Aspirin and Probenecid Inhibit Organic Anion Transporter 3–Mediated Renal Uptake of Cilostazol and Probenecid Induces Metabolism of Cilostazol in the Rat

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ABSTRACT

This study aimed to evaluate the transporter-mediated renal excretion mechanism of cilostazol and to characterize the mechanism of drug–drug interaction (DDI) between cilostazol and aspirin or probenecid. Concentrations of cilostazol and its metabolites OPC-13015 [6-4-[1-cyclohexyl-1H-tetrazol-5-yl]butoxy]-2(1H)-quinolinone] and OPC-13213 [3,4-dihydro-6-[4-[trans-4-hydroxycyclohexyl]-1H-tetrazol-5-yl]butoxy]-2(1H)-quinolinone] in rat biologic samples were measured by liquid chromatography–tandem mass spectrometry. Coadministration with probenecid, benzylpenicillin, or aspirin decreased the cumulative urinary excretion of cilostazol and renal clearance. Concentrations of cilostazol and OPC-13213 in plasma decreased, and the concentration of OPC-13015 increased in the presence of probenecid, benzylpenicillin, or aspirin. Cilostazol was distributed in the kidney and liver, with tissue to plasma partition coefficient ($K_{pu}$) values of 8.4 ml/g and 16.3 ml/g, respectively. Probenecid and aspirin reduced cilostazol distribution in the kidney. Probenecid did not affect cilostazol metabolism in the kidney but increased cilostazol metabolism in the liver, and aspirin had no effect on cilostazol metabolism. Benzylenepicillin, aspirin, and cyclo-trans-4-L-hydroxycyclohexyl-L-serine (JBP485) reduced cilostazol uptake in kidney slices and human organic anion transporter 3 (hOAT3)-human embryonic kidney 293 (HEK293) cells, whereas p-aminohippuric acid did not. Compared with the vector, hOAT3-HEK293 cells accumulated more cilostazol, whereas hOAT1-HEK293 cells did not. OAT3 and Oat3 play a major role in cilostazol renal excretion, whereas OAT1 and Oat1 do not. Oat3 and Cyp3a are both targets of the DDI between cilostazol and probenecid. Aspirin inhibits OAT3-mediated uptake of cilostazol and does not influence cilostazol metabolism.

Introduction

Transporters govern the transport of drugs in and out of cells and can affect a drug safety profile or its metabolite concentration in various tissues. Transporter-based drug interactions in the clinic may be inhibitory, inductive, or both (Endres et al., 2006). For example, coadministration of a drug that inhibits or induces a transporter may affect the pharmacokinetics of a drug that is a substrate for that transporter. Thus, it is important to determine whether transporters affect the absorption and distribution of an investigated drug and whether the drug can affect the absorption and distribution of other drugs by affecting transporters.

The kidney is a key organ for the excretion of drugs and drug metabolites. The transport systems responsible for renal tubular secretion of drugs are classified as either organic anion transporters (OATs) or organic cation transporters based on their preferential substrate selectivity (Burckhardt and Burckhardt, 2011). Multidrug and toxin compartment extrusion transporters and carnitine/organic cation transporters are also important for renal transport. Various anionic drugs and substances are absorbed into tubular cells by basolateral multispecific OATs (Burckhardt and Wolff, 2000). There are eight isoforms in the organic anion transporter family: OAT1–OAT7 and OAT10 (Ahn and Nigam, 2009). OAT1/SLC22A6, OAT2/SLC22A7, and OAT3/SLC22A8 were identified on the basolateral membrane of the human proximal tubules (Miyazaki et al., 2004). Cytochrome P450 (P450) is predominantly found in the liver, as well as in many other extrahepatic tissues such as the kidney. Most of the available P450 studies focus on hepatic forms, and much less is known about renal P450. The mammalian kidney is capable of...
metabolizing and conjugating a variety of drugs because it possesses xenobiotic-metabolizing enzymes (Hosseinpour et al., 2002). Renal metabolism of some substances may occur faster in the kidney than in the liver. For example, the glycation of benzoic acid is faster in the perfused rat kidney than in the liver, and the activity of γ-glutamyl transpeptidase activity is highest in the kidney (Poon and Pang, 1995). CYP3A4 and CYP3A5 in the human kidney were previously reported (Lasker et al., 2000; Aleksa et al., 2005), and CYP2C11 in the rat kidney was reported (Pfohl-Leschzynski et al., 1998).

Cilostazol [6-[4-[(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2 (1H)-quinolinone] is an effective and rapidly acting antithrombotic agent with the ability to inhibit phosphodiesterase 3 (Parker et al., 1999). P450 enzymes extensively metabolize cilostazol. However, OPC-13213 and OPC-13015 [6-[4-((trans-4-hydroxy cyclohexyl)-1H-tetrazol-5-yl)butoxy]-2(1H)-quinolinone] are the only 2 of the 11 known cilostazol metabolites that are quantifiably measurable in plasma and are considered to be pharmacologically active (Kim et al., 2009). To our knowledge, there are currently no available reports on the kidney’s role in the metabolism of cilostazol. In addition, it is unknown whether the metabolism of cilostazol in the kidney causes the small amount of renal excretion of unchanged cilostazol. Previous studies showed that both influx and efflux transporters influence cilostazol bioavailability. P-glycoprotein contributes, in part, to the intestinal secretion of cilostazol, although further investigation is needed to identify the absorptive transporter of cilostazol (Toyobuku et al., 2003). In the kidneys, OATs play a pivotal role in drug absorption and excretion. However, whether the renal excretion of cilostazol involves OATs has not been established in the literature.

