Title

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

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Running title: Mechanism of DDI between cilostazol and aspirin or probenecid

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ABBREVIATIONS

OATs, organic anion transporters; PAH, $p$-aminohippuric acid; PCG, penicillin-G; HEK293 cells, human embryonic kidney cells; AUC, area under the plasma concentration-time curve; CL, clearance; CYP, Cytochrome P450; P-gp, P-glycoprotein; DDI, drug-drug interactions
Abstract

The aim of this study was to evaluate transporter-mediated renal excretion mechanism for 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 and OPC-13213) in rat biological or cell samples were measured by LC-MS/MS. Co-administration with probenecid, benzylpenicillin or aspirin decreased cilostazol cumulative urinary excretion and renal clearance. Concentrations of cilostazol and OPC-13213 in plasma were decreased and OPC-13015 concentration was increased in presence of probenecid, whereas, in combination with benzylpenicillin or aspirin, rat plasma cilostazol sharply increased and concentrations of OPC-13015 and OPC-13213 did not change. In urine, OPC-13015 was below the level of detection. Cumulative urinary excretion of OPC-13213 decreased in presence of probenecid, benzylpenicillin or aspirin. Cilostazol was distributed in kidney and liver with Kp values of 8.4 mL/g and 16.3 mL/g. Probenecid and aspirin reduced cilostazol distribution in kidney. Probenecid did not affect cilostazol metabolism in kidney, but increased cilostazol metabolism in liver, and aspirin had no effect on cilostazol metabolism. Benzylpenicillin, aspirin and JBP485 reduced cilostazol uptake in kidney slices and hOAT3-HEK293 cells, while $\beta$-aminohippuric acid did not. Compared to vector, hOAT3-HEK293 cells accumulated more cilostazol whereas hOAT1-HEK293 cells did not. OAT3 and Oat3 play the major role in cilostazol renal excretion, while OAT1 and Oat1 do not. Oat3 and Cyp3a are both targets of 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 by affecting it or its metabolites concentration in various tissues. Transporter-based drug interactions in clinic may be inhibitory, inductive or both (Endres et al., 2006). For example, co-administration of a drug that is an inhibitor or inducer of 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 through affecting transporters.

The kidney is a key organ for excretion of drugs and drug metabolites. The transport systems responsible for renal tubular secretion of drugs have been classified as either organic anion transporters (OATs) or cation transporters (OCTs) based on their preferential substrate selectivity (Burckhardt and Burckhardt, 2011). Multidrug and toxin compound extrusion (MATE) transporter and carnitine/organic cation transporter (OCTN) are also important for renal transport. Various anionic drugs and substances are taken up into tubular cells by basolateral multispecific OATs (Burckhardt and Wolff, 2000). There are eight isoforms in the organic anion transporter family: OAT1-7 and OAT10 (Ahn and Nigam, 2009). OAT1/SLC22A6, OAT2/SLC22A7 and OAT3/SLC22A8 have been identified on the basolateral membrane of the human proximal tubules (Miyazaki et al., 2004).

Cytochrome P450 (CYP) is found predominantly in liver but also in many other extrahepatic tissues such as kidney. The majority of CYP studies has been focused on
hepatic forms and much less is known about renal CYP. 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 kidney than in liver. For example, the glycination of benzoic acid is faster in the perfused rat kidney than in the liver, and the activity of γ-glutamyl transferase activity is highest in the kidney (Poon and Pang, 1995). As reported previously, CYP3A4 and 3A5 (Aleksa et al., 2005; Lasker et al., 2000) have been reported in human kidney and CYP2C11 in rat kidney (Pfohl-Leszkowicz 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 (PDE3) (Parker et al., 2012). 72 h after an oral dose, 43 % of cilostazol and its metabolites are excreted in the urine (Vats et al., 2012). The metabolite of cilostazol in urine is mainly OPC-13213 and unchanged cilostazol is nearly 1% (Bramer and Forbes, 1999). Cilostazol is extensively metabolized by CYP enzymes. However, only 2 of its 11 known metabolites are quantifiably measurable in plasma and considered to be pharmacologically active: OPC-13015 and OPC-13213 (Kim et al., 2009). There is no report about the role the kidney plays in the metabolism of cilostazol. Whether the small amount of renal excretion of unchanged cilostazol is caused by the metabolism of cilostazol in kidney has not been investigated. Previous studies have shown that both influx and efflux transporters influence cilostazol bioavailability. P-Glycoprotein (P-gp) contributes, in part, to the
intestinal secretion of cilostazol though 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 settled in the literature.

