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Inhibitory Effect of Zinc on the Absorption of JBP485 via the Gastrointestinal Oligopeptide Transporter (PEPT1) in Rats

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Summary: The aim of this study was to investigate the pharmacokinetic mechanism of interaction between JBP485 and zinc. The plasma concentration of JBP485 after oral administration in vivo, the plasma concentration of JBP485 from the portal vein after jejunal perfusions in situ, the serosal fluid concentration of JBP485 in everted small intestine preparations and the uptake of JBP485 by HeLa-hPEPT1 cells in vitro were determined by LC-MS/MS. RT-PCR and Western blotting were used to determine the mRNA and protein levels of Pept1 in the intestinal mucosa. The AUCs of JBP485 in in vivo, in vitro and in situ studies were significantly decreased after zinc pre-administration. Kinetic analysis showed that zinc inhibits the uptake of JBP485 by decreasing the affinity of JBP485 for PEPT1 in HeLa-hPEPT1 cells. RT-PCR and Western blotting indicated that zinc had no effect on basal intestinal Pept1 expression. Our results are novel in demonstrating for the first time that zinc ions, but not zinc gluconate, can inhibit the transport activity of PEPT1. In addition, the uptake of JBP485 was not affected by changes in pH values after zinc treatment. Zinc decreases the absorption of JBP485 by inhibiting the transport activity of PEPT1; however, basal intestinal Pept1 expression does not change.

Keywords: JBP485; pharmacokinetic interaction; small intestine; zinc

Introduction

The H⁺-coupled peptide transporter (PEPT1) is a secondary active transporter of low affinity and high capacity and is highly expressed in the brush border membrane of intestinal epithelial cells. PEPT1 mediates the absorption of di- and tripeptides and plays important nutritional roles in protein homeostasis. Furthermore, as PEPT1 has a broad substrate specificity, peptide-like drugs structurally related to small peptides are also transported by PEPT1. Examples of such drugs include β-lactam antibiotics, the antivirus agent valacyclovir, and the angiotensin-converting enzyme inhibitor (ACE inhibitor). Thus, the intestinal peptide transporter PEPT1 plays important nutritional and pharmacokinetic roles.

JBP485 (cyclo-trans-4-L-hydroxyprolyl-L-serine) is a dipeptide which was first isolated from Laennec (a trade name for the hydrolyzate of human placenta) and has since been synthesized by chemical means. Animal experiments have indicated that JBP485 exhibits obvious liver protective effects and gastrointestinal protective effects after oral administration. JBP485 is well absorbed through the gastrointestinal tract, and previous studies have shown that its absorption can be inhibited by glycylsarcosine Gly-Sar, which is a substrate of PEPTs. These studies suggest that JBP485 is recognized by the peptide transporter system in the gastrointestinal tract.

Zinc is an essential metal ion necessary for growth, metabolism and maintenance of cell function. Zinc deficiency in humans results in growth retardation, male hypogonadism, skin changes, poor appetite, mental lethargy, childhood diarrhea and respiratory illnesses. As these symptoms dissipate when zinc is administered, the metal ion zinc is clinically used for zinc deficiency. Because zinc is often
administered orally and it can inhibit various membrane proteins, we hypothesized that the functions of PEPT1 might be affected by zinc taken orally. It has been suggested that intestinal peptide transporters contribute to protein homeostasis and the therapeutic efficacy of peptide-like drugs. 

Therefore, it is important to predict and avoid any potential food or drug interaction in patients treated with zinc.

