Peptide Cotransporter 1 in Intestine and Organic Anion Transporters in Kidney Are Targets of Interaction between JBP485 and Lisinopril in Rats

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Summary: The purpose of this study was to clarify the pharmacokinetic mechanism of interaction between JBP485 (cyclo-trans-4-L-hydroxyprolyl-L-serine, a dipeptide with antihepatitis activity) and lisinopril (an angiotensin-converting enzyme inhibitor) in vitro and in vivo. When JBP485 and lisinopril were administered orally simultaneously, the plasma concentrations of the two drugs were decreased significantly, but few changes were observed after simultaneous intravenous administration of the two drugs. The uptake of JBP485 and lisinopril in everted intestinal sacs and in HeLa cells transfected with human peptide cotransporter 1 (PEPT1), as well as absorption of JBP485 and lisinopril after jejunal perfusion were reduced after simultaneous drug administration, which suggested that the first target of drug interaction was PEPT1 in the intestine during the absorption process. The cumulative urinary excretions and renal clearance of the two drugs were decreased after intravenous co-administration, while uptakes of the two drugs in kidney slices and hOAT1/hOAT3-transfected HEK293 cells were decreased. These results indicated that the second target of drug-drug interaction was located in the kidney. These findings confirmed that the pharmacokinetic mechanism of interaction between JBP485 and lisinopril could be explained by their inhibition of the same transporters in the intestinal mucosa (PEPT1) and kidneys (OATs).

Keywords: JBP485; dipeptide; ACE inhibitor; drug interaction; transporters; pharmacokinetics; intestinal absorption; renal excretion

Introduction

The purpose of clinical drug–drug interaction (DDI) is to enhance the good effects or reduce the toxicity of the drugs in question. However, some drug combinations lead to increased toxic or reduced pharmacological effects. Small intestinal absorption and renal excretion are two of the main ways to study adsorption, distribution, metabolism and excretion (ADME). Di- and tripeptides are taken up into intestinal cells by the low-affinity H+/peptide cotransporter 1 (PEPT1). Angiotensin converting enzyme (ACE) inhibitors, β-lactam antibiotics and antivirals have been unequivocally demonstrated to use PEPT1 for intestinal absorption. Tubular secretion of xenobiotics, a transporter-mediated pathway, is a key function of the renal proximal tubule. Organic anion transporters (OATs) are the major drug transporter family that is highly expressed in the kidneys. Renal OAT1 and OAT3 are located on the basolateral membranes of proximal tubule cells, and have been shown to play a central role in the renal secretion of a wide range of anionic xenobiotics. Importantly, involvement of renal OATs in DDIs has been widely reported. For example, cephalosporins decreases the cumulative renal excretion of dipeptide through inhibition of OAT1 and OAT3.

JBP485 was first isolated by Laennec. It has clear antihepatitis and liver protective effects after oral administration, which has been indicated in animal experiments. Our previous studies have demonstrated that JBP485 is...
mainly absorbed by PEPT1 in the small intestine and excreted by OATs in the kidneys.\textsuperscript{21,22} 

Lisinopril is an ACE inhibitor that is primarily used for treatment of hypertension, congestive heart failure and heart attack, and prevention of renal and retinal complications of diabetes. Lisinopril has a number of properties that distinguish it from other ACE inhibitors: it is hydrophilic, has a long half-life and tissue penetration, is not metabolized by the liver, and is excreted unchanged, primarily in the urine.\textsuperscript{23} In almost every review on PEPTs, it has been stated that ACE inhibitors are peptide transporter substrates,\textsuperscript{24} suggesting that the intestinal PEPT takes up lisinopril and other ACE inhibitors. However, we have not found any study to suggest that renal excretion of lisinopril is mediated by OATs located on the basolateral membrane of proximal tubule epithelia.

To investigate whether combination of JBP485 and lisinopril results in a DDI, to clarify the pharmacokinetic mechanisms of any such interaction, and to provide a rationale for the clinical use of this drug combination, we used LC-MS/MS methods for determination of the two drugs in rat plasma, cell lysates, Krebs-Ringer buffer (KRB) and Krebs–bicarbonate slicing buffer. We utilized \textit{in vivo} oral administration, \textit{in situ} intestinal perfusion, \textit{in vitro} everted small-intestinal sac preparations, \textit{in vivo} urinary excretion, \textit{in vitro} kidney slices, and transfected-cell uptake studies to investigate the change in pharmacokinetics when JBP485 and lisinopril were administered together. These findings confirmed that the pharmacokinetic mechanisms of DDI between JBP485 and lisinopril could be explained by their inhibition of the same transporters in the intestinal mucosa (PEPT1) and kidneys (OAT1 and OAT3).