Dual antiplatelet treatment with aspirin and cilostazol has been investigated and may result in a more favorable patient risk-benefit analysis in the prevention of stent thrombosis (Jeon et al., 2010). However, the question still remains whether the beneficial DDI between cilostazol and aspirin is mediated by transporters.

In this study, we used renal clearance and distribution experiment in vivo, kidney and liver slices in vitro as well as 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

**Materials.** Cilostazol was purchased from Pharmaceutical Co., Ltd. Zhejiang Kinglyuan. JBP485 was supplied by Japan Bioproducts Industry Co., Ltd (Tokyo, Japan) and bestatin (internal standard) was provided by Pharmaceutical Co., Ltd. Shen Luck. Probenecid (>99.0% purity), \( p \)-aminohippuric acid (PAH), benzylpenicillin (PCG), aspirin, dexamethasone, ketoconazole and sulfaphenazole were purchased from Sigma-Aldrich (St. Louis, MO). OPC-13015 and OPC-13213 were purchased from Toronto Research Chemicals Inc. HPLC-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.** HEK293 cells were grown in low-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10\% (v/v) fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100\( \mu \)g/mL streptomycin and kept at 37\(^\circ\)C with a 5\% (v/v) CO\(_2\) atmosphere and 95\% relative humidity. Cell culture reagents were purchased from GIBCO\(^\circledR\) (Grand Island, NY).

**Animals.** Male Wistar rats (weighing 220 to 250 g) were obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008-0002), were allowed free access to water and fed a chow diet. Before pharmacokinetic experiments, the animals fasted for 12 h 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 h. For in vivo studies, rats were anesthetized with pentobarbital (60 mg/kg) by intraperitoneal injection before the onset of experiment. In distribution study, kidney and liver slices studies, rats were anesthetized with ether.

**In vivo renal clearance experiments.** Rats were divided randomly into four groups: 1) cilostazol alone (20μg/kg); 2) cilostazol (20μg/kg) + probenecid (100mg/kg); 3) cilostazol (20μg/kg) + PCG (200 mg/kg); and 4) cilostazol (20μg/kg) + aspirin (10mg/kg). Test drugs, diluted in normal saline, were administered intravenously via the jugular vein (injected through the left side of the jugular vein). After i.v. administration, blood samples (300μl) were collected through the right side of the jugular with heparinized syringes at the following time points: 1, 5, 10, 30, 60, 120, 240, 360, 480 and 600 min. Isotonic saline solution (300μl) was injected following 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 h after administration. Cilostazol, OPC-13015 and OPC-13213 concentrations were measured by LC-MS/MS. Cumulative urinary excretion, renal clearance and pharmacokinetic parameters were calculated.

**Kidney and liver distribution study.** Rats were divided randomly into three groups: 1) cilostazol alone (20μg/kg); 2) cilostazol (20μg/kg) + probenecid (100mg/kg); and 3) cilostazol (20μg/kg) + aspirin (10mg/kg). Drugs were administered intravenously to rats via the jugular vein. After administration, the animals were sacrificed by drawing whole blood from the abdominal aorta using a
heparinized syringe under ether anesthesia. Kidney and liver were removed and washed with normal saline to remove the blood or content, blotted on filter paper, and then weighed for wet weight, and stored at -70°C. Before analysis, tissues were homogenized in 300 μl chilled (4°C) normal saline for about 4 min with a tissue homogenizer (IKA-T10 model, Germany) in an ice bath. Subsequently, the homogenates were centrifuged at 11374 g for 10 min at 4°C to remove cellular debris and the supernatant was used for LC-MS/MS determination.

In vitro kidney slice culture. Rat kidney slices were prepared as previously described (Vickers et al., 1992). Kidney slices were prepared from cortex region of organ in oxygenated Krebs-Henseleit buffer (PH 7.4) at 4°C under constant oxygenation (O₂:CO₂, 95:5). 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), L-glutamine (2mM), Nu-Serum (10%), fungizone and gentamycin. Slices were equilibrated at 37 °C in an atmosphere of 95% O₂/5% CO₂ for 90 min in culture medium containing 0.1μM dexamethasone, 10μM sulfaphenazole, 8mM probenecid and 30μM aspirin. Following the preincubation period, the media was replaced with fresh media, including 2μM cilostazol in the absence or presence of 50μM dexamethasone, 10μM sulfaphenazole, 8mM probenecid and 30μM aspirin. Slices were harvested at 2, 4, 6, 8, 10 and 24 h. At the end of the incubation period, kidney 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 liver slice culture. Rat liver slices were prepared as previously described (Elferink et al., 2004). Briefly, following 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 (Zhixin Co., Ltd., Shanghai, China).