Dual antiplatelet treatment with aspirin and cilostazol may result in a more favorable patient risk-benefit analysis in the prevention of stent thrombosis (Jeon et al., 2010). However, questions still remain regarding whether transporters mediate the beneficial drug–drug interaction (DDI) between cilostazol and aspirin.

This study used renal clearance and distribution experiments in vivo, kidney and liver slices in vitro, and human OAT1- and OAT3-transfected cells to investigate the metabolism of cilostazol in rat kidney, the elimination mechanism of cilostazol at the molecular level, and the targets of the DDI between cilostazol and aspirin or probenecid.

**Materials and Methods**

Cilostazol was purchased from Zhejiang Kinglyuan Pharmaceutical Co., Ltd. (Shangyu, China). JBP485 (cyclo-trans-4-1-hydroxyprolyl-serine) was supplied by Japan Bioproducts Industry Co., Ltd. (Tokyo, Japan), and bestatin (internal standard) was provided by Pharmaceutical Co., Ltd. (Shen Luck, Shenzhen, China). Probenecid (>99.9% purity), p-aminophenic acid (PAH), benzylpenicillin (PCG), aspirin, dexamethasone, ketoconazole, and sulfaphenazole was purchased from Sigma-Aldrich (St. Louis, MO). OPC-13015 and OPC-13213 were purchased from Toronto Research Chemicals Inc. (Toronto, ON, CA). High-performance liquid chromatography–grade methanol and acetonitrile were obtained from Tedia (Carson City, NV). All other reagents were purchased from commercial sources and were of the highest purity grade available.

**Cell Culture.** Human embryonic kidney 293 (HEK293) cells were grown in low-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin and were kept at 37°C with a 5% (v/v) CO2 atmosphere and 95% relative humidity. Cell culture reagents were purchased from Gibco (Grand Island, NY).

**Animals.** Male Wistar rats (weighing 220–250 g) were obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008-0002), and were fed a chow diet and allowed free access to water. The animals fasted for 12 hours before pharmacokinetic experiments, with access to water ad libitum. All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

**In Vivo and In Vitro Pharmacokinetic Studies.** In all cases, rats fasted overnight for 12 hours. For in vivo studies, rats were anesthetized with pentobarbital (60 mg/kg) by intraperitoneal injection before the experiment onset. Rats were anesthetized with ether in distribution study and kidney or liver slices study.

**In Vivo Renal Clearance Experiments.** Rats were divided randomly into four groups: 1) cilostazol alone (20 µg/kg), 2) cilostazol (20 µg/kg) plus probenecid (100 mg/kg), 3) cilostazol (20 µg/kg) plus PCG (200 mg/kg), and 4) cilostazol (20 µg/kg) plus aspirin (10 mg/kg). Test drugs, diluted in normal saline, were administered intravenously through the jugular vein (injected through the left side). After intravenous administration, blood samples (300 µl) were collected through the right side of the jugular vein with heparinized syringes at the following time points: 1, 5, 10, 30, 60, 120, 240, 360, 480, and 600 minutes. Isotonic saline solution (300 µl) was injected after each blood sample collection. Bladders were cannulated with polyethylene tubing, the distal end of which flowed into an Eppendorf tube resting on a small pad of ice. Urine was collected directly from the bladder at 2, 4, 6, 8, 10, and 24 hours after administration. Cilostazol, OPC-13015, and OPC-13213 concentrations were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Cumulative urinary excretion, renal clearance (CLR), and pharmacokinetic parameters were calculated.

**Kidney and Liver Distribution Study.** Rats were divided randomly into three groups: 1) cilostazol alone (20 µg/kg), 2) cilostazol (20 µg/kg) plus probenecid (100 mg/kg), and 3) cilostazol (20 µg/kg) plus aspirin (10 mg/kg). Drugs were administered intravenously to rats via the jugular vein. After administration, the animals were humanely killed by drawing whole blood from the abdominal aorta using a heparinized syringe under ether anesthesia. The kidneys and livers were removed and washed with normal saline to remove the blood or content, blotted on filter paper, and were then weighed for wet weight and stored at −70°C. Before analysis, tissues were homogenized in 300 µl of chilled (4°C) normal saline for approximately 4 minutes with a tissue homogenizer (IKA-T10 model; IKA, Staufen, Germany) in an ice bath. The homogenates were subsequently centrifuged at 11,374 × g for 10 minutes at 4°C to remove cellular debris, and the supernatant was used for LC-MS/MS determination.

**In Vivo Kidney Slice Culture.** Rat kidney slices were prepared as previously described (Vickers et al., 1992). Kidney slices were prepared from the cortex region of the organ in oxygenated Krebs-Henseleit buffer (pH 7.4) at 4°C under constant oxygenation (95% O2/5% CO2). The slices were cultured in 24-well culture plates. Culture media consisted of Dulbecco’s modified Eagle’s medium supplemented with insulin (10−8 M), glucagon (10−9 M), t-glutamine (2 mM), Nu-Serum (10%; Becton, Dickinson and Company, Franklin Lakes, NJ), Fungizone (amphotericin B Sigma-Aldrich, St. Louis, MO), and gentamycin. Slices were equilibrated at 37°C in a 95% O2/5% CO2 atmosphere for 90 minutes in culture medium containing 0.1 µM dexamethasone, 10 µM sulfaphenazole, 8 mM probenecid, and 30 µM aspirin. After the preincubation period, the media were replaced with fresh media, including 2 µM cilostazol in the absence or presence of 50 µM dexamethasone, 10 µM sulfaphenazole, 8 mM probenecid, and 30 µM aspirin. Slices were harvested at 2, 4, 6, 8, 10, and 24 hours. Kidney slices were washed with ice-cold Krebs-Henseleit buffer at the end of the incubation period. The concentrations of cilostazol, OPC-13015, and OPC-13213 were determined by LC-MS/MS.