A previous study confirmed that zinc inhibited Gly-Sar uptake by oocytes expressing rat Pept1 and rat Pept2. In addition, zinc inhibited the absorption of cephoten, a substrate of PEPTs, by oral administration in vivo and jejunal perfusions in situ. To determine whether zinc can inhibit the intestinal absorption of JBP485 by PEPT1 and to examine the pharmacokinetic mechanisms of interaction between the two drugs, we examined the drug-drug interaction (DDI) between JBP485 and zinc using in vivo oral administration, in situ intestinal perfusions, in vitro everted small intestinal sac preparations, transfected-cell uptake studies, RT-PCR and Western blot analysis. Our results suggest that zinc can inhibit the intestinal absorption of JBP485. In addition, zinc decreased the absorption of JBP485 by inhibiting the transport activity of PEPT1 rather than by decreasing PEPT1 expression. These findings provide important information about DDI for the clinical application of dipeptides with zinc.

Materials and Methods

Materials and animals: JBP485 was provided by Japan Bioproducts Industry Company Limited (Tokyo, Japan). ZnSO₄ and diethylpyrocarbonate (DEPC) were purchased from Nacalai Tesque (Kyoto, Japan). Methanol was of high-performance liquid chromatography (HPLC) grade (Tedia, USA). The HeLa cells stably expressing PEPT1 were constructed by our laboratory. All other reagents and solvents were of analytical grade and were commercially available. Male Wistar rats (weighing 250–280 g) were obtained from the Experimental Animal Center in the Dalian Medical University (permit number SCXK 2008-0002). They were allowed free access to water and were fed a chow diet. Before the pharmacokinetic experiments, animals were fasted for 12 h with water available ad libitum. All animal experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Pharmacokinetic interaction studies in rats: Rats fasted overnight for 12 h were anesthetized with ether before each experiment. JBP485 and zinc were dissolved in normal saline or Krebs-Ringer bicarbonate buffer (KRB) and administered to the rats in aqueous solution.

In vivo absorption experiment in rats

Rats were initially given ZnSO₄ (20 mg/kg) or buffer and JBP485 (25 mg/kg) was administered 15 min later. The drugs were administered orally by gavage. Blood samples were collected at the following time points after the administration of JBP485: 1, 5, 10, 20, 30, 60, 120, 180, 240, 360, 480 and 600 min. Samples of 200–300 µl were collected in heparinized tubes and then centrifuged for 10 min at 5,000 g. The plasma obtained was then used for the determination of JBP485 by LC-MS/MS as described.

In vitro everted intestinal sac preparation

Animals were anesthetized using ether and the jejunum was removed (10 ± 1 cm, approximately 2 cm distal to the ligament of Treitz) by cutting across the upper end of the duodenum and the lower end of the ileum and manually stripping the mesentery. The jejunal segments were transferred to cold normal oxygenated saline followed by manual stripping off of the mesentery. After rinsing with saline using a syringe, the jejunal segments were everted as previously described with some modifications. The everted intestine was placed in a glucose-saline solution in a flat dish at room temperature. A thread ligature was tied around one end to facilitate subsequent identification and to check for perforation. The empty sac was filled with 1 ml KRB (pH 7.4), containing 0.5 mM MgCl₂, 4.5 mM KCl, 120 mM NaCl, 0.7 mM Na₂HPO₄, 1.5 mM NaH₂PO₄, 1.2 mM CaCl₂, 15 mM NaHCO₃, 10 mM glucose and 20 mg/l of phenolsulfonphthalein as a nonabsorbable marker. After pretreating with 1 mM ZnSO₄ or buffer for 15 min, the distended sac was placed in the incubation medium (mucosal solution) containing JBP485 (0.5 mM). 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 incubation medium during the incubation period. Samples from the lumen of the everted sac (50 µl) were collected for JBP485 determination by LC-MS/MS as described.

In situ jejunal perfusion technique

The abdomen of the animal was opened after ether anesthesia and a segment of the proximal jejunum was isolated (10 ± 1 cm, approximately 2 cm distal to the ligament of Treitz) followed by incisions at both the proximal and distal ends. An inflow cannula made of silastic tubing was inserted in the jejunum approximately 2 cm below the ligament of Treitz and 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 (pre-warmed to 37°C) to remove any residual intestinal contents. An oxygenated perfusion solution was 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. The solution for jejunal perfusion was the same as that of KRB above. After pre-perfusion with 1 mM ZnSO₄ or buffer for 15 min, JBP485 (0.5 mM) dissolved in KRB was administered. Portal vein blood (200–300 µl) was collected using a vein detained needle purchased from a clinic for JBP485 determination by LC-MS/MS as described.