The slices were placed into the culture medium consisted of Waymouth’s media containing 10% fetal bovine serum, 25mM HEPES, 5µg/mL insulin, 50µg/mL gentamicin, 2.5µg/mL amphotericin B, 25mM glucose and 2.4g/L sodium bicarbonate with a pH of 7.4. Prior to treatment, slices were equilibrated at 37 °C in an atmosphere of 95% O₂/5% CO₂ for 1h in culture medium containing 0.1μM, 1μM, 8mM and 30μM dexamethasone, ketoconazole, probenecid and aspirin, respectively. Treatment was commenced by replacing the culture medium with fresh medium containing the required concentrations of test compounds. Liver slices were incubated with cilostazol (2μM) in the absence or presence of 50μM, 1μM, 8mM and 30μM dexamethasone, ketoconazole, probenecid and aspirin for 1, 3, 5, 10 and 24 h. 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., Shanghai, China). After pre-incubation for 3 min 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 min, a 10 min 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 (2μM) in the presence or absence of PAH (0.1mM, 0.2mM, 0.5mM), PCG (0.1mM, 0.2mM, 0.5mM), JBP485 (0.2mM, 0.5mM, 1.0mM), probenecid (0.05mM, 0.1mM, 0.2mM) and aspirin (0.01mM, 0.03mM and 0.1mM). The following inhibitor concentrations were chosen: PCG (0.2mM), JBP485 (0.5mM), probenecid (0.1mM) and aspirin (0.03mM). At the end of the incubation period, kidney slices were washed with ice-cold Hanks’ balanced salt solution (HBSS) (pH7.5). Accumulated cilostazol in homogenized kidney was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and the uptake of cilostazol was reported as pmol/mg kidney.

**Uptake by transporter expression systems.** Uptake experiments with hOAT1-, hOAT3-HEK293 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 h before each experiment. Uptake was initiated by adding transport buffer (1 mL) with cilostazol (2μM) after the cells had been washed twice and incubated with transport buffer at 37°C for 15 min. The uptake was terminated upon the removal of the medium and 3 washes, each with 1mL of ice-cold HBSS, lysed with 0.3 mL of 0.1% (v/v) Triton X-100, then transferred to a polythene tube for quantization by
LC-MS/MS. The inhibitory effects of PCG (0.2mM), JBP485 (0.5mM) and aspirin (0.03mM) on cilostazol uptake were investigated. The uptake of cilostazol was measured in hOAT1-, hOAT3-HEK293 and mock cells. Samples were taken at 0.25, 0.5, 1, 3, 5 and 10 min and according to the results, a time of 1 minute was selected as representative of the uptake rate and used to examine the concentration-dependent uptake and effect of various inhibitors on cilostazol uptake. Protein was measured with the bicinchoninic acid (BCA) procedure (Solarbio, China) using bovine serum albumin for the standard.

**Sample preparation.** 50 μL of internal standard at 1μg/mL bestatin and 250 μL of acetonitrile were added to 50 μL of rat plasma or urine samples. The mixed samples were vortexed for 1 min and centrifuged at 11374 g for 10 min to remove the protein precipitate. 250 μL supernatants 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 at pH 7.4 after weighing, respectively and homogenized (IKA-T10 homogenizer; IKA, Staufen, Germany) on ice. Other preparations were handled the same way as the plasma samples. 10 μL of each sample was injected for LC-MS/MS analysis.

**LC-MS/MS analysis.** An Agilent LC system (Agilent HP1200, Agilent Technology Inc., Palo Alto, CA, USA) 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 mm × 4.6 i.d., 5 μm, Dalian Elite Analytical Instruments Co. Ltd, China).

The mobile phase consisted of solvent A (10mM 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 0-1 min. A linear gradient was performed over 4 min, 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 min. The total analysis time was 10 min. The flow rate was set at 0.4mL/min and the injection volume used was 10(L. The column was maintained at room temperature. An API 3200 triple-quadrupole mass spectrometer (Applied Biosystems, Concord, Ont, Canada) was operated with a TurboIonspray interface in positive ion mode. Analyst 1.4.1 software (Applied Biosystems) was used to control the equipment and for data acquisition and analysis. The optimized truncated MRM fragmentation transitions were m/z 370.40→m/z 288.10 with a collision energy (CE) of 22 eV for cilostazol, m/z 309.10→m/z 120.30 with a CE of 32 eV for bestatin, m/z 368.20→m/z 285.90 with a CE of 20eV for OPC-13015 and m/z 386.1→m/z 288.1 with a CE of 20eV for OPC-13213. The dwell time for each transition was 200 ms.