**In Vitro Liver Slice Culture.** Rat liver slices were prepared as previously described (Efterink et al., 2004). In brief, after anesthesia, intact liver lobes were removed and placed in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O2/5% CO2, pH 7.4) and then cut into slices (200–300 µm thickness, 10–14 mg) with a ZQP-86 tissue slicer (Zhixun Co., Ltd., Shanghai, China).

The slices were placed into the culture medium consisting of Waymouth’s media containing 10% fetal bovine serum, 25 mM HEPES, 5 µM/l insulin, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin B, 25 mM glucose, and 2.4 g/l
sodium bicarbonate (pH 7.4). Before treatment, slices were equilibrated at 37°C in an atmosphere of 95% O2/5% CO2 for 1 hour in culture medium containing 0.1 μM, 1 μM, 8 mM, and 30 μM dexamethasone, ketonazole, probenecid, and aspirin, respectively. Treatment began by replacing the culture medium with fresh medium containing the required concentrations of test compounds. Liver slices were incubated with cilostazol (2 μM; 1 hour) in the absence or presence of 50 μM dexamethasone, 1 μM ketonazole, 8 mM probenecid, and 30 μM aspirin for 1, 3, 5, 10, and 24 hours, respectively. At the end of the incubation period, liver slices were washed with ice-cold Krebs-Henseleit buffer. The concentrations of cilostazol, OPC-13015, and OPC-13213 were determined by LC-MS/MS.

In Vitro Uptake in Kidney Slices. After anesthesia, the kidneys of rats were incised, decapsulated, and immediately placed into oxygenated buffer at 4°C and then cut into slices with a ZQP-86 tissue slicer (300 μm thickness; Zhixin Co., Ltd.). After preincubation for 3 minutes at 37°C, kidney slices were transferred to 24-well culture plates containing fresh carbogen-saturated cilostazol for further incubation. According to cilostazol uptake measured at 0, 1, 3, 5, 10, 15, and 30 minutes, a 10-minute time point was selected as the linear uptake time and used to examine the concentration-dependent uptake and effect of various inhibitors on the uptake of cilostazol. Furthermore, kidney slices were incubated with cilostazol (2 μM) in the presence or absence of PAH (0.1, 0.2, and 0.5 mM), PCG (0.1, 0.2, and 0.5 mM), BFP485 (0.2, 0.5, and 1.0 mM), probenecid (0.05, 0.1, and 0.2 mM), and aspirin (0.01, 0.03, and 0.1 mM). The following inhibitor concentrations were chosen: PGC (0.2 mM), BFP485 (0.5 mM), probenecid (0.1 mM), and aspirin (0.03 mM). At the end of the incubation period, kidney slices were washed with ice-cold Hank’s balanced salt solution (pH 7.5). Accumulated cilostazol in homogenized kidneys was determined by LC-MS/MS, and the uptake of cilostazol was reported in picomoles per milligram of kidney.

Uptake by Transporter Expression Systems. Uptake experiments with hOAT1-HEK293 cells, hOAT3-HEK293 cells, or mock cells were performed as previously described (Zhu et al., 2012). HEK293 cells were seeded in 24-well culture plates with nearly confluent cells seeded 48 hours before each experiment. Uptake was initiated by adding transport buffer (1 ml) with cilostazol (2 μM) after the cells were washed twice and incubated with transport buffer at 37°C for 15 minutes. The uptake was terminated upon the removal of the medium and three washes, each with 1 ml of ice-cold Hank’s balanced salt solution, lysed with 0.3% Triton X-100, and then transferred to a polyethylene tube for quantitation by LC-MS/MS. The inhibitory effects of PCG (0.2 mM), BFP485 (0.5 mM), and aspirin (0.03 mM) on cilostazol uptake were investigated. The uptake of cilostazol was measured in hOAT1-HEK293 cells, hOAT3-HEK293 cells, and mock cells. Samples were taken at 0.25, 0.5, 1, 3, 5, and 10 minutes. According to the results, a time of 1 minute was selected as representative of the uptake rate and was used to examine the concentration-dependent uptake and effect of various inhibitors on cilostazol uptake. Protein was measured with the bicinchoninic acid procedure (Solarbio, Beijing, China) using bovine serum albumin for the standard.

Sample Preparation. Fifty microliters of the internal standard at 1 μg/ml bestatin and 250 μl of acetonitrile was added to 50 μl of rat plasma or urine samples. The mixed samples were vortexed for 1 minute and centrifuged at 11,374g for 10 minutes to remove the protein precipitate. The supernatants (250 μl) were transferred to another polythene tube and evaporated to dryness at 37°C under nitrogen. The residues were then reconstituted with 200 μl of the mobile phase.

Kidney slices or liver slices were mixed with 300 μl of normal saline or 0.1 M phosphate buffer (pH 7.4) after weighing, respectively, and were homogenized (IKA-T10 homogenizer) on ice. Other preparations were handled using the same method as the plasma samples. Ten microliters of each sample was injected for LC-MS/MS analysis.

