Pharmacokinetics and Mechanism of Intestinal Absorption of JBP485 in Rats

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Summary: To investigate the pharmacokinetics and mechanism of intestinal absorption of JBP485 in rats, the pharmacokinetics of JBP485 were investigated in vivo both intravenously and orally. The effects of glycyl-sarcosine (Gly-Sar) on the uptake and transepithelial transport of JBP485 were examined in everted intestinal sacs, in situ jejunal perfusion, Caco-2 cells and PEPT1 transfected Hela cells. The gastrointestinal absorption of JBP485 was rapid. $T_{1/2}$ was $2.25 \pm 0.06$ h, $CL_{\text{plasma}}$ was $2.99 \pm 0.002$ ml/min/kg, $V_d$ was $0.22 \pm 0.05$ l/kg and bioavailability was about 30% at a dosage of 25 mg/kg. JBP485 underwent rapid distribution in the tissues. Gly-Sar significantly decreased JBP485 uptake and transport in these models. A kinetic study showed that JBP485 was transported by PEPT1 in Caco-2 cells with $K_m$ and $V_{\text{max}}$ values of $0.33 \pm 0.13$ mM and $0.72 \pm 0.06$ nmol/mg protein/10 min, respectively. JBP485 appeared to have linear pharmacokinetics at intravenous doses of 6.25-100 mg/kg with minor first-pass effect, and JBP485 was mainly distributed in the kidney; JBP485 is a substrate for PEPT1 which is involved in the absorption of JBP485 in rat intestine.

Keywords: JBP485; dipeptide; PEPT1; Glycylsarcosine; Pharmacokinetics

Introduction

JBP485 (cyclo-trans-4-L-hydroxyprolyl-L-serine; Fig. 1), a dipeptide, was first isolated from Laennec (a trade name for the hydrolyzate of human placenta) by the Japan Bioproducts Industry Co., Ltd, as a mitogen from a baby hamster kidney cell line and was synthesized by chemical means.1) Laennec injections have been used clinically to treat chronic hepatic injuries for over 40 years in Japan. A previous study showed that Laennec stimulated liver regeneration and decreased cytosolic enzyme activities in serum in α-naphthylisothiocyanate (ANIT)-intoxicated rats.2) We reported first that JBP485 exhibited potent anti-hepatitis activity in 2000.3) We reported first that JBP485 exhibited potent anti-hepatitis activity in 2000.3) The anti-hepatitis activity was confirmed by our subsequent study.4,5) JBP485 was well absorbed by the intestine in rats after oral administration and it has been suggested that JBP485 is recognized by PEPT1, a peptide transport-er, in the rat gastrointestinal tract.5) The $H^+$/peptide cotransporters PEPT1 and PEPT2,
transport di/tri-peptides across the apical membranes of intestinal and renal epithelial cells. PEPT1, a low affinity, high-capacity transporter, is expressed principally in the small intestine. It plays an essential role in the absorption of small peptides arising from the digestion of dietary protein in the small intestine. Apart from the degradation products of nutritional proteins, several peptidomimetic drugs such as β-lactam antibiotics, certain angiotensin-converting enzyme (ACE) inhibitors like fosinopril and quinapril, valacyclovir and bestatin are recognized by PEPT1 and PEPT2 as substrates because of their steric resemblance to di/tri-peptides.

The human colon adenocarcinoma cell line, Caco-2, expresses PEPT1 and other solute transporters for sugars, amino acids, bile acids, nucleosides and efflux mechanisms. Caco-2 cells, grown on permeable supports, undergo differentiation, become polarized and display vectorial transport of nutrients. Caco-2 cell monolayers have thus provided a useful model system for studying PEPT1-mediated drug transport across the intestinal mucosa. In addition, HeLa cells transfected with peptide transporter 1 (HeLa/PEPT1) can stably express human PEPT1.

The purposes of the present study were to investigate the pharmacokinetics and mechanism of intestinal absorption of JBP485, and determine if the gastrointestinal absorption of JBP485 is involved with PEPT1 in rat intestine. The effects of glycylsarcosine (Gly-Sar) and JBP923 (another dipeptide, a derivative of JBP485) on the uptake and transepithelial transport of JBP485 were examined in vitro and in situ. The results of these investigations provided the pharmacokinetic characterization of JBP485 and important information on the drug-drug interactions of the dipeptide (JBP485) with other drugs which are substrates for PEPT1.

