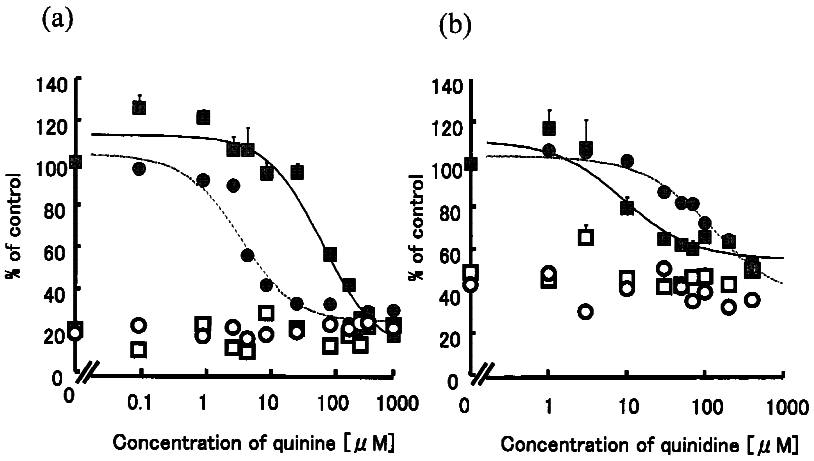

Fig. 4. Concentration-dependent effect of quinine (a) and quinidine (b) on the function of Oatp1 and Oatp2. Uptake of 0.1 μM [^{3}H]-$E_2$17βG (■) or 50 nM [^{3}H]-digoxin (●) by Oatp1- and Oatp2-expressing LLC-PK_1 cells, respectively, was examined in the presence or absence of quinine (a) or quinidine (b). Uptake of $E_2$17βG (□) and digoxin (○) in vector-transfected cells is also shown. Results are given as a percent of the control. Each point and bar represent the mean ± SE (n = 9 from three independent cell preparations). The solid and dotted lines represent the fitted line for the Oatp1-mediated uptake of $E_2$17βG and Oatp2-mediated uptake of digoxin, respectively.

Previously, Hagenbuch *et al* (29) estimated the contributions of Na^+-taurocholate transporting polypeptides (Ntcp) and Oatp1 to the uptake of taurocholate (TC) and sulfobromophthalein (BSP) by using *Xenopus laevis* oocytes injected with total rat liver mRNA and antisense oligonucleotides. Although this is a useful approach, the practical application of this method may be limited because there is often difficulty in observing a significant uptake of test compounds into oocytes injected with total rat liver mRNA. Kouzuki *et al* compared the uptake of compounds into cultured rat hepatocytes and COS-7 cells transiently expressing Ntcp and Oatp1 and estimated the relative contributions of Ntcp and Oatp1 to the total uptake in cultured rat hepatocytes by normalizing the uptake of test compounds with respect to that of representative substrates for these transporters (20,30). In determining the contribution of the cloned transporters to the cellular uptake, the use of selective inhibitors may be helpful, together with these previously proposed methods. Indeed, by comparing the uptake of temocaprilat into hepatocytes and Oatp1 expressing COS-7 cells, Ishizuka *et al* suggested that ~50% of the uptake of this ACE inhibitor into rat hepatocytes might be accounted for by Oatp1 (10). This result is consistent with the finding that ~50% of temocaprilat uptake into rat hepatocytes is inhibited by an excess (up to 40 μM) of $E_2$17βG (10). However, it is possible that the presence of other, as yet unidentified, transporters may also affect this kind of analysis. For accurate estimation of the contribution, molecular cloning of additional important transporters is required.

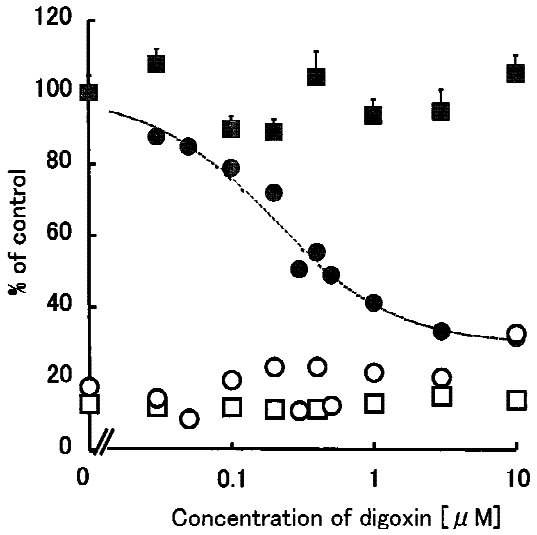

Fig. 5. Concentration-dependent effect of digoxin on the function of Oatp1 and Oatp2. Uptake of 0.1 μM [^{3}H]-$E_2$17βG (■) or 50 nM [^{3}H]-digoxin (●) by Oatp1- and Oatp2-expressing LLC-PK_1 cells, respectively, was examined in the presence or absence of different concentrations of digoxin. Uptake of $E_2$17βG (□) and digoxin (○) in vector-transfected cells is also shown. Results are given as a percent of the control. Each point and bar represent the mean ± SE (n = 9 from three independent cell preparations). The dotted line represents the fitted line for Oatp2-mediated uptake of digoxin.