**Materials and Methods**

**Materials:** JBP485 was provided by Japan Bioproducts Industry Co. Ltd. (Tokyo, Japan). Lisinopril, with purity >99.5\%, was purchased from Hubei Saibo Chemical Co. Ltd. (Wuhan, China). Paracetamol (internal standard, IS), with purity >99.0\%, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol was of HPLC grade (Tedia, Fairfield, OH, USA). Fetal bovine serum (FBS) and Dulbecco’s Modified Eagle’s Medium (DMEM) were obtained from Gibco (Grand Island, NY, USA). The stable transfectants expressing hPEPT1-HeLa and HeLa empty-vector cells were constructed and identified by our laboratory. The stable transfectants expressing hOAT1- or hOAT3-HEK293 and HEK293 empty-vector cells were a generous gift from Professor Yuichi Sugiyama, Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan) and Li-kun Gong (Shanghai Institute of Materia Medica, Chinese Academy of Science, China). All other reagents and solvents were of analytical grade, and were commercially available.

**Animals:** Male Sprague-Dawley rats weighing 220–250 g were obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number: SCXK 2008–0002). The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. All animals were fasted overnight before dosing, and access to water was provided. Animals were fed after collection of the 4-h blood samples.

**Drug stability:** The stability of JBP485 and lisinopril in different matrices was evaluated by analyzing relative error at the concentrations of 20.0 (QC-L) and 1,000 ng/ml (QC-H), which were exposed to different samples (plasma, urine and buffer solutions). The spiked samples were analyzed after storage at ambient temperature for 2 h, in an autosampler for 24 h at room temperature after protein precipitation, at −20 °C for 7 days, and after three freeze-thaw cycles from −20 °C to room temperature. These results were compared with those obtained for freshly prepared samples. The determination of JBP485 or lisinopril was not interfered with when the two drugs were together in the different biological samples mentioned above.

**Pharmacokinetic interaction in rats:** In all cases, rats were fasted overnight and were anesthetized with ether before the onset of each experiment. JBP485 and lisinopril were dissolved in normal saline or buffer solution and administered to the rats in aqueous solution.

**In vivo absorption in rats:** Rats were divided randomly into three groups: (1) JBP485 alone (25 mg/kg) as control; (2) lisinopril alone (5 mg/kg) as control; (3) JBP485 (25 mg/kg) + lisinopril (5 mg/kg) as the experimental group. The drugs were administered orally by gavage. Blood samples were collected at 1, 5, 10, 20, 30, 60, 120, 180, 240, 360, 480, 600 and 720 min for JBP485 and lisinopril determination, as described below.

**In situ jejunal perfusion:** A laparotomy was performed after ether anesthesia, and an inflow cannula made of silastic tubing was inserted in the jejunum approximately 1 cm below the ligament of Treitz.\textsuperscript{25} An outflow cannula was set up at a distance of 10 cm. The bile duct was ligated to prevent possible enterohepatic circulation. The portal vein was non-ligated and cannulated using a heparinized i.v. catheter (BD Inayte-W 1.1 × 30 mm), closed with an adapter (BD PRN Adapter 19 mm-LC), and maintained with a gelatin sponge. The jejunal segment was then flushed with saline solution (prewarmed to 37 °C) to remove residual intestinal contents. Oxygenated perfusion solution was delivered with a peristaltic pump at a flow rate of 4 mL/15 min through an inlet tube that was water-jacketed at 37 °C, before its entry into the jejunal segment. The solution for jejunal perfusion was the same as the KRB below. After a 30-min equilibration period, 0.5 mM JBP485 alone, 0.1 mM lisinopril alone, or 0.5 mM JBP485 + 0.1 mM lisinopril dissolved in KRB were administered. Portal vein blood was collected for JBP485 and lisinopril determination, as described below. Portal vein blood samples were collected \textit{via} the adapter at 1, 5, 15, 30, 45 and 60 min post dosing.
**In vitro everted intestinal sac preparation:** The abdomen was opened by a midline incision and the jejunum was removed by cutting across the upper end of the duodenum (i.e., ~2 cm distal to the ligament of Treitz) and the lower end of the ileum, and manually stripping the mesentery. The small intestine was washed out carefully with cold, normal, oxygenated saline, using a syringe equipped with a blunt end. Intestinal segments (10 ± 1 cm) were everted according to the conventional technique described by Wilson and Wiseman with modifications. The distended sac was placed in incubation medium for perforation. The empty sac was one end to facilitate subsequent identification and to check for perforation. The empty sac was filled with 1 ml KRB. The distended sac was placed in incubation medium (mucosal solution) containing: (1) 0.5 mM JBP485; (2) 0.1 mM lisinopril; or (3) 0.5 mM JBP485 + 0.1 mM lisinopril. The incubation medium was surrounded by a water jacket maintained at 37°C. A gas mixture of 95% O₂ and 5% CO₂ was bubbled through the external medium during the incubation period. At the end of the incubation period, the serosal fluid was drained through a small incision into a test tube. Samples from the lumen of the everted sac (50 µl) were collected at 1, 5, 15, 30, 45 and 60 min post dosing for JBP485 and lisinopril determination, as described below.