Materials and Methods

Materials: JBP485 and JBP923 were provided by Japan Bioproducts Industry Co., Ltd (Tokyo, Japan). Gly-Sar was purchased from Sigma (USA). HeLa cells transfected with vector and PEPT1 were a generous gift from Professor Akira Tsuji, Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University (Kanazawa Japan). Caco-2 cells were obtained from Professor Zhonggui He, Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University (Shenyang, China), and they purchased the cells from ATCC. Cell culture reagents were purchased from Gibco (Grand Island, NY, USA). Other reagents will be further specified when mentioned.

Animals: Male Wistar rats (250 ± 20 g) were supplied by the Laboratory Animal Center of Dalian Medical University (Dalian, China). The rats were housed under controlled environmental conditions (temperature at 23 ± 1°C; humidity at 55 ± 5%) that included lighting, commercial food diet and free access to water. The experiments started after the rats were acclimatized for at least one week. The studies were approved by the Animal Ethics Committee of Dalian Medical University. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Pharmacokinetic study: The rats were fasted overnight with free access to water for at least 12 h before the experiment. After i.v. (6.25, 25, 100 mg/kg), oral (25 mg/kg) and portal vein (6.25 mg/kg) injection of JBP485, 0.5 ml of blood was collected at the following time intervals: 0, 0.02, 0.05, 0.08, 0.17, 0.33, 0.5, 1, 2, 4, 6, 8, 10 h. The plasma was immediately separated by centrifugation at 5,000 rpm for 10 min at 4°C. All samples were stored at −20°C until analyzed.

Plasma clearance (CLplasma) was calculated by:

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

Where AUC_{i.v.} is the area under the plasma concentration-time profile after i.v. injection.

\[ AUC_{i.v.} = \frac{A}{\alpha} + \frac{B}{\beta} \]  

Where alpha is the distribution rate constant and beta the elimination rate constant. The hepatic availability (\(F_h\)) was calculated as:

\[ F_h = \frac{AUC_{pv}}{AUC_{i.v.}} \]  

Where AUC_{pv} is AUC after portal vein injection. AUC during the 10 h portal vein injection was calculated by trapezoidal rule. AUC after the end of injection was obtained by Eq. 2. The input data for the fitting were weighted as the reciprocal of the square of the observed values, and the algorithm used for the fitting was the damping Gauss-Newton method.

Tissue distribution study: The rats were anesthetized with diethyl ether. A dose of JBP485 (25 mg/kg) was administered intravenously to rats via the jugular vein. After administration, different tissues (heart, liver, spleen, lung, kidney, stomach, muscle, testis, fat, adrenal gland, eye, brain and plasma) were removed at time intervals of 5, 10, 20 min 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 12,500 rpm for 10 min at 4°C to remove cellular debris and the supernatant was used for LC/MS/MS determination.

In vitro everted intestinal sac preparation: Rats were anesthetized with ether after overnight fasting and
the abdomen was opened by a midline incision. 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 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\textsuperscript{15} with modifications. The everted intestine was placed in glucose-saline at room temperature in a flat dish. A thread ligature was tied around one end to facilitate subsequent identification and check for perforations. The end of incubation, the serosal fluid was drained through a small incision into a test tube. Samples were then collected for JBP485 determination by LC/MS/MS.

In situ jejunal perfusion technique: A laparotomy was performed after the rats were anesthetized using ether, and an inflow cannula made of silastic tubing was inserted into the jejunum approximately 1 cm below the ligament of Treitz.\textsuperscript{16} An outflow cannula was set up at a distance of 10 cm. The bile duct was ligated to prevent possible enterohepatic circulation. The jejunal segment was then flushed with saline solution (prewarmed to 37°C) to remove residual intestinal contents. Perfusion solutions were delivered with a peristaltic pump at a flow rate of 4 ml/15 min through an inlet tube water-jacketed at 37°C before its entry into the jejunal segment.\textsuperscript{17} The solution for jejunal perfusion was the same as the KRB buffer above. After a 30 min equilibration period, 0.5 mM JBP485 alone, 0.5 mM JBP485 + 20 mM Gly-Sar; (3) 0.5 mM JBP485 + 1 mM JBP923. The incubation medium was surrounded by a water jacket maintained at 37°C. A gas mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2} was bubbled through the external incubation medium during the incubation period. At the end of incubation, the serosal fluid was drained through a small incision into a test tube. Samples were then collected for JBP485 determination by LC/MS/MS.

Cell culture: Caco-2 cells were cultured as described previously\textsuperscript{18} with minor modification. The passage number used for this study ranged from 30 to 33. Caco-2 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) (heat-inactivated), 1% non-essential amino acid solution, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. All cell cultures were maintained in a humidified incubator at 37°C with 5% carbon dioxide in air atmosphere. When the cells reached confluence, they were plated on 24-well dishes at a density of 5 × 10\textsuperscript{5} cells/well, and the medium was changed 24 h after plating. Caco-2 cells used in our studies were grown for 14 days.