Uptake experiments using transporter expression systems: Uptake experiments using HeLa cells transfected with PEPT1 were performed at 37°C in the...
uptake buffer with the pH adjusted to 6.0. The composition of the uptake buffer was as follows: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM MES or HEPES. Cultured cells were washed and pre-incubated in the transport buffer (pH 6.0) for 30 min at 37°C with or without 1 mM ZnSO₄. Preliminary experiments showed that the inhibitory effect of zinc was maximal after 30 min of pre-incubation in 1 mM ZnSO₄. After pre-incubation for 30 min at 37°C, the experiment was terminated by removing the medium, followed by washing three times with 1 ml of transport buffer (pH 6.0). The uptake was then initiated by adding 0.5 ml transport buffer (pH 6.0) containing JBP485 (0.5–2.5 mM, concentration dependent on JBP485 uptake) or by adding 0.5 ml transport buffer (pH 5.5–7.0, pH dependent on JBP485 uptake) containing drugs including JBP485 (0.5 mM). After incubation for 3 min at 37°C, the experiment was terminated by removing the medium, followed by washing three times with 1 ml of ice-cold transport buffer. The uptake concentrations of the solubilized cells were subsequently determined. The inhibition of JBP485 uptake by DEPC (0.5 mM, pH 6.0), zinc gluconate (1 mM, pH 6.0), FeSO₄ (1 mM, pH 6.0) and CuSO₄ (1 mM, pH 6.0) were also investigated. Samples were then analyzed by LC-MS/MS after homogenization.

**Determination of JBP485 by LC-MS/MS:** Plasma samples (50 μl) were added to 50 μl of the internal standard solution (200 ng/ml paracetamol) and 400 μl methyl alcohol for de-proteinization. After centrifugation at 12,000 g for 10 min, the upper organic layer was transferred into a polyethylene tube and dried with nitrogen at 37°C. After incubation for 3 min at 37°C, the experiment was terminated by removing the medium, followed by washing three times with 1 ml of ice-cold transport buffer. The uptake concentrations of the solubilized cells were subsequently determined. The inhibition of JBP485 uptake by DEPC (0.5 mM, pH 6.0), zinc gluconate (1 mM, pH 6.0), FeSO₄ (1 mM, pH 6.0) and CuSO₄ (1 mM, pH 6.0) were also investigated. Samples were then analyzed by LC-MS/MS after homogenization.

**Reverse-transcription polymerase chain reaction (RT-PCR) analysis in intestinal mucosa:** After pre-treating with 1 mM ZnSO₄ or buffer for 15 min, total RNA was extracted from the jejunal mucosa of the perfused intestine using TRizol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s instructions. The yield of RNA from each preparation was determined by ultraviolet spectrophotometry. Two-step RT-PCR was performed according to the protocol provided with the kit (Takara, Dalian, China), and cDNA was subsequently amplified using a GeneAmp PCR system (Techne TCS12, UK). RNA samples (500 ng) were first reverse transcribed and then immediately amplified by PCR. Reverse transcription was performed for 10 min at 30°C and 30 min at 42°C and samples were subsequently heated for 5 min at 95°C to terminate the reverse transcription reaction. The conditions for PCR were as follows: denaturation, 94°C for 1 min; annealing, 65°C for 1 min; and extension, 72°C for 1 min (35 cycles). Primers (Takara, Dalian, China) specific for rat Pept1 were as follows: 5′-GTGTGGGGCCGCCACATCATACCGT-3′ (forward) and 5′-GTTTGTCTGGAGACAGGTTCAC-3′ (reverse) to obtain an expected product of 735 bp. The primers for β-actin were as follows: 5′-ATTGAACCGGCTTGGTCAC-3′ (forward) and 5′-CATCGGAAACCCTGTCATTG-3′ (reverse) to obtain a PCR product of 560 bp. The co-amplified PCR products were separated on a 1.5% agarose gel by electrophoresis and visualized by staining with 0.1 mg/l ethidium bromide. Quantity One (version 4.40) software was used to analyze the OD bands on the gel. The level of Pept1 mRNA expression in each sample was normalized by the corresponding β-actin mRNA level.