**Data analysis.** The main pharmacokinetic parameters were calculated according to the Practical Pharmacokinetic Program (3P97) edited by the Chinese Mathematical Pharmacological Society. The 3P97 program obtained C-T curves and main pharmacokinetic parameters automatically. The quality of the fit was judged by
evaluating the S.D. of parameter estimates the coefficient of determination (r²) and by visual inspection of the residual plots. The main pharmacokinetic parameters were calculated using equations (1) and (2). Plasma clearance (CLₚ) was calculated with the following equation:

\[ CL_p = \frac{\text{Dose}}{\text{AUC}_{i.v.}} \]  \hspace{1cm} (1)

where \(\text{AUC}_{i.v.}\) is the area under the plasma concentration-time profile after i.v. administration. Renal clearance (CLₐ) of cilostazol was calculated with the following equation:

\[ CL_R = \frac{\text{A}_{\text{total}}}{\text{AUC}_{i.v.}} \]  \hspace{1cm} (2)

where \(\text{A}_{\text{total}}\) is the total cumulative amount of cilostazol excreted in urine over 24 h.

Kinetic parameters were obtained using non-linear, least-squares regression analysis via the Michaelis–Menten equation:

\[ v = \frac{V_{\text{max}} S}{K_m + S} \]  \hspace{1cm} (3)

where \(v\) is the uptake velocity of the substrate (pmol/min per mg protein); \(K_m\) is the Michaelis constant (µM); \(S\) is the substrate concentration in the medium (µM); \(V_{\text{max}}\) is the maximum uptake rate (pmol/min per mg protein).

The inhibition constant (\(K_i\)) was calculated by IC₅₀, for competitive inhibition:

\[ \text{IC}_{50} = K_i \left( 1 + \frac{[S]}{K_m} \right) \]  \hspace{1cm} (4)

For noncompetitive inhibition:

\[ \text{IC}_{50} = K_i \left( 1 + \frac{K_m}{[S]} \right) \]  \hspace{1cm} (5)

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

The value of the tissue-to-plasma partition coefficient (\(K_p\)) was calculated with
following equation:

\[ K_p = \frac{C_t}{C_p} \quad (6) \]

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

**Statistical analysis.** Statistical analysis was performed using the SPSS11.5 package. Test results are expressed as the mean ± S.D. One-way analysis of variance (ANOVA) was performed to determine 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. When cilostazol (20μg/kg) and probenecid (100mg/kg) (a well-known inhibitor of OAT1 and OAT3 (Giacomini et al., 2010) were intravenously co-administered, changes in cilostazol cumulative urinary excretion were assessed (Fig. 1A). Compared with the control group (cilostazol alone), when cilostazol and probenecid were co-administered, the cumulative urinary excretion of cilostazol decreased significantly and the renal clearance rate (CLR) decreased by 41.8% (Fig. 1D).

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

To examine whether the metabolism of cilostazol was influenced when cilostazol was coadministered with probenecid, PCG and aspirin, we determined the
concentrations of OPC-13015 and OPC-13213 in plasma and urine. Compared to control group, the coadministration of cilostazol and probenecid increased OPC-13015 concentration in plasma and AUC increased by 42.7% (Fig. 3C), whereas the concentration of OPC-13213 and AUC decreased by 75.1% (Fig. 3B). Coadministration of PCG or aspirin did not influence the concentrations of the two metabolites in plasma (Fig. 3B and 3C). In urine, OPC-13015 concentrations were below the limit of detection. However, the cumulative urinary excretion of OPC-13213 was higher than both that of cilostazol and OPC-13015. Probenecid, PCG and aspirin all reduced OPC-13213 cumulative urinary excretion with CLR decreased by 17.3%, 44.5% and 41.5%, respectively.

**Distribution of cilostazol in kidney and liver.** To examine whether cilostazol was taken into kidney in intact form and probenecid or aspirin affected the distribution of cilostazol in kidney and liver, we investigated cilostazol distribution following i.v. administration with cilostazol alone or in combination with probenecid or aspirin in rats (Fig. 4). In control group (cilostazol alone), 120 min after administration, the Kp values of cilostazol in kidney and liver were 8.4 mL/g and 16.3 mL/g, respectively (Fig. 4A and 4B). The co-administration of cilostazol and probenecid or aspirin decreased the distribution of cilostazol in kidney (Fig. 4A), however, did not change cilostazol distribution in liver (Fig. 4B).