HeLa/PEPT1 and mock cells were grown in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine, and 1 mg/ml geneticin (G418) as described by Nakanishi.\textsuperscript{19} For the uptake assay, each cell line was seeded on eight-well plates and cultured for 3 days.

Uptake studies: Caco-2 cells were placed on a shaking plate, preheated to 37°C. The culture medium was removed, and the Caco-2 cell monolayers were washed twice with preincubation medium consisting of HBSS buffer. After washing, the monolayers were preincubated with preincubation medium for 10 min at 37°C. The medium was removed after preincubation, and the monolayers were incubated with 1 ml fresh preincubation medium containing JBP485 (1 mM) with or without Gly-Sar (50 mM) for the indicated time at 37°C. The uptake was measured at 5, 10, 20 and 30 min. At the end of incubation, the uptake was terminated by ice-cold HBSS, and the cell monolayers were washed twice with ice-cold pH 7.4 HBSS to remove extracellular JBP485. Accumulated concentrations of JBP485 were determined by LC/MS/MS after the cells were lysed. Concentration dependence of JBP485 uptake was determined in the presence or absence of 50 mM Gly-Sar. To examine the kinetics of JBP485 uptake in Caco-2 cells, uptake was measured at pH 6.0 over a concentration range of 0 to 1 mM at 37°C and at 4°C. Cell lysates were collected for JBP485 determination as described below.

An uptake study with HeLa/PEPT1 and mock cells was also performed at 37°C in HBSS adjusted to pH 6.0. Cultured cells were washed and preincubated in the buffer without JBP485 for 10 min at 37°C. Uptake was initiated by adding HBSS (1 ml) containing [\textsuperscript{3}H]JBP485 (144 nM) and [\textsuperscript{3}H]Gly-Sar (1.31 nM), respectively. After incubation for designated times at 37°C, the experiment was terminated by removing the medium, followed by washing twice with 2 ml ice-cold HBSS. Samples were then analyzed by liquid scintillation spectrophotometry using a scintillation counter (LS6000SE, Beckman Coulter Inc., Fullerton, CA).

LC/MS/MS conditions: Analysis of the samples was performed using a triple quadrupole mass spectrometer with electrospray ionization (ESI) on a turbo ionspray source (API 3200; Applied Biosystems, Foster City, CA, USA) coupled to a liquid chromatography system (Agilent HP1200, Agilent Technology Inc., Palo Alto, CA, USA) and a C\textsubscript{18} column 2.1 × 150 mm (Elite Hypersil, China). The mobile phase consisted of 5% methanol and 95% water with 0.1% formic acid, and was delivered at a flow rate of 0.5 ml/min. Multiple reaction monitoring (MRM) mode was utilized to detect compounds of interest. Analytes were detected in the positive mode, and protonated molecular ions at m/z 201.2 → 86.1 for JBP485 and m/z 201.2 → 86.1 for Gly-Sar.
219.2.1 → 86.1 for the internal standard (JBP923) were monitored.

**Statistical analysis:** Data were expressed as means ± S.D., and analyzed by analysis of variance (ANOVA) and the Student’s t-test using the statistical software SPSS 11.5. Differences were considered significant at P < 0.05.

**Results**

**Pharmacokinetic characterization of JBP485 in rats:** The plasma concentration-time curves for different doses of JBP485 are shown in Figures 2 and 3. The plasma concentration of JBP485 (25 mg/kg) gradually decreased after i.v. administration with a terminal phase half-life of 2.25 ± 0.06 h (Fig. 2, Table 1). JBP485 was found in plasma with kₐ of approximately 0.008 min⁻¹ only 1 min after oral administration (Fig. 2), suggesting that the gastrointestinal absorption of JBP485 was rapid. JBP485 appeared to have a linear pharmacokinetic characterization for intravenous doses of 6.25–100 mg/kg because the t₁/₂b, CLplasma and Vd were not significantly changed (Fig. 3, Table 1). AUC after portal vein administration was comparable with that after i.v. administration at 6.25 mg/kg (Table 1), suggesting that the hepatic first-pass removal of JBP485 was minor (Fig. 3). The bioavailability of JBP485 was about 30% at a dosage of 25 mg/kg. Table 1 shows the pharmacokinetic parameters of JBP485.

Tissue distribution of JBP485 was investigated following i.v. administration in rats at a dosage of 25 mg/kg.