LC-MS/MS Analysis. An Agilent liquid chromatography system (Agilent HPLC200; Agilent Technologies, Palo Alto, CA) was used for LC-MS/MS analysis of cilostazol, OPC-13015, and OPC-13213. Isocratic chromatographic separation was performed on a Hypersil BDS-C18 column (150 x 4.6 mm inner diameter, 5 μm; Dalian Elite Analytical Instruments Co., Ltd., Dalian, China). The mobile phase consisted of solvent A (10 mM ammonium acetate solution), solvent B (acetonitrile), and solvent C (methanol). The initial condition was 80% solvent A, 10% solvent B, and 10% solvent C from 0 to 1 minute. A linear gradient was performed over 4 minutes, with mobile phase B increasing from 10% to 40% and mobile phase C increasing from 10% to 30%. The mobile phase was returned to the initial condition and re-equilibrated for 5 minutes. The total analysis time was 10 minutes. The flow rate was set at 0.4 ml/min, and the injection volume used was 10 μl. The column was maintained at room temperature. An API 3200 triple-quadrupole mass spectrometer (Applied Biosystems, Concord, ON, Canada) was operated with a TurboSpray interface in positive ion mode. Analyst 1.4.1 software (Applied Biosystems) was used to control the equipment and perform data acquisition and analysis. The optimized truncated multiple reaction monitoring fragmentation transitions were as follows: cilostazol, m/z 370.40 → 288.10 with a collision energy (CE) of 22 electron volts (eV); bestatin, m/z 309.10 → 120.30 with a CE of 32 eV; OPC-13015, m/z 368.20 → 285.90 with a CE of 20 eV; and OPC-13213, m/z 386.11 → 288.11 with a CE of 20 eV. The dwell time for each transition was 200 milliseconds.

Data Analysis. The main pharmacokinetic parameters were calculated according to the Practical Pharmacokinetic Program (3P97 ) edited by the Chinese Mathematical Pharmacological Society (Beijing, China). The 3P97 program automatically obtained C-T curves and main pharmacokinetic parameters. The quality of the fit was judged by evaluating the S.D. of parameter estimates and the coefficient of determination (r²) and by performing a visual inspection of the residual plots. The main pharmacokinetic parameters were calculated using eqs. 1 and 2. Plasma clearance (CLp) was calculated with eq. 1 as follows:

\[ CL_p = \frac{Dose}{AUC_{i.v.}} \]

where \( AUC_{i.v.} \) is the area under the plasma concentration-time profile after intravenous administration. The CLq of cilostazol was calculated with eq. 2 as follows:

\[ CL_q = \frac{A_{total}}{AUC_{i.v.}} \]

where \( A_{total} \) is the total cumulative amount of cilostazol excreted in urine over 24 hours.

Kinetic parameters were obtained using nonlinear, least-squares regression analysis via the following Michaelis–Menten equation (eq. 3):

\[ v = \frac{(V_{max} S)}{(K_m + S)} \]

where \( v \) is the uptake velocity of the substrate (in picomoles per minute per milligram of protein), \( K_m \) is the Michaelis constant (in micromoles), and \( S \) is the substrate concentration in the medium (in micromoles), and \( V_{max} \) is the maximum uptake rate (in picomoles per minute per milligram of protein). The inhibition constant (K) was calculated by IC50 values for competitive inhibition, using eq. 4 as follows:

\[ IC50 = K_i (1 + [S]/K_m) \]

For noncompetitive inhibition, the Kc value was calculated using eq. 5 as follows:

\[ IC50 = K_i (1 + K_m/[S]) \]

where \([S]\) is the substrate concentration.

The tissue to plasma partition coefficient (Kp) value was calculated with eq. 6 as follows:

\[ K_p = C_t/C_p \]

where \(C_t\) and \(C_p\) are drug concentrations in the tissues and arterial plasma, respectively.

Statistical Analysis. Statistical analysis was performed using the SPSS 11.5 package (SPSS Inc, Chicago IL). Test results are expressed as the mean ± S.D. One-way analysis of variance was performed to determine the statistical significance among multiple treatments for a given parameter. P values <0.05 or <0.01 were considered statistically significant.

**Results**

Effect of Probenecid, PCG, and Aspirin on Cilostazol Renal Excretion, Plasma Concentration, and Metabolism. Changes in cilostazol cumulative urinary excretion were assessed when cilostazol (20 μg/kg) and probenecid (100 mg/kg; a well-known inhibitor of OAT1
and OAT3; Giacomini et al., 2010), were intravenously coadministered (Fig. 1A). The cumulative urinary excretion of cilostazol decreased significantly when cilostazol and probenecid were coadministered and the CLR decreased by 41.8% compared with the control group (cilostazol alone) (Fig. 1D).

We determined the plasma concentrations of cilostazol to clarify whether the concentration of cilostazol in plasma increased when probenecid decreased the CLR of cilostazol. The plasma concentration and AUC of cilostazol did not increase when the two drugs were administered in combination. However, the plasma concentration and AUC of cilostazol decreased compared with the control group (Fig. 2A). The AUC of cilostazol decreased by 62.4% (Fig. 2D). However, when PCG (an inhibitor of OAT1 and OAT3; Deguchi et al., 2004) or aspirin was coadministered with cilostazol, the cilostazol AUC increased by 75.5% or 58%; CLp decreased by 41.7% or 37.6% (Fig. 2, B, C, E, and F; Table 1), and cumulative urinary excretion was significantly reduced (Fig. 1, B and C), with an 87.8% or 70.6% decrease in CLR (Fig. 1, E and F; Table 1), respectively. In all groups, the CLR of cilostazol was only 0.4%–1.9% of CLp.