**In vivo plasma concentration and renal excretion:** Rats were anesthetized with ether and administered the following drugs via the jugular vein: (1) JBP485 (25 mg/kg); (2) lisinopril (5 mg/kg); or (3) JBP485 (25 mg/kg) + lisinopril (5 mg/kg). Blood samples were collected at 1, 5, 10, 30, 60, 120, 240, 360, 480, 600 and 720 min. The bladder was 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 10, 20, 30, 60, 120, 240, 360, 720 and 1,440 min. The concentrations of JBP485 and lisinopril were measured. The cumulative urinary excretion and renal clearance were calculated.

**In vitro uptake in kidney slices:** Rats were anesthetized with ether and fixed in the supine position on the operating table; kidneys were incised, decapsulated and immediately placed into oxygenated buffer at 4°C as described by Nozaki et al. In brief, kidneys were cut into slices accurately using a ZQP-86 tissue slicer (Zhixin Co. Ltd., Xiangshan, Zhejiang, China) (thickness 300 µm, surface area ~0.15 cm²) and prepared in buffer. After 3 min preincubation under a carbogen atmosphere at 37°C in six-well culture plates, with gentle shaking, kidney slices were transferred to 24-well culture plates containing 1 ml fresh carbogen-saturated JBP485 (0.5 mM) and/or lisinopril (0.1 mM). In addition, JBP485 or lisinopril in the presence or absence of probenecid (1 mM) was used. The uptake was measured at 5, 10, 15, 30, 45 and 60 min. At the end of the incubation period, kidney slices were washed with ice-cold Hanks’ balanced salt solution (HBSS; pH 7.4). Accumulated concentrations of JBP485 and lisinopril were determined by LC-MS/MS after the kidney slices were homogenized.

**In vitro transporter uptake assays:** hPEPT1-HeLa, hOAT1-HEK293 and hOAT3-HEK293 transfected cells were grown in DMEM with 10% FBS with antibiotics in an atmosphere of 5% CO₂/95% air at 37°C, respectively. The assays were carried out in 24-well culture plates, with nearly confluent cells seeded 48 h before each experiment. Immediately before the experiment, the cells were washed twice with 1 ml HBSS buffer at room temperature and then incubated with 1 ml HBSS for 15 min at 37°C. After removal of the medium, the cells were incubated with 1 ml HBSS (pH 5.8 for hPEPT1-HeLa cells and pH 7.4 for hOAT1-/hOAT3-HEK293 transfected cells) containing drugs including: (1) JBP485 alone (0.5 mM for hPEPT1-HeLa cells) as a control for JBP485; (2) lisinopril alone (0.1 mM for hPEPT1-HeLa cells) as a control for lisinopril; (3) JBP485 (0.5 mM) + lisinopril (0.1 mM) for hPEPT1-HeLa cells as the inhibitory group; (4) JBP485 alone (0.5 mM for hOAT1-/hOAT3-HEK293 cells) as a control for JBP485; (5) lisinopril alone (0.1 mM for hOAT1-/hOAT3-HEK293 cells) as a control for lisinopril; or (6) JBP485 (0.5 mM) + lisinopril (0.1 mM for hOAT1-/hOAT3-HEK293 cells) as the inhibitory group. After incubation for the designated times at 37°C with gentle shaking, the experiment was terminated by removing the medium, followed by washing three times with 1 ml ice-cold HBSS and lysed in 0.3 ml well HBSS with 1% Triton X-100 for 60 min. The aliquots were subjected to LC-MS/MS. The uptake assays of the two agents were measured in a time-dependent manner, at the uptake time of 1, 3, 5, 10 and 15 min for PEPT1; and at 0.5, 1, 3 and 5 min for OATs.