**Cilostazol metabolism in kidney slices.** To examine the reason why concentration of unchanged cilostazol were lower in urine, we explored whether cilostazol could be metabolized by kidney and whether probenecid or aspirin affected
cilostazol renal metabolism, we used fresh kidney slices to prove the hypothesis (Fig. 5). Cilostazol was administered with dexamethasone (an inducer of CYP3A4/3A2/2C11 (Teo et al., 2012; Rowlands et al., 2000)) (50μM), sulfaphenazole (an inhibitor of CYP2C11) (10μM), probenecid (8mM) and aspirin (30μM) 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), but the concentration of OPC-13213 significantly increased while the concentrations of cilostazol decreased compared with control in the dexamethasone treatment group (Fig. 5A and 5B). In contrast, the metabolism of cilostazol to OPC-13213 was significantly inhibited following treatment with sulfaphenazole (Fig. 5B). Correspondingly, the concentration of cilostazol increased with sulfaphenazole treatment compared with the control group (Fig. 5A). In probenecid or aspirin treatment group, the concentration of OPC-13213 or cilostazol was not different from that in control group (Fig. 5A and 5B).

**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 μM), ketoconazole (a typical inhibitor of CYP3A4/3A2 (Lee et al., 2006; Chen et al., 2004)) (1 μM), probenecid (8 mM) and aspirin (30 μM). The results showed that the concentration of OPC-13015 significantly increased (Fig. 6C), while the concentrations of cilostazol and OPC-13213 decreased (Fig. 6B) compared with control when liver slices were treated with dexamethasone. In the probenecid
treatment group, changes in the concentrations of cilostazol, OPC-13015 and OPC-13213 were similar to those of the dexamethasone treatment group (Fig. 6A-C). In contrast, the metabolism of cilostazol to OPC-13015 was significantly inhibited following treatment with ketoconazole (Fig. 6C). Correspondingly, the concentrations of cilostazol and OPC-13213 increased with ketoconazole treatment compared with the control group (Fig. 6A and 6B). There was no significant difference in the concentrations of cilostazol, OPC-13015 and OPC-13213 between the aspirin treatment group and the control group.

**Effect of Oats substrates or inhibitor and aspirin on the uptake of cilostazol in kidney slices.** To exclude the impact of changing physiological conditions, we used fresh rat kidney slices to further investigate the target transporters involved in the renal clearance of cilostazol (Fig. 7A and 7B). Because we observed that the uptake of cilostazol increased linearly over a period of 10 min (Fig. 7A), the concentration-dependent uptake of cilostazol was examined at 10 min (Fig. 7B). To determine the effect of concentrations of substrates or inhibitor and aspirin on uptake of cilostazol, we measured the concentration-dependent inhibitory effect of PAH (a substrate of OAT1), PCG, probenecid, JBP485 (a dipeptide (Liu et al., 2000), with anti-hepatitis and gastrointestinal protective effects (Liu et al., 1998; Wu et al., 2008), first isolated from Laennec (Yang et al., 2009), a substrate of OAT1 and OAT3 (Zhang et al., 2010)) and aspirin on cilostazol uptake (Fig. 7B). 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 (0.2mM), JBP485 (0.5mM), probenecid (0.1mM) and aspirin (0.03mM) 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 HEK 293 Cells.** To further examine whether cilostazol is a substrate of OAT1 or OAT3, time-dependent cilostazol uptake in hOAT1- or hOAT3- and vector-HEK293 cells was measured. Uptake of cilostazol by hOAT1-transfected HEK293 cells was not significantly different from vector-HEK293 cell uptake (Fig. 8A), while cilostazol uptake in hOAT3-transfected HEK293 cells was significantly greater than uptake in vector-HEK293 cells. Cilostazol uptake occurred linearly for up to 1 min (Fig. 8B). The addition of PCG, JBP485 and aspirin significantly inhibited the uptake of cilostazol (Fig. 8B). The effects of PCG, JBP485 and aspirin on concentration-dependent uptake of cilostazol by hOAT3-transfected HEK293 cells were examined (Fig. 9A) and Eadie-Hofstee plot analysis was conducted (Fig. 9B). Km and Vmax values for the transport of cilostazol by hOAT3 were found to be 23.6±0.0140µM and 0.726±0.00600 pmol/mg protein/min, respectively.