We determined the concentrations of OPC-13015 and OPC-13213 in plasma and urine to examine whether the metabolism of cilostazol was influenced when cilostazol was coadministered with probenecid, PCG, and aspirin. Compared with the control group, the coadministration of cilostazol and probenecid increased the OPC-13015 concentration in plasma and the AUC increased by 42.7% (Fig. 3C), whereas the OPC-13213 concentration and AUC decreased by 75.1% (Fig. 3B). The coadministration of PCG or aspirin did not influence the concentrations of the two metabolites in plasma (Fig. 3, B and C). OPC-13015 concentrations were below the limit of detection in urine. However, the cumulative urinary excretion of OPC-13213 was higher than that of both cilostazol and OPC-13015. Probenecid, PCG, and aspirin all reduced OPC-13213 cumulative urinary

Fig. 1. Cumulative urine excretion curves (A–C) and renal clearances (D–F) of cilostazol after intravenous injection of 20 μg/kg cilostazol and probenecid, PCG, or aspirin in rats. (A and D) Coadministration of 100 mg/kg probenecid (A and D), 200 mg/kg PCG (B and E), and 10 mg/kg aspirin (C and F). Data are expressed as the mean ± S.D. (n = 5). *P < 0.05 versus control.
excretion, with $\text{CLR}$ values that decreased by 17.3%, 44.5%, and 41.5%, respectively.

**Distribution of Cilostazol in the Kidney and Liver.** To examine whether cilostazol was absorbed in the kidney intact and whether probenecid or aspirin affected the distribution of cilostazol in the kidney and liver, we investigated cilostazol distribution after intravenous administration with cilostazol alone or in combination with probenecid or aspirin in rats (Fig. 4). In the control group (cilostazol alone), the $K_p$ values of cilostazol in the kidney and liver were 8.4 and 16.3 ml/g, respectively, 120 minutes after administration (Fig. 4). The coadministration of cilostazol and probenecid or aspirin decreased the distribution of cilostazol in the kidney (Fig. 4A); however, it did not change the distribution of cilostazol in the liver (Fig. 4B).

**Cilostazol Metabolism in Kidney Slices.** To examine why the concentrations of unchanged cilostazol were lower in urine, we explored whether cilostazol could be metabolized by the kidney and whether probenecid or aspirin affected cilostazol renal metabolism. We used fresh kidney slices to prove our hypothesis (Fig. 5). Cilostazol was administered with dexamethasone (50 μM; an inducer of CYP3A4/CYP3A2/CYP2C11; Rowlands et al., 2000; Teo et al., 2012), sulfaphenazole (10 μM; an inhibitor of CYP2C11), probenecid (8 mM), and aspirin (30 μM) in kidney slices. The concentration of...
OPC-13015 was not changed significantly in the absence or presence of dexamethasone, sulfaphenazole, probenecid, or aspirin (Fig. 5C). However, the concentration of OPC-13213 significantly increased, whereas the concentrations of cilostazol decreased compared with the control in the dexamethasone treatment group (Fig. 5, A and B). By contrast, the metabolism of cilostazol to OPC-13213 was significantly inhibited after treatment with sulfaphenazole (Fig. 5B). Correspondingly, the concentration of cilostazol increased with sulfaphenazole treatment compared with the control group (Fig. 5A). In the probenecid or aspirin treatment group, the concentration of OPC-13213 or cilostazol was not different from that in the control group (Fig. 5, A and B).

**Cilostazol Metabolism in Rat Liver Slices.** To explore other targets involved in the DDI between cilostazol and probenecid, we used rat liver slices to investigate the effect of probenecid on cilostazol metabolism (Fig. 6). Cilostazol was administered with dexamethasone (50 \( \mu \)M), ketoconazole (1 \( \mu \)M; a typical inhibitor of CYP3A4/CYP3A2; Chen et al., 2004; Lee et al., 2006), probenecid (8 mM), and aspirin (30 \( \mu \)M). The results showed that the concentration of OPC-13015 significantly increased (Fig. 6C), whereas the concentrations of cilostazol and OPC-13213 decreased (Fig. 6B) compared with controls when liver slices were treated with dexamethasone. In the probenecid treatment group, changes in the concentrations of cilostazol, OPC-13015,

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cilostazol</th>
<th>Cilostazol Plus PCG</th>
<th>Cilostazol Plus Aspirin</th>
<th>Cilostazol Plus Probenecid</th>
</tr>
</thead>
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<tr>
<td>( C_0 ) (ng/ml)</td>
<td>353.2 ± 3.4</td>
<td>365.2 ± 2.2</td>
<td>382.3 ± 3.1</td>
<td>235.4 ± 2.6</td>
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<td>( K_e ) (min(^{-1}))</td>
<td>0.0026 ± 0.0004</td>
<td>0.0031 ± 0.0003</td>
<td>0.0029 ± 0.0002</td>
<td>0.0021 ± 0.0001</td>
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<tr>
<td>( AUC_{0-\infty} ) (ng·min/ml)</td>
<td>8453 ± 150</td>
<td>14833 ± 108(^*)</td>
<td>13356 ± 95(^*)</td>
<td>5776 ± 124(^*)</td>
</tr>
<tr>
<td>( t_{1/2} ) (min)</td>
<td>266.6 ± 1.7</td>
<td>223.5 ± 1.6(^*)</td>
<td>238.8 ± 1.2(^*)</td>
<td>332.8 ± 1.4(^*)</td>
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<tr>
<td>( V_d ) (L/kg)</td>
<td>0.057 ± 0.006</td>
<td>0.055 ± 0.007</td>
<td>0.052 ± 0.005</td>
<td>0.085 ± 0.004</td>
</tr>
<tr>
<td>( CL_d ) (ml/min per kg)</td>
<td>2.4 ± 0.4</td>
<td>1.4 ± 0.3(^*)</td>
<td>1.5 ± 0.5(^*)</td>
<td>3.2 ± 0.7(^*)</td>
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\(^*\)Statistically significant difference (\( p < 0.01 \)).