**Biological sample preparation:** The concentrations of JBP485 and lisinopril in plasma, urine and cell lysates were determined by LC-MS/MS. A 50-µl aliquot of IS (paracetamol, 200 ng/ml) or 500 µl methanol was added to a 25 µl biological sample. The mixture was vortexed for 1 min and centrifuged at 16,000 × g for 10 min to remove the protein precipitate; the upper organic layer was transferred into a polycarbonate tube and dried with nitrogen at 37°C. The dried residue was dissolved in 200 µl water with 0.1% formic acid, and 10 µl was injected into the LC-MS/MS. Urine samples were diluted 20 times with the mobile phase. The other preparations were the same as the plasma samples. The kidney slices were mixed with 300 µl normal saline after weighing, and then homogenized (T10 ULTRA-TURRAX; IKA) in an ice bath. The other preparations were carried out as for the plasma samples.

**LC-MS/MS analysis:** An Agilent LC system (Agilent HP1200, Agilent Technology Inc., Palo Alto, CA, USA) was used. 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 3% methanol and 97% water with 0.1% formic acid.
0.1% formic acid for JBP 485, and 30% methanol and 70% water with 0.1% formic acid for lisinopril, and the flow rate was 0.5 mL/min. The column was maintained at room temperature. An API 3200 triple-quadrupole mass spectrometer (Applied Biosystems, Concord, Ont, Canada) was operated with a Turbolonspray interface in positive ion mode. Analyst 1.4.1 software (Applied Biosystems) was used for the control of equipment, data acquisition and analysis. For the optimization of MS/MS parameters, the instrument was operated with an ion spray voltage at +4.5 kV, heater gas temperature at 450°C, nebulizer gas (Gas 1) at 0.34 MPa, heater gas (Gas 2) at 0.34 MPa, curtain gas at 0.060 MPa and collision gas at 0.030 MPa. All gases used were nitrogen. Declustering potential was set at 35 V for both the analyte and IS. Multiple reaction monitoring (MRM) was employed for data acquisition. The optimized “truncated” MRM fragmentation transitions were m/z 201.1 → m/z 86.1 with collision energy (CE) of 33 eV for JBP 485, m/z 406.1 → m/z 206.0 with CE of 30 eV for lisinopril and m/z 152.1 → m/z 110.1 with CE of 24 eV for paracetamol (IS). The dwell time for each transition was 200 ms.

**Data analysis:** The 3P97 program was used to calculate the main pharmacokinetic parameters. A two-compartmental data analysis was performed for each individual JBP485 and lisinopril concentration-time profile. The quality of the fit was judged by evaluating the standard error of parameter estimates and the coefficient of determination (r²), and by the visual inspection of residual plots. The plasma clearance (CLp) was calculated using the following equation:

\[ \text{CL}_{p} = \frac{\text{dose}}{\text{AUC}_{1-\text{v}}} \]  

where AUC_{1-\text{v}} is the area under the plasma concentration–time profile after i.v. injection.

\[ \text{AUC}_{1-\text{v}} = A/\alpha + B/\beta \]  

\[ V_{1} = \text{dose}/(A + B) \]

where \( \alpha \) and \( \beta \) are the apparent rate constants, \( A \) and \( B \) are the corresponding zero-time intercepts, and \( V_{1} \) is the distribution volume of the central compartment.