Fig. 10 shows the concentration-dependent inhibitory affect 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 Ki values of 126±19.0µM, 274±13.0µM and 15.0±8.10µM, respectively (Table 2).
Discussion

We investigated that cilostazol was the substrate of OAT3/Oat3 in rat and human and OAT3/Oat3 was also the target of DDI between cilostazol and aspirin or probenecid by in vivo renal clearance and distribution study as well as in vitro uptake study using rat kidney slices and human OAT1- and OAT3-transfected cells. 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 have been shown to play a vital role in renal secretion of anionic xenobiotics. The similarities of Oat1 and Oat3 in rat to OAT1 and OAT3 in human are 98% and 90%. 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 make a comparison of 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. So we thought that the functions of these transporters are similar. The results confirmed that cilostazol is mainly metabolized to OPC-13213 in kidney (Fig. 4) and OAT3/Oat3 is involved in the renal excretion of cilostazol and is the target of DDI between cilostazol and aspirin. The targets of DDI between cilostazol and probenecid are Oat3 and Cyp 3a 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 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 rat are the most similar to CYP3A4 and CYP2C19 in human (Bogaards et al., 2000) (VanAlstine and Hough, 2011). Presently, it has been suggested that CYP3A1, CYP3A2, and CYP2C11 are expressed in rat kidney (Pfohl-Leszkowicz et al., 1998) and CYP3A4, 3A5, 2C19 are detected in human kidney (Aleksa et al., 2005; Lasker et al., 2000).

In the in vivo renal clearance experiment, the low cumulative urinary excretion and $CL_R$ of cilostazol and the decrease in all co-administrated groups compared with control group over 24h suggest that cilostazol renal excretion is inhibited by probenecid, PCG or aspirin. The increase in cilostazol plasma concentration and AUC with a decrease in $CL_p$ (Fig. 2B-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 kidney. PCG or aspirin did not change OPC-13213 or OPC-13015 plasma concentration (Fig. 3B and 3C). 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 dose and higher than that of cilostazol. Probenecid, PCG and aspirin decreased OPC-13213 cumulative urinary excretion (Fig. 3A). Since clinical dosing is oral, we used this route of administration for cilostazol, aspirin and probenecid in the rat and observed
similar results as with i.v. administration (results were shown in supplemental Table 1). The plasma concentration of cilostazol was increased when aspirin was co-administered. AUC was increased by 51.1%. No change of Cmax and Tmax values showed that aspirin only inhibited the clearance of cilostazol. Co-administration of probenecid decreased the plasma concentration of cilostazol, AUC was decreased by 56.0%. The decrease Cmax indicated that probenecid affected both the processes of distribution and clearance. We have demonstrated that OPC-13213 was not the substrate of OAT1 or OAT3 (data not show).

We further investigated the influence of probenecid or aspirin on cilostazol distribution in kidney to clarify whether the decrease of OPC-13213 cumulative urinary excretion was caused by the reduction of unchanged cilostazol taken into kidney.

The distribution experiment results indicated the disposition of unchanged cilostazol in liver and kidney was higher than that in plasma. The decrease mediated by probenecid and aspirin on cilostazol distribution in kidney and no change in liver (Fig. 4) showed that cilostazol uptake in kidney could be inhibited by probenecid and aspirin. Kidney slices study demonstrated kidney contributed to cilostazol metabolism and probenecid or aspirin had no effect on cilostazol renal metabolism. There was no significant difference in 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 likely not only within the kidney. According to
conclusions made by Kim KA (Kim et al., 2005), probenecid has an inducing effect on carbamazepine’s (CBZ) biotransformation to CBZ-E, likely mediated by CYP3A4 activation. Because the metabolism of cilostazol can also be mediated by CYP3A4, we speculated that the other target of the cilostazol/probenecid DDI is in liver. In the in vivo experiment, probenecid-mediated increase in OPC-13015 concentration and decrease in OPC-13213 concentration indicated that probenecid could increase cilostazol metabolism. Liver slices metabolism experiment demonstrated this point. In dexamethasone and probenecid treatment group, cilostazol concentration in liver slices was lower than that in control group. Treatment with ketoconazole increased 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 while ketoconazole decreased OPC-13015 concentration. Aspirin did not affect the concentration of OPC-13015. Opposite results were obtained in determination of OPC-13213 concentration in all co-administrated groups.

