Screening of Antibiotics That Interact with Organic Anion-Transporting Polypeptides 1B1 and 1B3 Using Fluorescent Probes

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Hepatic organic anion transporters OATP1B1 and OATP1B3 are expressed at the sinusoidal membrane of hepatocytes and contribute to the hepatic uptake of a wide variety of clinically used drugs. To identify the antibiotics that interact with the human organic anion transporters OATP1B1 and OATP1B3, we applied a screening system using fluorescent probes. Twenty-six antibiotics with a variety of mechanisms of action were examined. The screening demonstrated that four antibiotics inhibited OATP1B1-mediated transport and 11 antibiotics inhibited OATP1B3-mediated transport in a concentration-dependent manner. Antibiotics that inhibited OATP1B3-mediated transport tended to exhibit higher affinity than those that inhibited OATP1B1-mediated transport. To clarify whether the antibiotics that interacted with OATP1B1 and/or OATP1B3 were substrates for these transporters, an uptake study was performed. Rifampicin and penicillin were transported by both OATP1B1 and OATP1B3. Moreover, OATP1B3 was involved in the transport of ceftriaxone, cefmetazole, cefoperazone, and cefotaxime. Macrolides were not significantly transported by either transporter. In conclusion, the results demonstrated that our system is a useful method for the rapid screening of transporter–antibiotic interaction, and we found novel substrates. Our results indicate that OATP1B1 and/or OATP1B3 contribute to the transport process of some antibiotics, and that drug–drug interactions associated with these transporters could occur after the administration of antibiotics.

Key words antibiotic; interaction; screening; organic anion-transporting polypeptide 1B1; organic anion-transporting polypeptide 1B3

For safe and effective treatment of bacterial infections, it is important to understand the pharmacokinetic disposition of antibiotics. Membrane transporters play pivotal roles in the absorption, distribution, and excretion of many drugs. Several reports described the contribution of transporters to the pharmacokinetics of antibiotics. P-glycoprotein (ATP-binding cassette B1 (ABCB1)) is known to limit the oral bioavailability of grepafloxacin and salinomycin.[1—3] Breast cancer resistance protein (BCRP/ABCG2) affects the oral bioavailability of quinolone antibacterial drugs.[4] P-glycoprotein and BCRP expressed at the brush-border membrane in the intestine work as an absorption barrier of these drugs. ABC transporters expressed at canalicular membrane in the liver (multidrug resistance-associated protein 2 (MRP2/ABCC2), P-glycoprotein, and BCRP) are involved in the biliary excretion of macrolides, quinolones, and β-lactams.[5—8] Renal excretion of β-lactams is mediated by the renal uptake *via* the basolateral organic anion transporter OAT3 and by the secretory transport *via* the brush-border membrane transporter MRP4 (ABCC4).[9—11]

Organic anion-transporting polypeptides (OATPs) are sodium-independent organic anion transporters found in a variety of tissues, including the liver, kidney, intestine, and brain. OATPs contribute to the transport of bile acids, thyroid hormones, steroid conjugates, anionic oligopeptides, eicosanoids, various drugs, and other xenobiotic compounds across membranes.[12,13] OATP1B1 and OATP1B3 are members of the liver-specific subfamily of OATPs, which are localized to the sinusoidal membrane of hepatocytes.[14—17] OATP1B1 and OATP1B3 transport a wide variety of clinically used drugs, although only a few reports described the role of these transporters in the hepatic handling of antibiotics. In the present study, we attempted to identify the antibiotics that interact with OATP1B1 and OATP1B3 using an automated image acquisition and analysis system. In addition, to determine novel substrates of these transporters, a transport study was conducted.

MATERIALS AND METHODS

Materials Lithocholyl-($N\varepsilon$-NBD)-lysine (LCA-NBD) and chenodeoxycholyl-($N\varepsilon$-NBD)-lysine (CDCA-NBD) were synthesized as previously described.[18] Anti-OATP2 antibody was purchased from Affinity BioReagents, Inc. (Golden, CO, U.S.A.). All other chemicals were commercially available and of the highest purity possible.