The oral availability (\( F \)) was calculated as:

\[ F = \frac{\text{AUC}_{p.o.}}{\text{AUC}_{1-\text{v}}} \]

where AUC_{p.o.} is the AUC after oral administration. It was calculated by the trapezoidal rule. The renal clearance of JBP485 or lisinopril (CLr) were calculated as

\[ \text{CL}_{r} = \frac{A_{\text{total}}}{\text{AUC}_{1-\text{v}}} \]

where \( A_{\text{total}} \) is the cumulative amount of JBP485 or lisinopril excreted in urine over 24 h. The uptake clearance of JBP485 or lisinopril (CL_{uptake}) was calculated as

\[ \text{CL}_{\text{uptake}} = \frac{A_{\text{total}}}{\text{AUC}_{0-60}} \]

where \( A_{\text{total}} \) is the cumulative uptake amount of JBP485 or lisinopril in kidney slices over 60 min, and \( \text{AUC}_{0-60} \) is the area under the JBP485 or lisinopril medium concentration–time curve from 0 to 60 min, as determined by the trapezoidal rule.

**Statistical analysis:** Statistical analysis was carried out using the SPSS11.5 package. Test results were expressed as mean ± SD. To test for statistically significant differences among multiple treatments for a given parameter, one-way analysis of variance (ANOVA) was performed. The statistical significance of differences between mean values was calculated using the non-paired t-test. If \( p \) was <0.05 or <0.01, differences were considered statistically significant.

**Results**

**Pharmacokinetic interaction after intravenous or oral administration:** To establish whether the first target of DDI was in the gastrointestinal tract, JBP485 and lisinopril were administered simultaneously by the intravenous or oral routes. When the two drugs were co-administered orally, their plasma concentrations and AUCs were decreased significantly compared to the control groups (Figs. 1A and 1B). The AUCs of JBP485 and lisinopril were decreased to 39.4% and 48.5%, respectively. The \( C_{\text{max}} \) of JBP485 and lisinopril were also decreased by 71.4% and 73.4%, respectively (Table 1). However, when they were co-administered intravenously, their plasma concentrations and pharmacokinetic parameters were almost unchanged compared to the corresponding control groups (Figs. 1C and 1D, Table 1). This indicated that the DDI target of JBP485 and lisinopril might be in the intestine.

**DDI in situ single-pass intestinal perfusion studies:** To investigate further if the target of DDI was located in the intestine, we used in situ jejunal perfusion to observe the DDIs between JBP485 and lisinopril. When the two drugs were perfused simultaneously, the portal vein concentrations of the two drugs were significantly decreased (Figs. 2A and 2B), AUCs of JBP485 and lisinopril in the inhibitory group were decreased by 47.8% and 62.3%, respectively (Fig. 2). It also indicated that the DDI target of JBP485 and lisinopril might be in intestine.

**DDI in everted gut sac model in vitro:** To exclude the impact of changes in physiological conditions, and to confirm the DDI target between JBP485 and lisinopril, we used an everted gut sac model to investigate the DDI of the two drugs in vitro (Fig. 3). The serosal concentrations of JBP485 and lisinopril in the co-administered groups were both decreased significantly compared to those in the corresponding control group (Figs. 3A and 3B). The AUCs of JBP485 and lisinopril in the inhibitory group were decreased to 73.9% (for JBP485) and 60.2% (for lisinopril), respectively. The *in vivo* and *in vitro* results suggested that the target of DDI between JBP485 and lisinopril was located in the intestine.

**DDI in hPEPT1-transfected HeLa cells:** To confirm whether the target of DDI between JBP485 and lisinopril was related to PEPT1 in the intestine, we constructed...
Fig. 1. Mean plasma concentration-time curves of JBP485 and lisinopril after p.o. and i.v. administration in rats
The plasma concentration of JBP485 after oral administration (A); the plasma concentration of lisinopril after oral administration (B); the plasma concentration of JBP485 after intravenous administration (C); the plasma concentration of lisinopril after intravenous administration (D). Data are expressed as mean ± S.D. (*p < 0.05 vs. control, **p < 0.01 vs. control; n = 3).