![Fig. 2. Mean plasma concentration-time profiles of JBP485 after intravenous and oral administration in rats. Each point represents the mean ± S.D., n = 3.](image)

**Fig. 3.** Mean plasma concentration-time profiles of JBP485 after jugular vein (A) and portal vein (B) administration in rats. Each point represents the mean ± S.D., n = 3.

| Table 1: Main pharmacokinetic parameters of JBP485 in rats (Mean ± SD) (n = 3) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | 100 mg/kg       | 25 mg/kg        | 6.25 mg/kg      | Unit            |
| T₁/₂b           | 2.30 ± 0.04     | 2.25 ± 0.06     | 2.39 ± 0.03     | h               |
| CLplasma        | 2.95 ± 0.012    | 2.99 ± 0.002    | 2.89 ± 0.007    | ml/min/kg       |
| Vd              | 0.17 ± 0.03     | 0.15 ± 0.05     | 0.16 ± 0.05     | l/kg            |
| MRT             | 113.57 ± 2.14   | 114.22 ± 1.83   | 114.21 ± 0.77   | min             |
| Cmax            | 600.00 ± 16.89  | 171.67 ± 6.46   | 38.56 ± 3.45    | µg/ml           |
| AUC0–10h(i.v.)  | 33309.02 ± 316.56 | 8373.85 ± 253.74 | 2118.40 ± 34.79 | µg·min/ml       |
| AUC0–10h(i.v)   | —               | 2285.34 ± 286.58 | —               | µg·min/ml       |
| FPO             | —               | —               | 1847.88 ± 46.76 | µg·min/m        |
| FPV             | 27.30 ± 1.53    | —               | 87.23 ± 4.85    | %               |
|
The results indicated that JBP485 underwent rapid distribution in the tissues/organs throughout the whole body within the time course examined. It was shown that JBP485 was mainly distributed in tissues such as kidney, lung and liver (Fig. 4), implying that the distribution of JBP485 depended on the blood flow or perfusion rate of the organ, the ability of the drug to penetrate organ membranes, tissue specificity, protein binding. The high distribution in kidney demonstrated that kidney might be the primary excretory organ for JBP485. The lowest level found in the brain implied that JBP485 had difficulty crossing the blood-brain barrier because of its high polarity (Fig. 4).

Effects of Gly-Sar and JBP923 on the uptake of JBP485 in everted gut sac of rats in vitro: To understand the mechanism of JBP485 absorption by rat intestine, we investigated the effects of Gly-Sar on the uptake of JBP485 (0.5 mM) by an everted gut sac model in vitro. The uptake of JBP485 was markedly inhibited by 20 mM Gly-Sar in rat everted gut sac. A similar phenomenon was found in the presence of 1 mM JBP923 (Fig. 5).

Effects of Gly-Sar and JBP923 on absorption of JBP485 by single-pass intestinal perfusion in vivo: To further examine the mechanism of JBP485 (0.5 mM) absorption by rat intestine, we also investigated the effects of Gly-Sar on absorption of JBP485 by single-pass intestinal perfusion in vivo. The concentration of JBP485 absorbed into the portal vein markedly decreased by 20 mM Gly-Sar and 1 mM JBP923 (Fig. 6).

Uptake of JBP485 in Caco-2 and HeLa/PEPT1 cells: We also examined the effects of Gly-Sar on JBP485 uptake by Caco-2 cells. The concentration dependence of the initial uptake of JBP485 was studied over the range 0 to 1 mM (Fig. 7A). The profile of uptake appeared to be saturable at 37°C and the uptake was greatly reduced at 4°C. The accumulation of JBP485 into monolayers increased with the duration of culture, with Km and Vmax values of 0.33 ± 0.13 mM and 0.72 ± 0.06 nmol/mg protein/10 min, respectively (Fig. 7A inset). However, this accumulation was significantly inhibited by the presence of 50 mM Gly-Sar, suggesting that Gly-Sar markedly influenced JBP485 transport across Caco-2 cells (Fig. 7B).

To understand exactly whether JBP485 was the substrate for PEPT1 we used HeLa/PEPT1 cells to examine the uptake of [3H]JBP485 and effects of Gly-Sar on the uptake of [3H]JBP485. The uptake of [3H]JBP485 in HeLa/PEPT1 cells increased in a time-dependent manner and the uptake of JBP485 was much higher than that in HeLa/mock cells (Fig. 8A). However, the uptake of [3H]JBP485 in HeLa/PEPT1 was significantly inhibited by excess Gly-Sar (10 mM) (Fig. 8A). A similar phenomenon was found in the uptake of [3H]Gly-Sar (Fig. 8B) under the same conditions with excess Gly-Sar. It is suggested that JBP485 is also the substrate for PEPT1 similar to Gly-Sar.