Cell Culture and Transfection Studies HEK293 cells, which are derived from human embryonic kidneys, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum under an atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were transfected with a pcDNA3.1(+) plasmid vector (Invitrogen, Carlsbad, CA, U.S.A.) encoding OATP1B1 using LipofectAMINE 2000

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(Invitrogen) according to the manufacturer's instructions. After 3 weeks of selection in G418 (0.5 mg/ml), single colonies were screened for OATP1B1 expression by immunoblot analysis and transport studies. OATP1B3/HEK293 cells transduced with OATP1B3 and the empty vector-transfected mock cells were previously established.[19]

Immunoblot Analysis Membrane fractions (15 μg/lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (8%). OATP1B1 was detected with a monoclonal anti-OATP2 antibody diluted by a factor of 100, and detection was performed using Peroxidase-conjugated AffiniPure Goat Anti-Mouse immunoglobulin G (IgG)+IgM (H+L) (Jackson ImmunoResearch, West Grove, PA, U.S.A.) diluted 1 : 10000. HepG2 cells infected with recombinant adenoviruses encoding OATP1B1 was used as a positive control.

Transport Study The cellular uptake of LCA-NBD in monolayer cultures grown on 24-well plates was measured. After washing once, cells were preincubated in Krebs–Henseleit buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 5.0 mM glucose, and 1.53 mM $CaCl_2$, pH 7.4). Uptake was initiated by adding LCA-NBD to the medium. At the indicated times, uptake was terminated by replacement of the uptake buffer with ice-cold Krebs–Henseleit buffer containing 1% bovine serum albumin (BSA). After washing two times in ice-cold BSA-free Krebs–Henseleit buffer, cells were lysed in lysis buffer (20 mM Tris with 0.4% Triton X-100, adjusted to pH 9.0). The fluorescence contained in each aliquot was measured using a microplate spectrofluorometer (SPECTRAmax GEMINI XS; Molecular Devices, Sunnyvale, CA, U.S.A.) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

The uptake of antibiotics by monolayer cultures grown in 24-well plates was measured by using liquid chromatography/tandem mass spectrometry (LC/MS/MS). After terminating drug uptake, the cells were scraped and homogenized in 1 ml of water. Known quantities of the internal standard were added to each cell lysate sample. Samples were injected into the LC/MS/MS at 100-μl volumes.

The protein content of the solubilized cells was determined by using a Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.).

LC/MS/MS Conditions An on-line column-switching LC/MS/MS system was used for the identification of antibiotics. The HPLC system used was a Nanospace SI-2 (Shiseido Co., Ltd., Tokyo, Japan). The samples were injected and the solutes were concentrated on the MAYI-ODS (G) column (10 mm×4.6 mm i.d., Shimadzu Corp., Kyoto, Japan) for 5 min with an extraction mobile phase that contained 20 mM formic acid (pH 3.0) or 20 mM acetic acid (pH 7.0). Following the removal of the plasma proteins and other matrix components, the target antibiotics enriched on the MAYI-ODS (G) column were transferred to the analytical column (YMC-Pack proC18, 150 mm×2.0 mm i.d., 5 μm, YMC Co., Ltd., Kyoto, Japan) by switching a six-port valve. The antibiotics were eluted using isocratic conditions and a flow rate of 200 μl/min. The mobile phases were the following: for cefazolin, cefmetazole, cefoperazone, and cefotaxime, methanol/20 mM ammonium formate (40 : 60, v/v, pH 3.0 with formic acid); for penicillin and rifampicin, methanol/20 mM ammonium formate (60 : 40, v/v, pH 3.0 with formic acid); for ceftriaxone and cephalothin, methanol/20 mM ammonium formate (55 : 45, v/v, pH 7.0 with aqueous ammonia); and for erythromycin, clarithromycin, and roxythromycin, acetonitrile/methanol/20 mM ammonium formate (15 : 46 : 39, v/v/v, pH 3.0 with formic acid). Column effluents were introduced in the mass spectrometer (API 5000, Applied Biosystems Inc., Foster City, CA, U.S.A.) *via* an electrospray interface. Detection was performed by selected ionization monitoring in positive ion mode (*m/z* 455 $([M+H]^+)$>*m/z* 323, *m/z* 472 $([M+H]^+)$>*m/z* 356, *m/z* 646 $([M+H]^+)$>*m/z* 530, *m/z* 456 $([M+H]^+)$>*m/z* 324, *m/z* 335 $([M+H]^+)$>*m/z* 176, *m/z* 823 $([M+H]^+)$>*m/z* 791, *m/z* 555 $([M+H]^+)$>*m/z* 396, *m/z* 414 $([M+H]^+)$>*m/z* 337, *m/z* 734.5 $([M+H]^+)$>*m/z* 158, *m/z* 748.5 $([M+H]^+)$>*m/z* 158, and *m/z* 419.5 $([M+2H]^{2+})$>*m/z* 158 for cefazolin, cefmetazole, cefoperazone, cefotaxime, penicillin, rifampicin, ceftriaxone, cephalothin, erythromycin, clarithromycin and roxythromycin, respectively).