Table 1. Pharmacokinetic parameters of JBP485 and lisinopril following p.o. or i.v. administration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>JBP485</th>
<th>JBP485 + lisinopril</th>
<th>Lisinopril</th>
<th>Lisinopril + JBP485</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.o.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cmax (µg/ml)</td>
<td>15.7 ± 0.5</td>
<td>4.5 ± 0.8</td>
<td>6.4 ± 0.9</td>
<td>1.7 ± 0.6</td>
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<tr>
<td>Kc (min⁻¹)</td>
<td>0.0221 ± 0.0025</td>
<td>0.0174 ± 0.0015</td>
<td>0.012 ± 0.003</td>
<td>0.0106 ± 0.0023</td>
</tr>
<tr>
<td>Kc (min⁻¹)</td>
<td>0.0070 ± 0.0014</td>
<td>0.0056 ± 0.0007</td>
<td>0.0041 ± 0.0008</td>
<td>0.0035 ± 0.0007</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>185 ± 19</td>
<td>211 ± 25</td>
<td>154 ± 13</td>
<td>217 ± 21</td>
</tr>
<tr>
<td>AUC₀→∞ (µg min/ml)</td>
<td>2,783 ± 162</td>
<td>1,097 ± 67</td>
<td>588 ± 63</td>
<td>285 ± 33</td>
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<td>V₁/F (L/kg)</td>
<td>1.28 ± 0.03</td>
<td>3.804 ± 0.025</td>
<td>2.13 ± 0.07</td>
<td>4.39 ± 0.06</td>
</tr>
<tr>
<td>F (%)</td>
<td>27 ± 3</td>
<td>10.7 ± 1.4</td>
<td>21.9 ± 2.8</td>
<td>11.2 ± 2.1</td>
</tr>
<tr>
<td>i.v.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₀ (µg/ml)</td>
<td>109 ± 9</td>
<td>105 ± 7</td>
<td>38 ± 4</td>
<td>38 ± 4</td>
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<tr>
<td>Kc (min⁻¹)</td>
<td>0.0058 ± 0.0006</td>
<td>0.0066 ± 0.0008</td>
<td>0.0047 ± 0.0012</td>
<td>0.0041 ± 0.0014</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>173 ± 18</td>
<td>171 ± 21</td>
<td>139 ± 7</td>
<td>130 ± 16</td>
</tr>
<tr>
<td>AUC₀→∞ (µg min/ml)</td>
<td>10,169 ± 130</td>
<td>10,217 ± 115</td>
<td>2,681 ± 69</td>
<td>2,538 ± 63</td>
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<tr>
<td>T₁/₂α (min)</td>
<td>114 ± 12</td>
<td>106 ± 13</td>
<td>145 ± 21</td>
<td>168 ± 13</td>
</tr>
<tr>
<td>V₁ (L/kg)</td>
<td>0.410 ± 0.018</td>
<td>0.353 ± 0.022</td>
<td>0.372 ± 0.016</td>
<td>0.486 ± 0.011</td>
</tr>
<tr>
<td>CLp (ml/min/kg)</td>
<td>2.47 ± 0.03</td>
<td>2.45 ± 0.04</td>
<td>1.86 ± 0.12</td>
<td>1.98 ± 0.11</td>
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<tr>
<td>CLR (ml/min/kg)</td>
<td>2.19 ± 0.06</td>
<td>1.47 ± 0.07</td>
<td>1.80 ± 0.16</td>
<td>1.46 ± 0.13</td>
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MRT, mean residence time. *p < 0.01, †p < 0.001 compared with single administration.
hPEPT1-transfected HeLa cells and examined the uptake of JBP 485 and lisinopril in the transporter-transfected cells. The uptake concentration of JBP485 or lisinopril in hPEPT1-transfected HeLa cells was significantly reduced (Figs. 4A and 4B) when the two drugs were co-administered. On the other hand, the uptake of JBP485 or lisinopril by the mock cells (empty vector-containing cells from parental HeLa cells) was negligible compared with the uptake by hPEPT1-
transfected HeLa cells. This result indicated that the first target of DDI between JBP485 and lisinopril was related, at least in part, to PEPT1.

**DDI in urinary excretion in vivo:** To establish whether the DDI between JBP485 and lisinopril occurred in the kidney, we examined the cumulative urinary excretion of JBP485 and lisinopril. The cumulative urinary excretion at 24 h was 89.4% (for JBP485) (Fig. 5A) and 96.5% (for lisinopril) (Fig. 5B) of the dose when JBP485 (25 mg/kg) or lisinopril (5 mg/kg) was administered intravenously. This result indicated that renal excretion was the major route of excretion for the two drugs. When they were co-administered intravenously, the cumulative urinary excretion decreased to 60.4% (for JBP485) (Fig. 5A) and 73.9% (for lisinopril) (Fig. 5B) of the dose, and the renal clearance reduced to 67.1% (for JBP485) (Fig. 5A, inset) and 81.1% (for lisinopril) (Fig. 5B, inset) of that of the control group, respectively.