\(^\bullet\)Statistically significant difference (\( p < 0.05 \)).

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**Fig. 3.** Cumulative urine excretion curves of OPC-13213 (A) and mean plasma concentration–time curves of OPC-13213 (B) and OPC-13015 (C) after intravenous administration of 20 \( \mu \)g/kg cilostazol and 100 mg/kg probenecid, 200 mg/kg PCG, or 10 mg/kg aspirin in rats. Statistical differences between each set of points were compared with the control groups by a two-tailed unpaired \( t \) test. Data are expressed as the mean ± S.D. (\( n = 5 \)). *\( p < 0.05 \) (limit of significance).
and OPC-13213 were similar to those of the dexamethasone treatment group (Fig. 6). By contrast, the metabolism of cilostazol to OPC-13015 was significantly inhibited after treatment with ketoconazole (Fig. 6C). Correspondingly, the concentrations of cilostazol and OPC-13213 increased with ketoconazole treatment compared with the control group (Fig. 6, A and B). There was no significant difference in the

Fig. 4. Distribution of cilostazol in the rat kidney (A) and liver (B) after intravenous administration of 100 mg/kg probenecid or 10 mg/kg aspirin in rats. Statistical differences between each set of points were compared with the control groups by a two-tailed unpaired t test. Data are expressed as the mean ± S.D. (n = 5). *P < 0.05 (limit of significance).

Fig. 5. The concentration of cilostazol (A), OPC-13213 (B), and OPC-13015 (C) in the absence or presence of dexamethasone, sulfaphenazole, probenecid, and aspirin in rat kidney slices. The concentrations of cilostazol, dexamethasone, sulfaphenazole, probenecid, and aspirin are 2, 50 μM, 10 μM, 8 mM, and 30 μM, respectively. Data are expressed as the mean ± S.D. (n = 3) *P < 0.05; **P < 0.01 versus control.
concentrations of cilostazol, OPC-13015, and OPC-13213 between the aspirin treatment group and the control group.

**Effect of Oat Substrates or Inhibitors and Aspirin on the Uptake of Cilostazol in Kidney Slices.** To exclude the impact of changing physiologic conditions, we used fresh rat kidney slices to further investigate the target transporters involved in the renal clearance of cilostazol (Fig. 7). Because we observed that the uptake of cilostazol increased linearly over a period of 10 minutes (Fig. 7A), the concentration-dependent uptake of cilostazol was examined at 10 minutes (Fig. 7B). To determine the effect of concentrations of substrates or inhibitors and aspirin on the uptake of cilostazol, we measured the concentration-dependent inhibitory effect of PAH (a substrate of OAT1), PCG, probenecid, JBP485, and aspirin on cilostazol uptake (Fig. 7B). JBP485 is a dipeptide (Liu et al., 2000) with antihepatitis and gastrointestinal protective effects (Liu et al., 1998; Wu et al., 2008) that was first isolated from Laennec (Yang et al., 2009), a substrate of OAT1 and OAT3 (Zhang et al., 2010). We found that PCG, probenecid, JBP485, and aspirin inhibited the uptake of cilostazol in a concentration-dependent manner (Fig. 7B). PAH did not have an inhibitory effect. PCG (0.2 mM), JBP485 (0.5 mM), probenecid (0.1 mM), and aspirin (0.03 mM) significantly inhibited cilostazol uptake in a time-dependent manner (Fig. 7A). These findings indicate that the target transporter involved in the renal excretion of cilostazol involves Oat3, but not Oat1, in rats.

**Cilostazol Uptake by hOAT1-/hOAT3-Transfected HEK293 Cells.** Time-dependent cilostazol uptake in hOAT1-HEK293 cells, or hOAT3-HEK293 cells, and vector-HEK293 cells was measured to further examine whether cilostazol is a substrate of OAT1 or OAT3. Cilostazol uptake by hOAT1-transfected HEK293 cells was not significantly different from vector-HEK293 cell uptake (Fig. 8A), whereas cilostazol uptake in hOAT3-transfected HEK293 cells was significantly greater than uptake in vector-HEK293 cells. Cilostazol uptake occurred linearly for up to 1 minute (Fig. 8B). The addition of PCG, JBP485, and aspirin significantly inhibited the uptake of cilostazol (Fig. 8B). We examined the effects of PCG, JBP485, and aspirin on concentration-dependent uptake of cilostazol by
hOAT3-transfected HEK293 (Fig. 9A), and we also conducted Eadie-Hofstee plot analysis (Fig. 9B). $K_m$ and $V_{\text{max}}$ values for the transport of cilostazol by hOAT3 were 23.6 ± 0.0140 μM and 0.726 ± 0.00600 pmol/mg protein per minute, respectively.