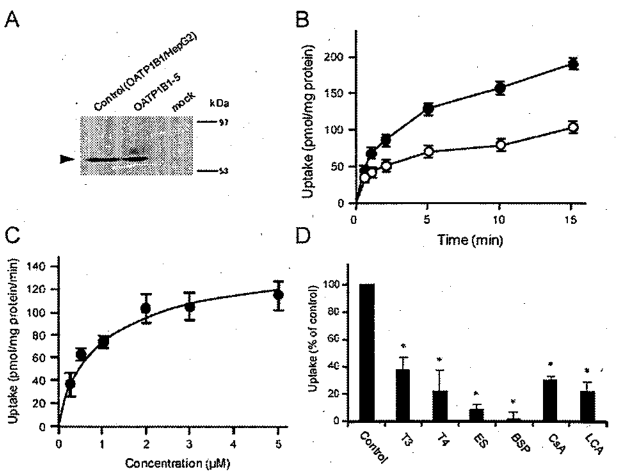

Fig. 1. Characteristics of LCA-NBD Transport *via* OATP1B1

(A) Immunoblot analysis of OATP1B1. (B) Time course of LCA-NBD uptake *via* OATP1B1. OATP1B1-expressing cells (closed circles) or mock cells (open circles) were incubated at 37 °C with 0.25 μM LCA-NBD. Each point represents the mean±S.E. of three independent determinations. (C) Kinetic analysis of LCA-NBD uptake *via* OATP1B1. Cells were incubated for 1 min at 37 °C with varying concentrations of LCA-NBD. OATP1B1-mediated uptake was calculated after subtraction of the nonspecific uptake by mock cells. Each point represents the mean±S.E. of three independent experiments. (D) Inhibitory effects of various compounds on LCA-NBD uptake *via* OATP1B1. Cells were incubated for 1 min at 37 °C with 0.25 μM LCA-NBD in the presence or absence of various compounds (10 μM). OATP1B1-mediated uptake was calculated after subtracting nonspecific uptake by mock cells. Data are expressed as percentage of the control value. Each column represents the mean±S.E. of three independent determinations. $*p<0.05$, significantly different from control. T3, triiodothyronine; T4, thyroxine; ES, estrone-3-sulfate; BSP, bromosulfophtalein; CsA, cyclosporin A; LCA, lithocholic acid.

Screening of Antibiotics Cells seeded in 96-well plates (8×10^3 cells/well) were incubated for 24 h. Cell nuclei were stained with 100 μl Hoechst 33258 (Sigma, St. Louis, MO, U.S.A.) solution (20 μM in DMEM) for 20 min under a 5% CO_2 and 95% air atmosphere at 37 °C. Cells were washed once after nuclear staining, then preincubated in Krebs–Henseleit buffer for 10 min. Uptake was initiated by the addition of LCA-NBD (0.5 μM, for OATP1B1) or CDCA-NBD (0.1 μM, for OATP1B3) in the presence or absence of varying concentrations of antibiotics (1 to 10000 μM). BSP (sulfobromophtalein) (Sigma-Aldrich, St. Louis, MO, U.S.A.), a rep-