**DDIs in transporters in kidney slices:** To confirm the target of DDI between JBP485 and lisinopril in the kidney, we used fresh rat kidney slices to investigate the DDI mechanism of the two drugs in excretion. The uptake clearance rate for JBP485 in the presence of lisinopril was 75.5% of that in the control group, as calculated by equation.23 JBP485 (0.5 mM) also inhibited lisinopril (0.1 mM) uptake in renal slices, and the uptake clearance rate of lisinopril was 67.4% of that in the control group. To clarify the excretion mechanism of JBP485 and lisinopril, we observed the effect of 1 mM probenecid (an OAT inhibitor) on JBP485 and lisinopril uptake.28 Probenecid could significantly inhibit the uptake of JBP485 and lisinopril (Fig. 6). This finding indicated that the second target of DDI between JBP485 and lisinopril was related to OATs in the kidneys.

**Uptake interaction of JBP485 and lisinopril in hOAT1-/hOAT3-HEK293 cells:** To confirm that the target of DDI between JBP485 and lisinopril is OATs in the kidneys, we investigated the uptake interaction of JBP485 and lisinopril in hOAT1-/hOAT3-HEK293 cells. Lisinopril inhibited the uptake of JBP485 in hOAT1-HEK293 cells (Fig. 7A) and hOAT3-HEK293 cells (Fig. 7C). On the other hand, JBP485 also markedly inhibited the uptake of JBP485 in hOAT1-HEK293 cells (Fig. 7B).
lisinopril in hOAT1-HEK293 cells and hOAT3-HEK293 cells. The uptake of JBP485 or lisinopril was significantly greater in hOAT1-/hOAT3-HEK293 cells than in the mock cells (empty vector-containing cells derived from HEK293 cells). These results indicated that the targets of DDI between JBP485 and lisinopril were, at least in part, OAT1 and OAT3 in the kidneys.

**Discussion**

DDIs influence pharmacokinetics and pharmacodynamics (both benefits and risks) by affecting drug ADME. Pharmacokinetics is now challenged by the growing importance of transporters, a relatively new and potentially major factor in drug ADME. Transporter-mediated DDIs play an important role in drug therapy. Oligopeptides are known to be absorbed through brush border membrane PEPT1 into intestinal epithelial cells. ACE inhibitors sterically resemble Ala-Pro dipeptide or Xaa-Ala-Pro tripeptide structures. It has been hypothesized that they share the same intestinal transport route as di- and tripeptides. Thus, when combined with each other, their pharmacological effect will be affected after oral administration. Renal OAT-mediated DDIs between dipeptides and ACE inhibitors have attracted increasing attention for the discovery and development of new therapeutics. With this in mind, in this study, we established sensitive and efficient LC-MS/MS methods for simultaneous detection of JBP485 (dipeptides) and lisinopril (ACE inhibitor) to interpret the mechanisms of their interaction, thus providing a strong basis for clinical therapy.

When JBP485 (25 mg/kg) and lisinopril (5 mg/kg) were co-administered orally, their plasma concentration, AUC and Cmax were significantly decreased in comparison with those in the control groups (Figs. 1A and 1B, Table 1). These results imply that there is mutual inhibition in intestinal absorption when the drugs are co-administered orally. The values of AUCl∞ were approximately 2.56-fold (JBP485) and 2.07-fold (lisinopril) of their corresponding control values after combined oral administration (Table 1), indicating that absorption of the two drugs was delayed significantly. These results show clearly that when dipeptide drugs and ACE inhibitors are co-administered orally, their absorption is inhibited. Nonetheless, there was almost no change in plasma concentration when JBP485 and lisinopril were administered intravenously (Figs. 1C and 1D). There were no significant differences in pharmacokinetic parameters (Table 1).
indicated that the small intestine is the first interaction target of the DDIs between JBP485 and lisinopril.