In conclusion, we have performed comparative studies of the inhibitory effects of a wide range of compounds on Oatp1- and Oatp2-mediated transport and found that ibuprofen and quinidine preferentially inhibit Oatp1, whereas digoxin and quinine preferentially inhibit Oatp2. At appropriate concentrations, they are able to act as selective inhibitors of Oatp1 or Oatp2. These inhibitors may be used to estimate the contribution of Oatp1 and Oatp2.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a Kitasato University Research Grant for Young Researchers. We are grateful for Dr. Hiroshi Suzuki at the University of Tokyo for his kind help and fruitful advice in completing this work.

REFERENCES

1. M. Yamazaki, H. Suzuki, and Y. Sugiyama. Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics. *Pharm. Res.* **13**:497–513 (1996).
2. O. Takenaka, T. Hori, H. Suzuki, and Y. Sugiyama. Carrier-mediated active transport of the glucuronide and sulfate of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) into rat liver: quantitative comparison of permeability in isolated hepatocytes, perfused liver and liver in vivo. *J. Pharmacol. Exp. Ther.* **280**:948–958 (1996).
3. M. Ishigami, T. Tokui, T. Komai, K. Tsukahara, M. Yamazaki, and Y. Sugiyama. Evaluation of the uptake of pravastatin by perfused rat liver and primary cultured rat hepatocytes. *Pharm. Res.* **12**:1741–1745 (1995).
4. E. Jacquemin, B. Hagenbuch, B. Stieger, A. W. Wolkoff, and P. J. Meier. Expression cloning of a rat liver Na^+-independent organic anion transporter. *Proc. Natl. Acad. Sci. USA* **91**:133–137 (1994).
5. B. Noé, B. Hagenbuch, B. Stieger, and P. J. Meier. Isolation of multispecific organic anion and cardiac glycoside transporter from rat brain. *Proc. Natl. Acad. Sci. USA* **94**:10346–10350 (1997).
6. V. Cattori, B. Hagenbuch, N. Hagenbuch, B. Stieger, R. Ha, K. E. Winterhalter, and P. J. Meier. Identification of organic anion transporting polypeptide 4 (Oatp4) as a major full-length isoform of the liver-specific transporter-1 (rlst-1) in rat liver. *FEBS Lett* **474**:242–245 (2000).
7. M. Kakyo, M. Unno, T. Tokui, R. Nakagomi, T. Nishio, H. Iwasaki, D. Nakai, M. Sei, M. Suzuki, T. Naitoh, S. Matsuno, H. Yawo, and T. Abe. Molecular characterization and functional regulation of a novel rat liver-specific organic anion transporter rlst-1. *Gastroenterology* **117**:770–775 (1999).
8. T. Sekine, S. H. Cha, M. Tsuda, N. Apiwattanakul, N. Nakajima, Y. Kanai, and H. Endou. Identification of multispecific organic anion transporter 2 expressed predominantly in the liver. *FEBS Lett*. **429**:179–182 (1998).
9. H. Kusuhara, T. Sekine, N. Utsunomiya-Tate, M. Tsuda, R. Kojima, S. H. Cha, Y. Sugiyama, Y. Kanai, and H. Endou. Molecular cloning and characterization of a new multiple organic anion transporter from rat brain. *J. Biol. Chem.* **274**:13675–13680 (1999).
10. H. Ishizuka, K. Konno, H. Naganuma, K. Nishimura, H. Kouzuki, H. Suzuki, B. Stieger, P. J. Meier, and Y. Sugiyama. Transport of temocaprilat into rat hepatocytes: role of organic anion transporting polypeptides. *J. Pharmacol. Exp. Ther.* **287**:37–42 (1998).
11. B. Hsiang, Y. Zhu, Z. Wang, Y. Wu, V. Sasseville, W-P Yang, and T. G. Kirchgessner. A novel human hepatic organic anion transporting polypeptide (OATP2). *J. Biol. Chem.* **274**:37161–37168 (1999).
12. N. Kanai, R. Lu, A. W. Wolkoff, and V. L. Schuster. Transient expression of oatp organic anion transporter in mammalian cells: identification of candidate substrates. *Am. J. Physiol.* **270**:F319–F325 (1996).
13. U. Eckhardt, A. Schroeder, B. Stieger, M. Höchli, L. Landmann, R. Tynes, P. J. Meier, and B. Hagenbuch. Polyspecific substrate uptake by the hepatic organic anion transporter Oatp1 in stably transfected CHO cells. *Am. J. Physiol.* **276**:G1037–G1042 (1999).