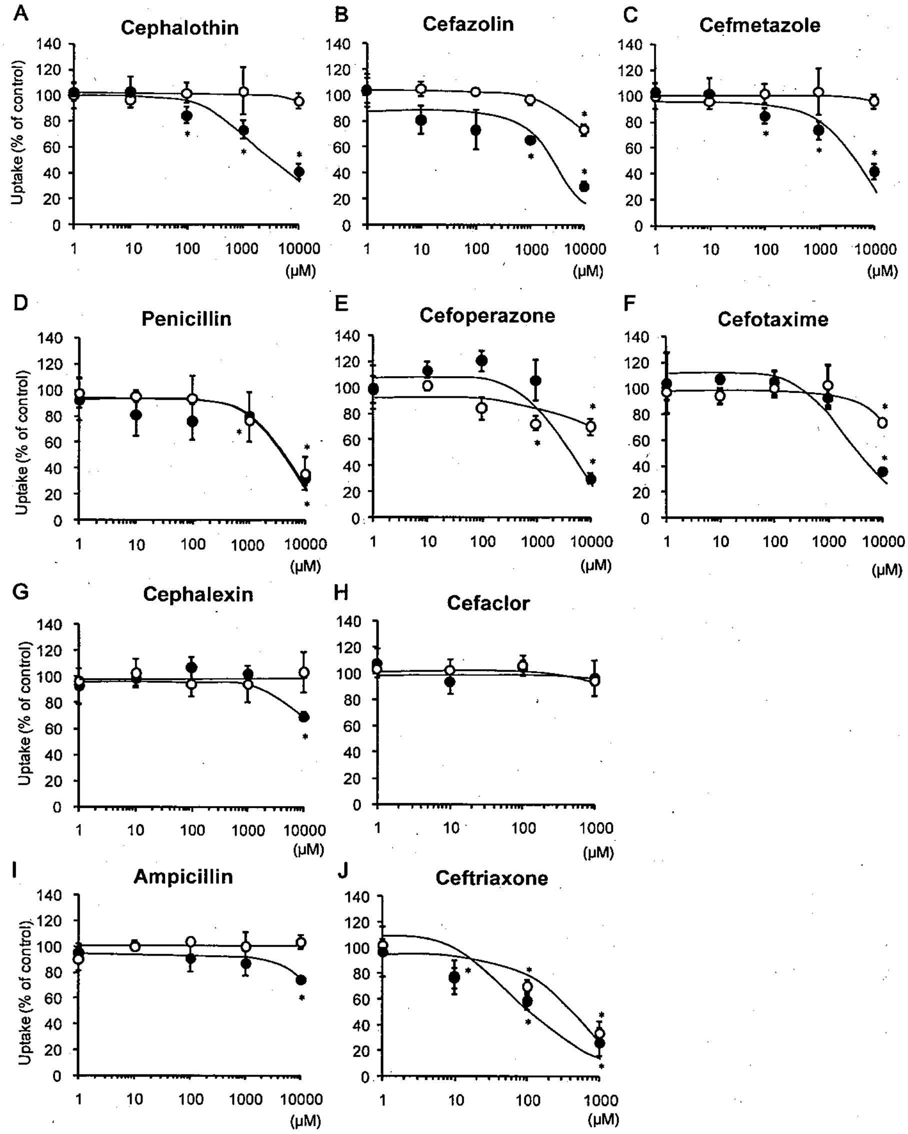

Fig. 2. Inhibitory Effects of Antibiotics on Fluorescent Probe Uptake *via* OATP1B1 and OATP1B3

Cells were incubated for 20 min at 37 °C with 0.5 μM LCA-NBD or 0.1 μM CDCA-NBD in the presence or absence of antibiotics. Open circles and closed circles represent the inhibitory effects of antibiotics on the OATP1B1- and OATP1B3-mediated uptake fluorescent probe, respectively. OATP1B1- or OATP1B3-mediated uptake was calculated after subtracting nonspecific uptake by mock cells. Each point represents the mean±S.E. of three independent determinations. (A) Cephalothin, (B) cefazolin, (C) cefmetazole, (D) penicillin, (E) cefoperazone, (F) cefotaxime, (G) cephalexin, (H) cefaclor, (I) ampicillin, (J) ceftriaxone, (K) erythromycin, (L) roxythromycin, (M) clarithromycin, (N) tetracycline, (O) minomycin, (P) kanamycin, (Q) norfloxacin, (R) levofloxacin, (S) itraconazole, (T) sparfloxacin, (U) ofloxacin, (V) fluconazole, (W) rifampicin, (X) meropenem, (Y) isoniazide, (Z) aztreonam. $*p<0.05$, significantly different from control.

resentative OATPs substrate, was used as a positive control. After 20 min of uptake, we measured LCA-NBD or CDCA-NBD and Hoechst 33258 fluorescence intensity using an automated image acquisition and analysis system (IN Cell Analyzer 1000, GE Healthcare) at excitation wavelengths of 460 and 360 nm and emission wavelengths of 535 and 475 nm, respectively. Fluorescence intensities were analyzed using the "Object Intensity Module" of the IN Cell Analyzer 1000.[20] The IC_{50} values were estimated by nonlinear regression analysis of the competition curves with a one-compartment model with the following equation: $V=100\times IC_{50}/(IC_{50}+[I])+A$, where V is the transport amount (% of control), [I] the concentration of antibiotics and A the nonspecific transport (% of control) using software Origin8 (Lightstone Corp., Tokyo, Japan).

Statistical Analysis Data are expressed as mean±S.E. When appropriate, the differences between groups were tested for significance using the unpaired Student's *t*-test. Statistical significance was indicated by *p* values of less than 0.05.