We used jejunal perfusion \textit{in situ} and everted gut sacs to confirm the target of the DDI between JBP485 and lisinopril. When JBP485 and lisinopril were perfused simultaneously, the portal vein and serosal concentrations of the two drugs were significantly lower than those in the corresponding control group (Figs. 2 and 3). The results suggest that JBP485 and lisinopril compete for a target in the small intestine, which inhibits absorption of the two drugs. Finally, to confirm the exact target of the DDI between JBP485 and lisinopril, we used hPEPT1-transfected HeLa cells to further investigate the interaction (Figs. 4A and 4B). When the two drugs were used alone, the uptake concentration of JBP485 or lisinopril was significantly higher than that in the co-administered groups. This result is consistent with \textit{in vivo} and \textit{in vitro} studies. It is well known that dipeptides and ACE inhibitors are co-substrates of PEPT1 and OATs.\textsuperscript{6,15,30} Furthermore, OATs have high expression levels in the kidneys. Therefore, the second target for DDI between JBP485 and lisinopril might be in the kidneys.

To confirm this speculation, we used bladder catheterization to observe the cumulative urinary excretion of the two drugs during the 24 h after JBP485 and lisinopril were given simultaneously \textit{via} intravenous administration. Cumulative urinary excretion was found to be significantly decreased (Figs. 5A and 5B) in comparison to that in the control groups, and renal clearance (Figs. 5A and 5B, inset) was significantly reduced in comparison with that in the control groups. Those findings indicated that the second target of DDI between JBP485 and lisinopril was located in the kidneys. This result led us to clarify the mechanism of the DDI in the kidneys. It is well known that OATs in the kidneys are responsible for active secretion from the renal tubules into the urine.\textsuperscript{1} We have reported previously that JBP485 is not only a substrate of PEPT1 in the intestine, but also is transported by OAT1 and OAT3 in the kidneys.\textsuperscript{19} We did not find any report that the transport of lisinopril was related to OATs; we first speculated that lisinopril might be the substrate of OATs.

To confirm that lisinopril is a substrate of OATs, and that the targets of DDI between JBP485 and lisinopril in the kidney are OATs, we investigated the DDI in kidney slices. The uptake of JBP485 (Fig. 6A) or lisinopril (Fig. 6B) was inhibited markedly by the addition of the two drug simultaneously. When probenecid (a typical substrate of OATs) was co-administered with JBP485 or lisinopril, the uptake of JBP485 or lisinopril was inhibited (Figs. 6A and 6B). This indicated that JBP485 and lisinopril are both substrates of OATs. In addition, to confirm this, we used hOAT1/-hOAT3-transfected HEK293 cells to perform the same experiment as with kidney slices. The similarities of oat1 in rats to OAT1 in humans and oat3 in rats to OAT3 in humans is 98% and 90%, respectively (the gene sequences of OAT1/OAT3 in humans and oat1/oat3 in rats were obtained from GenBank). Because the gene sequences are almost identical, the functions of transporter are similar. Therefore, we used hOAT1/-hOAT3-HEK293 cells as an \textit{in vitro} model to further investigate the second possible target of DDI between JBP485 and lisinopril in the kidney. The uptake of JBP485 or lisinopril was inhibited significantly when the two drugs were added simultaneously to the transfected cells (Fig. 7).

To explain why the cumulative urinary excretion and renal clearance of JBP485 and lisinopril were decreased, but the plasma concentrations of the two drugs were unchanged after intravenous administration, we determined the concentration ratios of the two drugs in the renal cortex and plasma after intravenous administration. The concentration ratio of JBP485 or lisinopril in the renal cortex and plasma (Kp) at 6 h was significantly increased from 0.85 ± 0.07 ml/g to 2.1 ± 0.8 ml/g for JBP485, and from 0.117 ± 0.015 ml/g to 0.54 ± 0.04 ml/g for lisinopril, respectively. This indicated that the two drugs were accumulated in the renal cortex when JBP485 and lisinopril were co-administered intravenously.

Taken together, our results confirm that the kidneys are the second target of DDI between JBP485 and lisinopril, and OAT1 and OAT3 in the kidneys are the target proteins that mediate excretion of JBP485 and lisinopril.

In conclusion: (1) there is a DDI between JBP485 and lisinopril when they are co-administered; (2) the first target of the DDI is PEPT1 that is located in the small intestine, and the second targets are OAT1 and OAT3 in the kidneys; and (3) our results are novel in demonstrating for the first time that lisinopril is the substrate of OAT1 and OAT3.

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\textbf{References}