14. N. Kanai, R. Lu, Y. Bao, A. W. Wolkoff, M. Vore, and V. L. Schuster. Estradiol 17β-D-glucuronide is a high-affinity substrate for oatp organic anion transporter. *Am. J. Physiol.* **270**:F326–F331 (1996).
15. J. E. van Montfoort, B. Hagenbuch, K. E. Fattinger, M. Müller, G. M. M. Groothuis, D. K. F. Meijer, and P. J. Meier. Polyspecific organic anion transporting polypeptides mediate hepatic uptake of amphipathic type II organic cations. *J. Pharmacol. Exp. Ther.* **291**:147–152 (1999).
16. T. Abe, M. Kakyo, H. Sakagami, T. Tokui, T. Nishio, M. Tanemoto, H. Nomura, S. C. Hebert, S. Matsuno, H. Kondo, and H. Yawo. Molecular characterization and tissue distribution of a new organic anion transporter subtype (oatp3) that transports thyroid hormones and taurocholate and comparison with oatp2. *J. Biol. Chem.* **273**:22395–22401 (1998).
17. C. Reichel, J. E. van Montfort, V. Cattori, C. Rahner, B. Stieger, T. Kamisako, and P. J. Meier. Localization and function of the organic anion-transporting polypeptide oatp2 in rat liver. *Gastroenterology* **117**:688–695 (1999).
18. L. Li, P. J. Meier, and N. Ballatori. Oatp2 mediated bidirectional organic solute transport: a role for intracellular glutathione. *Mol. Phamacol.* 58:335–340 (2000).
19. R. J. Bertz and G. R. Granneman. Use of in vitro and in vivo data to estimation the likelihood of metabolic pharmacokinetic interactions. *Clin. Pharmacokinet.* **32**:210–258 (1997).
20. H. Kouzuki, H. Suzuki, K. Ito, R. Ohashi, and Y. Sugiyama. Contribution of organic anion transporting polypeptide to uptake of its possible substrates into rat hepatocytes. *J. Pharmacol. Exp. Ther.* **288**:627–634 (1999).
21. H. Niwa, K. Yamamura, and J. Miyazaki. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**:193–200 (1991).
22. A. Schroeder, U. Eckhardt, B. Stieger, R. Tynes, C. D. Schteingart, A. F. Hofmann, P. J. Meier, and B. Hagenbuch. Substrate specificity of the rat liver Na+-bile salt cotransporter in Xenopus laevis oocytes and in CHO cells. *Am. J. Physiol.* **274**:G370–G375 (1998).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
24. K. Yamaoka, Y. Tanigawara, Y. Nakagami, and T. Uno. A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobio-Dyn.* **4**:879–885 (1981).
25. H. Kouzuki, H. Suzuki, and Y. Sugiyama. Pharmacokinetic study of the hepatobiliary transport of indomethacin. *Pharm. Res.* **17**: 432–438 (2000).
26. H. Kouzuki, H. Suzuki, B. Stieger, P. J. Meier, and Y. Sugiyama. Characterization of the transport properties of organic anion transporting polypeptide 1 (oatp1) and Na+/taurocholate cotransporting polypeptide (Ntcp): comparative studies on the inhibitory effect of their possible substrates in hepatocytes and cDNA-transfected COS-7 cells. *J. Pharmacol. Exp. Ther.* **292**: 505–511 (2000).
27. K. Fattinger, V. Cattori, B. Hagenbuch, P. J. Meier, and B. Stieger. Rifamycin SV and rifampicin exhibit differential inhibition of the hepatic rat organic anion transporting polypeptides, Oatp1 and Oatp2. *Hepatology* **32**:82–86 (2000).
28. A. Hedman and D. K. F. Meijer. Stereoselective inhibition by the diastereomers quinidine and quinine of uptake of cardiac glycosides into isolated rat hepatocytes. *J. Pharm. Sci.* **87**:457–461 (1997).
29. B. Hagenbuch, B. F. Scharschmidt, and P. J. Meier. Effect of antisense oligonucleotides on the expression of hepatocellular bile acid and organic anion uptake systems in *Xenopus laevis* oocytes. *Biochem. J.* **316**:901–904 (1996).
30. H. Kouzuki, H. Suzuki, K. Ito, R. Ohashi, and Y. Sugiyama. Contribution of sodium taurocholate co-transporting polypeptide to the uptake of its possible substrates into rat hepatocytes. *J. Pharmacol. Exp. Ther.* **286**:1043–1050 (1999).