RESULTS

Transport of LCA-NBD by OATP1B1-Transfected Cells As the first step, we established stable clones overexpressing OATP1B1. Clones that survived selection in 0.5 mg/ml G418 were expanded. Analysis for OATP1B1 overproduction by immunoblotting and LCA-NBD uptake (Figs. 1A, B) resulted in the isolation of one clone, OATP1B1-5 (OATP1B1/HEK293). Transport of LCA-NBD was rapid

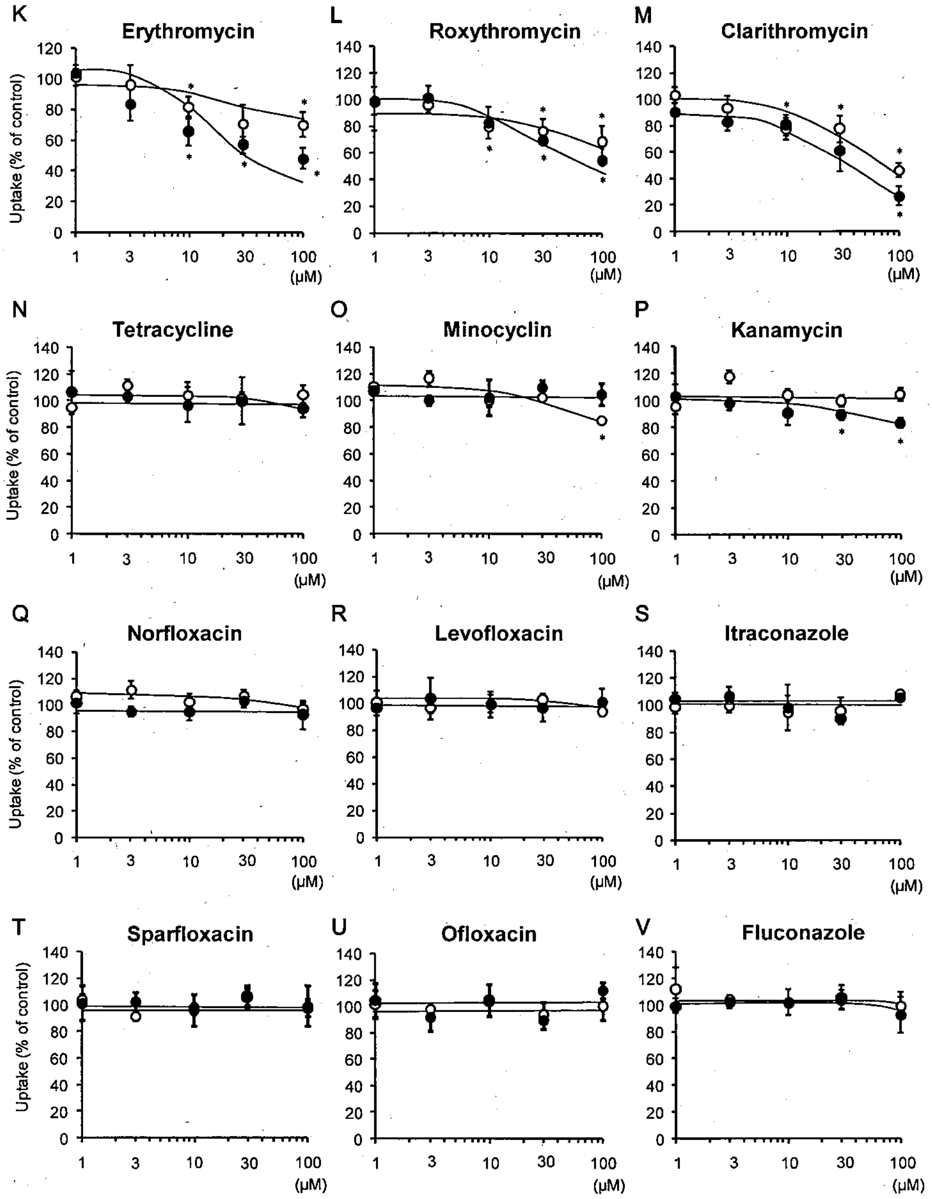

Fig. 2. Continued.

and almost reached a steady state 10 min after the start of the incubation. Kinetic parameters in OATP1B1 were determined after exposure to varying concentrations of LCA-NBD (Fig. 1C). The apparent Michaelis–Menten constant (K_m) for OATP1B1-mediated LCA-NBD uptake was $0.58\pm0.08\,\mu$M, and the maximum velocity (V_{max}) was 128 ± 5 pmol/mg protein/min. OATP1B1-mediated LCA-NBD transport was reduced by several previously reported substrates or inhibitors (Fig. 1D).