OPC-13015 is 3 times more potent than cilostazol with regard to inhibition of platelet aggregation, whereas OPC-13213 is 3 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. To sum up, though cilostazol concentration decreased, OPC-13015 concentration increased correspondingly. So there may be beneficial drug-drug interaction between cilostazol and probenecid. Aspirin did not affect cilostazol metabolism (Mallikaarjun et al., 1999b).
Our in vitro and in situ data indicated that OAT3/Oat3 but not OAT1/Oat1 plays a role in cilostazol excretion. Analysis of kidney slices showed that PAH did not affect cilostazol uptake whereas PCG, JBP485, probenecid and aspirin all inhibited cilostazol uptake (Fig. 7A, 7B). Transfected cells uptake experiment further supported this finding and investigated that cilostazol uptake was inhibited in the presence of PCG, JBP485 and aspirin in hOAT3-HEK293 cells (Fig. 8A, 8B). Our data demonstrates that particular attention must be paid when cilostazol is concomitantly used with others drugs that share OAT3 for urinary excretion. We also demonstrate that there is potential PCG and aspirin to interact with cilostazol at therapeutic dosages.

Our results demonstrate that cilostazol can be taken into kidney in intact form and metabolized to OPC-13213, likely by CYP2C11 in rat kidney. Cilostazol is a substrate of OAT3/Oat3 and not OAT1/Oat1. Oat3 and drug metabolizing enzymes are the targets of the DDI between cilostazol and probenecid in kidney and liver, respectively. At the same time, aspirin can inhibit cilostazol renal excretion while not impacting cilostazol metabolism.
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Authorship Contributions

Participated in research design: Kexin Liu, Chong Wang and Qi Liu

Conducted experiments: Kexin Liu, Chong Wang and Changyuan Wang

Performed data analysis: Qiang Meng, Jian Cang and Xiaokui Huo

Wrote or contributed to the writing of the manuscript: Kexin Liu and Chong Wang

Contributed new reagents or analytical tools: Jinyong Peng, Huijun Sun and Xiaochi Ma
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Zhang X, Galinsky RE, Kimura RE, Quinney SK, Jones DR, and Hall SD (2010)

Footnotes

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Legend to Figures

Fig. 1. Cumulative urine excretion curves (A, B, C) and renal clearances (D, E, F) of cilostazol after intravenous injection of 20 μg/kg cilostazol and probenecid, PCG or aspirin in rats.

A, D: Co-administration of 100 mg/kg probenecid. B, E: Co-administration of 200 mg/kg PCG. C, F: Co-administration of 10 mg/kg aspirin. Data are expressed as mean ± S.D. (* p<0.05 vs. control; n=5).

Fig. 2. Mean plasma concentration–time curves (A, B, C) and plasma clearances (D, E, F) of cilostazol after intravenous administration of 20 μg/kg cilostazol and probenecid, PCG or aspirin in rats.

A, D: Co-administration of 100 mg/kg probenecid. B, E: Co-administration of 200 mg/kg PCG. C, F: Co-administration of 10 mg/kg aspirin. Statistical differences between each set of points were compared with the control groups by a two-tailed unpaired t-test, with p< 0.05 as the limit of significance (* p<0.05). Data are expressed as mean ± S.D. (n=5).

Fig. 3. Cumulative urine excretion curves of OPC-13213 (A) and mean plasma concentration–time curves of OPC-13213 and OPC-13015 (B, C) after intravenous administration of 20 μg/kg cilostazol and 100 mg/kg probenecid,
**200mg/kg PCG or 10mg/kg aspirin in rats.** Statistical differences between each set of points were compared with the control groups by a two-tailed unpaired t-test, with p< 0.05 as the limit of significance (*p<0.05). Data are expressed as mean ± S.D. (n=5).

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

**Fig. 5.** The concentration of cilostazol(A), OPC-13213(B), 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 2μM, 50μM, 10μM, 8mM and 30μM, respectively. Data are expressed as mean±S.D. (*p<0.05; **p<0.01 vs. control; n=3).

**Fig. 6.** The concentration of cilostazol(A), OPC-13213(B), OPC-13015(C), in the absence or presence of probenecid, dexamethasone, ketoconazole and aspirin in rat liver slices.

The concentrations of cilostazol, dexamethasone, ketoconazole, probenecid and
aspirin are 2μM, 50μM, 10μM, 1μM and 30μM, respectively. Data are expressed as mean± S.D. (* p<0.05; ** p<0.01 vs. control; n=3).

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

Fig. 8. The time-dependent inhibitory effects of various inhibitors on cilostazol uptake.
A: The uptake of 2μM cilostazol in hOAT1- or vector-HEK293 cells. B: The uptake of 2μM cilostazol in the presence and absence of PCG (0.2mM), JBP485 (0.5mM), and aspirin (0.03mM) in hOAT3- or vector-HEK293 cells. Data are expressed as mean± S.D. (* p<0.05; ** p<0.01 vs. control; n=3).