Discussion

Recently, people are placing more emphasis on the research of the micromolecule oligopeptide because it possesses significant pharmaco-activity.4,5) Most studies
show that transporters play major roles in micromolecule 
oligopeptide absorption.\textsuperscript{10,19} Clarification of the me-
chanism involved in intestinal absorption of such oligopep-
tides has significance for the development of new medi-
cines.

We found that JBP485 was rapidly eliminated from 
plasma after i.v. injection with a terminal phase half-life 
of 2 h. On comparing the plasma concentration-time pro-
file of JBP485 after i.v., oral and portal vein injection, 
JBP485 appeared to follow a two-compartment model 
(Fig. 2) and have linear pharmacokinetic characteriza-
tion at intravenous doses of 6.25–100 mg/kg (Fig. 3A) as 
there were no marked changes in $\text{CL}_{\text{plasma}}$, $t_{1/2}$ and $V_d$. 
AUC after portal vein administration was also compara-
ble with that after i.v. administration at 6.25 mg/kg (Fig. 3B) with $F_h$ of 87\% (Table 1), suggesting that hepatic 
first-pass elimination was unremarkable. The concentra-
tion ratios of JBP485 in kidney and plasma ($K_p$) was 3.2, 
suggesting that JBP485 could be concentrated in the kid-
ney. Our recent results showed that accumulated urinary 
excretion of JBP485 was over 80\% of the dose ad-
ministered (data not shown), and there was concurrence
between renal excretion and high distribution in the kidney. These results demonstrate that kidney is the main excretory organ of prototype JBP485 and the anti-hepatitis activity is mainly produced by prototype JBP485. However, the bioavailability of JBP485 was only about 30%. We plan to enhance the anti-hepatitis activity by improving bioavailability in future work.

Micromolecular and hydrosoluble JBP485 was found in plasma at only 1 min after oral administration (Fig. 2), suggesting that the gastrointestinal absorption of JBP485 was rapid. This rapid absorption of JBP485 suggests that a certain specific mechanism may contribute to the absorption of JBP485 by the small intestine. We investigated whether the gastrointestinal absorption of JBP485 was involved with PEPT1 in relation to Gly-Sar (a typical PEPT1 substrate) and JBP923 (another dipeptide, a derivative of JBP485 with the similar chemical structure as JBP485) and β-lactam antibiotics.0 First, we found that JBP485 could be transported from mucosal lateral to serosal lateral by using an everted intestinal sac model in vitro (Fig. 5) and that JBP485 was inhibited by Gly-Sar and JBP923. These findings strongly suggest that JBP485 may compete at the same binding site as Gly-Sar and JBP923. A similar phenomenon was found in Caco-2 cells, Gly-Sar significantly inhibited JBP485 transport across Caco-2 cells (Fig. 7). Secondly, we investigated the effects of Gly-Sar on absorption of JBP485 by single-pass intestinal perfusion in rats in situ. The absorption of JBP485 markedly decreased by Gly-Sar (Fig. 6). Both the in vitro and in situ studies suggested that JBP485 may be a substrate for PEPT1. Zhang’s paper22) found that the plasma concentration of JBP485 and Cephalexin were both decreased after oral dosing of the combinations. These findings suggest that JBP485 may compete at the same binding site as Cephalexin in Caco-2 cell. Zhang’s paper was more focused on the research of the drug-drug interactions of the dipeptide (JBP485) with β-lactam antibiotics (Cephalexin), for clinical medication. To understand directly whether the gastrointestinal absorption of JBP485 may be involved with PEPT1, we investigated the effects of Gly-Sar on the uptake of [3H]JBP485 in PEPT1-expressing HeLa cells. Uptake of the tracer [3H]JBP485 markedly increased and was inhibited by excess Gly-Sar (10 mM) at the indicated times (Fig. 8A). We also used the same method to validate the dependability of the method using Gly-Sar (Fig. 8B). Finally, we confirmed that the gastrointestinal absorption of JBP485 involves PEPT1. This is the original finding of the present study.

Conclusions

(1) JBP485 appeared to have linear pharmacokinetics at intravenous doses of 6.25–100 mg/kg with minor first-pass effect, and JBP485 was mainly distributed in the kidney; (2) JBP485 is a substrate for the H+/peptide symporter PEPT1. PEPT1 is involved in the active transport of JBP485 in rat intestine. This mechanism allows rapid intestinal absorption of JBP485 in rats.

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References

PEPT1 is Involved in the Absorption of JBP485

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