Screening of Antibiotics in OATP1B1- and OATP1B3-Transfected Cells In this study, we examined the inhibitory effects of antibiotics on OATP1B1- and OATP1B3-mediated transport of fluorescent substrates using the IN Cell Analyzer 1000 system. LCA-NBD and CDCA-NBD, which are efficiently transported by OATP1B1 and OATP1B3, were used as fluorescent probes.[18,19] Twenty-six antibiotics with a variety of mechanisms of action were selected, and BSP was used as a positive control. The fluorescence intensity of LCA-NBD and CDCA-NBD was measured using the IN Cell Analyzer 1000. The result of the screening showed that six and 11 antibiotics inhibited OATP1B1- and OATP1B3-mediated transport, respectively, in a concentration-dependent manner (Fig. 2). OATP1B1- and OATP1B3-mediated transport was significantly inhibited by BSP to $25\pm0.5\%$ and $32\pm1.7\%$ of the control, respectively. The IC_{50} values of antibiotics inhibiting transport mediated by OATP1B1 and OATP1B3 are summarized in Table 1. Seven β-lactams (cepharothin, cefazolin, cefmetazole, cefoperazone, cefotaxime, ceftriaxone, and penicillin) decreased OATP1B3-mediated transport, whereas only two β-lactams (ceftriaxone and penicillin) decreased OATP1B1-mediated transport in a dose-dependent manner. Macrolides (erythromycin, roxythromycin, and clarithromycin) inhibited the transport mediated by OATP1B3, and clarithromycin also inhibited the

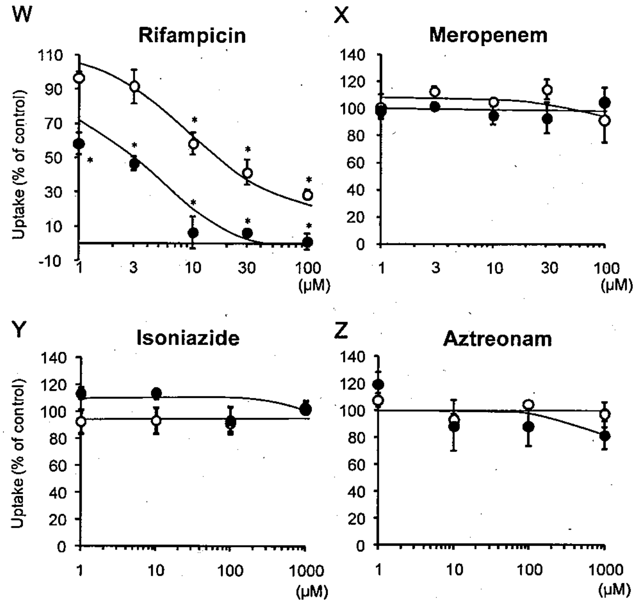

Fig. 2. Continued.

Table 1. IC_{50} Values of Antibiotics for OATP1B1 and OATP1B3

Antibiotics	IC_{50} (μM) OATP1B1	IC_{50} (μM) OATP1B3
β-Lactams		
Cephalothin	>10000	1600
Cefazolin	>10000	3900
Cefmetazole	>10000	5200
Penicillin	4900	5000
Cefoperazone	>10000	2800
Cefotaxime	>10000	2400
Cephalexin	>10000	>10000
Cefaclor	>1000	>1000
Ampicillin	>10000	>10000
Ceftriaxone	470	100
Meropenem	>100	>100
Macrolides		
Erythromycin	>100	27
Roxythromycin	>100	75
Clarithromycin	75	56
Tetracyclines		
Tetracycline	>100	>100
Minocyclin	>100	>100
Aminoglycoside		
Kanamycin	>100	>100
Newquinolones		
Norfloxacin	>100	>100
Levofloxacin	>100	>100
Sparfloxacin	>100	>100
Ofloxacin	>100	>100
Antifungals		
Itraconazole	>100	>100
Fluconazole	>100	>100
Antituberculosis drugs		
Rifampicin	8.8	3.9
Isoniazide	>1000	>1000
Monobactam		
Aztreonam	>1000	>1000

IC_{50}, 50% inhibitory concentration.

transport by OATP1B1. Rifampicin showed the strongest inhibition of both OATP1B1- and OATP1B3-mediated transport with IC_{50} values of 8.8 and 3.9 μM, respectively. Antibiotics that interacted with OATP1B3 tended to show higher affinity than those that interacted with OATP1B1.

Transport of Antibiotics by OATP1B1- and OATP1B3-Expressing Cells To clarify whether antibiotics that interact with OATP1B1 and/or OATP1B3 were substrates for these transporters, we performed an uptake study. The results of this study demonstrated that rifampicin and penicillin were substrates for both OATP1B1 and OATP1B3 (Fig. 3). Moreover, OATP1B3 contributed to the transport of ceftriaxone, cefmetazole, cefoperazone, and cefotaxime. Macrolides were not significantly transported by either transporter.