Fig. 9. The concentration-dependent inhibitory effects of various inhibitors on cilostazol uptake in hOAT3-HEK293 cells.
A: The uptake of cilostazol between 0.2 μM and 20 μM in the presence and absence of PCG (0.2mM), JBP485 (0.5mM) and aspirin (0.03mM) in hOAT3- or vector-HEK293 cells. B: Eadie-Hofstee plots. Data are expressed as mean± S.D. (n=3).

Fig. 10. The inhibitory effects of PCG, JBP485 and aspirin on cilostazol uptake in hOAT3-HEK293 cells.
Table 1. Pharmacokinetic parameters of cilostazol (20 μg/kg) after i.v. administration in rats when administered alone or in combination with PCG (200mg/kg), aspirin (10mg/kg) or probenecid (100 mg/kg)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cilostazol</th>
<th>Cilostazol+PCG</th>
<th>Cilostazol+Aspirin</th>
<th>Cilostazol+Probenecid</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>$Ke$ (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>
</tr>
<tr>
<td>$AUC_{0→∞}$ (ng·min/mL)</td>
<td>8453±150</td>
<td>14833±108</td>
<td>13356±95</td>
<td>5776±124</td>
</tr>
<tr>
<td>$T_{1/2β}$ (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>
</tr>
<tr>
<td>$Vd$ (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>$CL_p$ (mL/min/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>
</tr>
<tr>
<td>$CL_R$ (mL/min/kg)</td>
<td>0.047±0.004</td>
<td>0.0059±0.0008</td>
<td>0.014±0.002</td>
<td>0.031±0.005</td>
</tr>
</tbody>
</table>

$^a$ Statistically significant difference: $p < 0.01$.

$^b$ Statistically significant difference: $p < 0.05$. 
Table 2. IC$_{50}$ and K$_i$ values of cilostazol uptake in the presence of PCG, JBP485 and aspirin in hOAT3-HEK293 Cells. Values are mean± S.D. (n=3)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$(µM)</th>
<th>K$_i$(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>17.0±5.20</td>
<td>15.0±8.10</td>
</tr>
<tr>
<td>JBP485</td>
<td>297±15.0</td>
<td>274±13.0</td>
</tr>
<tr>
<td>PCG</td>
<td>137±17.0</td>
<td>126±19.0</td>
</tr>
</tbody>
</table>
Figure 1

A. Cumulative Urinary Excretion of Cilostazol (%)

B. Cumulative Urinary Excretion of Cilostazol (%)

C. Cumulative Urinary Excretion of Cilostazol (%)

D. CLr of Cilostazol (ml/min/kg)

E. CLr of Cilostazol (ml/min/kg)

F. CLr of Cilostazol (ml/min/kg)
Figure 2

A

B

C

D

E

F

Time (min)

Time (min)

Time (min)
Figure 5

A

Concentration of Cilostazol (pmol/mg kidney)

- Cilostazol
- Cilostazol+Dexamethasone
- Cilostazol+Sulfaphenazole
- Cilostazol+Aspirin
- Cilostazol+Probencid

Time (h)

B

Concentration of OPC-13213 (pmol/mg kidney)

- Cilostazol
- Cilostazol+Dexamethasone
- Cilostazol+Sulfaphenazole
- Cilostazol+Aspirin
- Cilostazol+Probencid

Time (h)

C

Concentration of OPC-13015 (pmol/mg kidney)

- Cilostazol
- Cilostazol+Dexamethasone
- Cilostazol+Sulfaphenazole
- Cilostazol+Aspirin
- Cilostazol+Probencid

Time (h)
Figure 7

A

Uptake of Cilostazol (pmol/mg kidney)

Time (min)

0 10 20 30

Cilostazol
Cilostazol + Aspirin
Cilostazol + JBP485
Cilostazol + PCG
Cilostazol + Probencid

B

Uptake of Cilostazol (% Control)

Control
PAH (0.1 mM)
PAH (0.2 mM)
PAH (0.5 mM)
PCG (0.1 mM)
PCG (0.2 mM)
PCG (0.5 mM)
JBP485 (0.2 mM)
JBP485 (0.5 mM)
JBP485 (1.0 mM)
PRO (0.05 mM)
PRO (0.1 mM)
PRO (0.2 mM)
ASP (0.01 mM)
ASP (0.03 mM)
ASP (0.1 mM)
Figure 10

Uptake of Cilostazol (% of control) vs. Inhibitor (µM)

- Cilostazol + Aspirin
- Cilostazol + JBP485
- Cilostazol + PCG