Figure 10 shows the concentration-dependent inhibitory effect of PCG, JBP485, and aspirin on cilostazol uptake in hOAT3-transfected HEK293 cells. Cilostazol uptake in hOAT3-transfected HEK293 cells was inhibited by PCG, JBP485, and aspirin, with corresponding $K_i$ values of 126 ± 19.0, 274 ± 13.0, and 15.0 ± 8.10 μM, respectively (Table 2).

**Discussion**

We investigated whether cilostazol was the substrate of OAT3/Oat3 in rats and humans and whether OAT3/Oat3 was also the target of the DDI between cilostazol and aspirin or probenecid by in vivo renal clearance and distribution studies as well as in vitro uptake studies using rat kidney slices and human OAT1- and OAT3-transfected cells. Our in vitro metabolism study using kidney and liver slices showed that probenecid could affect the metabolism of cilostazol. OAT1 and OAT3 are expressed on the basolateral membrane of proximal tubules and are shown to play a vital role in renal secretion of anionic xenobiotics. The similarities of Oat1 and Oat3 in rats to OAT1 and OAT3 in humans are 98% and 90%, respectively. To further clarify this, we examined the uptake of cilostazol, PAH (a typical substrate of OAT1), and estrone-3-sulfate (a typical substrate of OAT3) using rat and human kidney slices, respectively, to compare substrate affinities. The results showed that there were no significant differences between the uptakes of cilostazol, PAH, and estrone-3-sulfate in rat and human kidney slices. Therefore, we posited that the functions of these transporters are similar. Our results confirmed that cilostazol is mainly metabolized to OPC-13213 in the kidney (Fig. 4) and that OAT3/Oat3 is involved in the renal excretion of cilostazol and is the target of the DDI between cilostazol and aspirin. Oat3 and Cyp3a are the targets of the DDI between cilostazol and probenecid in rats.

In rats, nearly 43% of cilostazol and metabolites are excreted in urine (Vats et al., 2012). The majority of the metabolite is OPC-13213 (Mallikaarjun et al., 1999a). In humans, cilostazol is metabolized to

Fig. 7. The inhibition effects of various inhibitors on cilostazol uptake in kidney slices. (A) The inhibition effects of PCG (0.2 mM), JBP485 (0.5 mM), probenecid (0.1 mM), and aspirin (0.03 mM) on cilostazol (2 μM) uptake in kidney slices. (B) Effects of various concentrations of PAH (0.1, 0.2, and 0.5 mM), PCG (0.1, 0.2, and 0.5 mM), JBP485 (0.2, 0.5, and 1.0 mM), probenecid (0.05, 0.1, and 0.2 mM), and aspirin (0.01, 0.03, and 0.1 mM) on cilostazol (2 μM) uptake in kidney slices. Data are expressed as the mean ± S.D. (n = 3). *P < 0.05; **P < 0.01 versus control.

Fig. 8. The time-dependent inhibitory effects of various inhibitors on cilostazol uptake. (A) The uptake of 2 μM cilostazol in hOAT1-HEK293 or vector-HEK293 cells. (B) The uptake of 2 μM cilostazol in the presence and absence of PCG (0.2 mM), JBP485 (0.5 mM), and aspirin (0.03 mM) in hOAT3-HEK293 or vector-HEK293 cells. Data are expressed as the mean ± S.D. (n = 3) *P < 0.05; **P < 0.01 versus control.
OPC-13015 via CYP3A4 and to OPC-13213 via CYP2C19 (Kim et al., 2009). In rats, the metabolism of cilostazol to OPC-13015 or OPC-13213 is mediated by CYP3A2 or CYP2C11 (Kamada et al., 2011). CYP3A2 and CYP2C11 expressed in rats are the most similar to CYP3A4 and CYP2C19 in humans (Bogaards et al., 2000; VanAlstine and Hough, 2011). It was previously suggested that CYP3A1, CYP3A2, and CYP2C11 are expressed in the rat kidney (Pfohl-Leszkowicz et al., 1998) and that CYP3A4, CYP3A5, and CYP2C19 are detected in the human kidney (Lasker et al., 2000; Aleksa et al., 2005).

In the in vivo renal clearance experiment, the low cumulative urinary excretion and CL\text{R} of cilostazol and the decrease in all groups who received the coadministration compared with the control group over 24 hours suggest that cilostazol renal excretion is inhibited by probenecid, PCG, or aspirin. The increase in the cilostazol plasma concentration and AUC with a decrease in CL\text{p} (Fig. 2, B–F) indicates that PCG and aspirin inhibit cilostazol plasma elimination. The decrease in cumulative urinary excretion of cilostazol showed that both PCG and aspirin could reduce the uptake of cilostazol by the kidney. PCG or aspirin did not change the OPC-13213 or OPC-13015 plasma concentration (Fig. 3, B and C). This finding illustrated that PCG and aspirin did not influence the metabolism of cilostazol.