DISCUSSION

In the present study, we evaluated the interaction of antibiotics with OATP1B1 and OATP1B3, transporters localized specifically in the liver. We applied an imaging technique for the screening of antibiotics. The system that we have established is rapid and efficient because the process of washing and cell lysis is not required.

First, we established stable cell lines that overexpress OATP1B1. Clone OATP1B1-5 (OATP1B1/HEK293 cells) showed protein expression of OATP1B1 by immunoblotting and demonstrated high transport activity of the known OATP1B1 substrate estrone sulfate (17.5 nM [^{3}H]estrone sulfate transport by OATP1B1-5 cells, 625.1 fmol/mg protein/5 min (n=3); mock cells, 25.5 fmol/mg protein/5 min (n=3), respectively). When we tested the transport of five kinds of NBD-labeled bile acids (CDCA-NBD, LCA-NBD, cholyl-($N\varepsilon$-NBD)-lysine, deoxycholyl-($N\varepsilon$-NBD)-lysine, and ursodeoxycholyl-($N\varepsilon$-NBD)-lysine), the extent of LCA-NBD transport was the greatest (data not shown). Transport characteristics of LCA-NBD mediated by OATP1B1 were similar to those of other substrates (Fig. 1D). Therefore we decided to use LCA-NBD as a fluorescent probe for OATP1B1.

From the screening results, OATP1B1-mediated LCA-NBD transport was inhibited by four antibiotics (penicillin, ceftriaxone, clarithromycin, and rifampicin) in a dose-dependent manner (Fig. 2). Transport of CDCA-NBD by OATP1B3 was decreased by 11 antibiotics (cephalothin, cefazolin, cefmetazole, penicillin, cefoperazone, cefotaxime, ceftriaxone, erythromycin, roxythromycin, clarithromycin, and rifampicin) (Fig. 2). Rifampicin showed high affinities for both OATP1B1 and OATP1B3 with IC_{50} values of 8.8 and 3.9 μM, respectively (Table 1). Vavricka *et al.*[21] reported that the apparent K_m values of rifampicin transport were 13 μM for OATP1B1 and 2.3 μM for OATP1B3. This result strongly indicates that our screening system works well and could be used with good accuracy to estimate affinities for transporters. Macrolides also affected the transport by OATP1B1 and OATP1B3. The IC_{50} values for OATP1B3-mediated CDCA-NBD transport were 27 μM for erythromycin, 75 μM for roxythromycin, and 56 μM for clarithromycin (Table 1). The IC_{50} values for OATP1B1-mediated LCA-NBD transport were higher than those for OATP1B3-mediated CDCA-NBD transport. These data are consistent with results reported by Seithel *et al.*,[22] who discussed the feasibility of clinically relevant drug–drug interactions *via* OATP1B3. Our results pro-

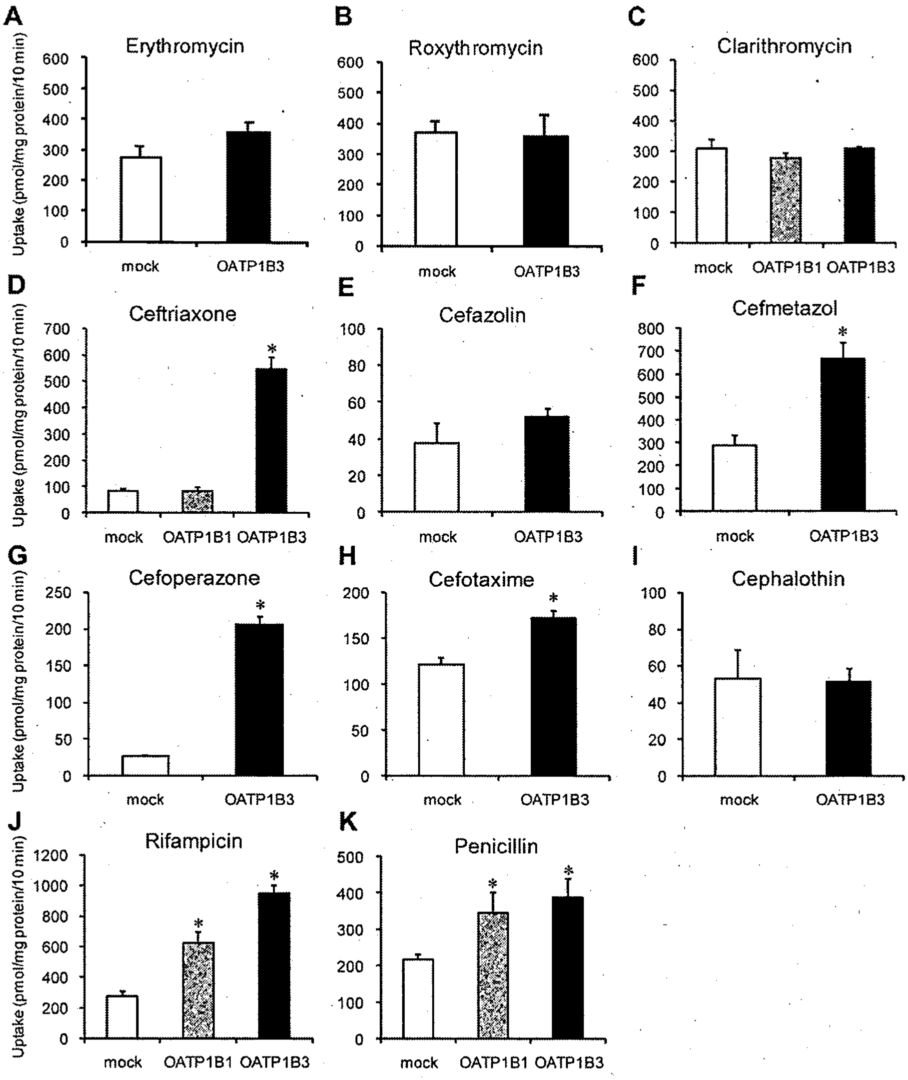

Fig. 3. Uptake of Antibiotics by OATP1B1- and OATP1B3-Expressing HEK293 Cells

OATP1B1- (shaded columns), OATP1B3-expressing cells (closed columns), or mock cells (open columns) were incubated at 37 °C for 10 min with 100 or 10 μM antibiotics. Each column represents the mean±S.E. of three independent determinations. $*p<0.05$, significantly different from mock cells. (A) 10 μM erythromycin, (B) 10 μM roxythromycin, (C) 10 μM clarithromycin, (D) 100 μM ceftriaxone, (E) 100 μM cefazolin, (F) 100 μM cefmetazole, (G) 100 μM cefoperazone, (H) 100 μM cefotaxime, (I) 100 μM cephalothin, (J) 10 μM rifampicin, (K) 100 μM penicillin.

vide support for a possible role of OATP1B3 in such drug–drug interactions. The inhibitory effects of β-lactams depended on the transporter. Cephalothin, cefazolin, cefmetazole, cefoperazone, and cefotaxime inhibited the transport mediated by OATP1B3 (IC_{50} values 1600, 3900, 5200, 2800, 2400 μM, respectively) but not by OATP1B1 (Fig. 2, Table 1). Penicillin and ceftriaxone altered both OATP1B1-mediated transport (IC_{50} values 4900, 470 μM, respectively) and OATP1B3-mediated transport (IC_{50} values 5000, 100 μM, respectively).

An important issue to consider is whether the drugs that inhibit OATP1B1- and/or OATP1B3-mediated transport are also substrates for the transporters. Identifying whether the drug behaves as "a substrate" or "an inhibitor" leads to a better understanding of its pharmacokinetics. In the present study, we found that ceftriaxone and cefotaxime were novel substrates for OATP1B3. These drugs are in part excreted from the bile, and in particular cefotaxime is metabolized to the deacetylated form in the liver.[23,24] Therefore OATP1B3 is thought to be partly involved in the sinusoidal uptake process of these drugs. Other substrates (rifampicin and penicillin for OATP1B1; rifampicin, penicillin, cefmetazole, and cefoperazone for OATP1B3) were consistent with previous reports.[21,25,26] The report by Nakakariya *et al.*,[26] which demonstrated that cefoperazone is transported by OATP1B1 and that cefazolin is transported by both OATP1B1 and OATP1B3, was inconsistent with our results. We did not perform the uptake study if the transporter-mediated uptake did not decrease to 50% of the control uptake at the maximum inhibitory concentration. Further, we used cultured cells for all the experiments, whereas Nakakariya's group used *Xenopus* oocytes. The discrepancy in the data might result from differences in the experimental conditions used in the two studies.

In conclusion, we identified antibiotics that interact with OATP1B1 and OATP1B3 using an automated image acquisition and analysis system. Our results indicate that OATP1B1 and/or OATP1B3 are involved in the transport process of some antibiotics, and that drug–drug interactions *via* these transporters could occur after the administration of antibiotics in the clinical setting.

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