The concentration of OPC-13015 was too low to be determined in urine. However, the cumulative urinary excretion of OPC-13213 was nearly 23% of the dose and was higher than that of cilostazol. Probenecid, PCG, and aspirin decreased OPC-13213 cumulative urinary excretion (Fig. 3A). Because clinical dosing is oral, we used this route of administration for cilostazol, aspirin, and probenecid in the rat and observed results that were similar to those obtained with intravenous administration (Supplemental Table 1). The plasma concentration of cilostazol increased when aspirin was coadministered. The AUC increased by 51.1%. No change in \(C_{\text{max}}\) and \(T_{\text{max}}\) values showed that aspirin only inhibited the clearance of cilostazol. Coadministration of probenecid decreased the plasma concentration of cilostazol, and the AUC decreased by 56.0%. The decrease in \(C_{\text{max}}\) indicated that probenecid affected both distribution and clearance. This study demonstrated that OPC-13213 was not the substrate of OAT1 or OAT3 (data not shown).

We further investigated the influence of probenecid or aspirin on cilostazol distribution in the kidney to clarify whether the decrease of OPC-13213 cumulative urinary excretion was caused by the reduction of unchanged cilostazol absorbed into the kidney. The results of the distribution experiment indicated that the disposition of unchanged cilostazol in the liver and kidney was higher than that in the plasma. The decrease mediated by probenecid and aspirin on cilostazol distribution in kidney (Fig. 4) showed that cilostazol uptake in kidney could be inhibited by probenecid and aspirin. Our study of kidney slices demonstrated that the kidney contributed to cilostazol metabolism and that probenecid or aspirin had no effect on cilostazol renal metabolism. There was no significant difference in the concentrations of OPC-13015 in all groups (Fig. 5C).

The probenecid-mediated decrease in cilostazol plasma concentrations suggests that probenecid does not inhibit cilostazol plasma elimination. Therefore, the target of the cilostazol/probenecid DDI is not likely to be only within the kidney. According to Kim et al. (2005), probenecid has an inducing effect on carbamazepine’s biotransformation to carbamazepine-E, which is probably mediated by CYP3A4 activation. Because CYP3A4 can also mediate the metabolism of cilostazol, we speculated that the other target of the cilostazol/probenecid DDI is in the liver. In the in vivo experiment, the probenecid-mediated increase in the OPC-13015 concentration and the decrease in the OPC-13213 concentration indicated that probenecid could increase cilostazol metabolism. The experiment on the metabolism of liver slices demonstrated this point. In the...
dexamethasone and probenecid treatment groups, the cilostazol concentration in liver slices was lower than that in the control group. Treatment with ketoconazole increased the cilostazol concentration in liver slices, and aspirin had no effect.

To further clarify our results, we simultaneously measured OPC-13015 and OPC-13213. Dexamethasone and probenecid both increased, whereas ketoconazole decreased the OPC-13015 concentration. Aspirin did not affect the concentration of OPC-13015. Opposite results were obtained upon determination of OPC-13213 concentration in all groups who received the coadministration.

OPC-13015 is three times more potent than cilostazol with regard to inhibition of platelet aggregation, whereas OPC-13213 is three times less potent than cilostazol (Okuda et al., 1993). Our results show that probenecid increased the metabolism of cilostazol to OPC-13015 mediated by CYP3a. In summary, although the cilostazol concentration decreased, the OPC-13015 concentration correspondingly increased. Thus, there may be a beneficial DI between cilostazol and probenecid. Aspirin did not affect cilostazol metabolism (Mallikarjun et al., 1999b).

Our in vitro and in situ data indicated that OAT3/Oat3, but not OAT1/Oat1, plays a role in cilostazol excretion. The analysis of kidney slices showed that PAH did not affect cilostazol uptake, whereas PCG, JBP485, probenecid, and aspirin all inhibited cilostazol uptake (Fig. 7). The experiment on transfected cell uptake further supported this finding and showed that cilostazol uptake was inhibited in the presence of PCG, JBP485, and aspirin in hOAT3-HEK293 cells (Fig. 8). Our data demonstrate that particular attention must be paid when cilostazol is concomitantly used with other drugs that share transporters in drug development. Nat Rev Drug Discov 9:215-236.


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Mechanism of DDI between Cilostazol and Aspirin or Probenecid 1007
Title

Aspirin and probenecid inhibit OAT3-mediated renal uptake of cilostazol and probenecid induces metabolism of cilostazol in rat

Chong Wang, Changyuan Wang, Qi Liu, Qiang Meng, Jian Cang, Huijun Sun, Jinyong Peng, Xiaochi Ma, Xiaokui Huo and Kexin Liu

Drug metabolism and disposition

Appendix

Detailed Pharmacokinetic parameters of cilostazol after p.o. administration in rats when administered alone or in combination with aspirin or probenecid.

Table Legent

Supplemental Table 1. Pharmacokinetic parameters of cilostazol (30 mg/kg) after p.o. administration in rats when administered alone or in combination with aspirin (10mg/kg) or probenecid (30 mg/kg)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cilostazol</th>
<th>Cilostazol+Aspirin</th>
<th>Cilostazol+Probenecid</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (μg/ml)</td>
<td>3.16±0.46</td>
<td>3.56±0.12</td>
<td>2.48±0.27</td>
</tr>
<tr>
<td>$AUC_{0\rightarrow\infty}$ (μg·min/ml) p.o.</td>
<td>580.08±0.75</td>
<td>1185.30±0.12$^a$</td>
<td>255.00±0.31$^a$</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>0.73±0.02</td>
<td>0.81±0.04</td>
<td>0.48±0.06</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (h)</td>
<td>3.43±0.07</td>
<td>4.09±0.03$^b$</td>
<td>5.74±0.05</td>
</tr>
</tbody>
</table>


\textsuperscript{a} Statistically significant difference: \( p < 0.01 \).

\textsuperscript{b} Statistically significant difference: \( p < 0.05 \).