**Western blot analysis in intestinal mucosa:** For Western blot analysis, the upper jejunum of rats was removed after the rats were euthanized. Then the upper jejunum was flushed with ice-cold saline and the mucosa was scraped gently with a glass slide into cold phosphate-buffered saline (PBS). Tissue samples that had been frozen and stored in rapid immunoprecipitation assay buffer (RIPA, Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitors were thawed on ice and then homogenized (IKA-T10 homogenizer; IKA, Staufen, Germany). The homogenate was centrifuged at 12,000 g for 30 min to remove cell debris and the supernatant was collected. Protein was measured according to the bichinchoninic acid (BCA, Solarbio, Beijing, China) procedure, with bovine serum albumin as the standard. Equal amounts (25 μg) of proteins were resuspended in electrophoresis sample buffer containing β-mercaptoethanol and separated by electrophoresis on a precast 10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA), followed by electrotransfer to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Membranes were blocked using 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST). β-actin served as the loading control. Membranes were incubated overnight at 4°C with a 1:500 dilution of polyclonal antibody for PEPT1 (Santa Cruz Biotechnology, Santa Cruz, CA) and with a 1:2000 dilution of monoclonal antibody for β-actin (Santa Cruz Biotechnology). After washing three times in TBST, membranes were incubated for 1 h at 37°C with a 1:5000 dilution of anti-rabbit or a 1:1000 dilution of anti-mouse horseradish peroxidase-conjugated secondary antibody (Invitrogen, Shanghai, China). After extensive washing with TBST, membranes were exposed to the enhanced chemiluminescence-plus reagents (ECL) from Beyotime Institute of Biotechnology (Haimen, China) according to the manufacturer’s protocol. Emitted light was documented with a BioSpectrum-410 multispectral imaging system with a Chemi HR camera 410. Protein bands were visualized and photographed under transmitted ultraviolet light. The image was used for semiquantitative measurements based on band densitometry.
Pharmacokinetics of JBP485 plasma concentrations

The main pharmacokinetic parameters were calculated using the 3P97 program. A two-compartmental data analysis was performed for each individual JBP485 concentration-time profile. The quality of the fit was judged by evaluating the standard error of parameter estimates and the coefficient of determination (data were weighted as $1/C^2$ and $r^2 = 0.9950$). The maximum plasma drug concentration ($C_{\text{max}}$) and the time to reach $C_{\text{max}}$ ($T_{\text{max}}$) were obtained directly from the experimental data. The area under concentration-time curve (AUC) was calculated using Eqs. 1 to 5.

$$C = N \cdot e^{-k_a t} + L \cdot e^{-\alpha t} + M \cdot e^{-\beta t}$$

where $k_a$, $\alpha$ and $\beta$ were the absorption rate constant, distribution rate constant and elimination rate constant, respectively.

$$N = \frac{k_a F X_0 (k_{21} - k_a)}{V_c (\alpha - k_a) (\beta - k_a)}$$

$$L = \frac{k_a F X_0 (k_{21} - \alpha)}{V_c (k_a - \alpha) (\beta - \alpha)}$$

$$M = \frac{k_a F X_0 (k_{21} - \beta)}{V_c (k_a - \beta) (\alpha - \beta)}$$

where $F$, $X_0$, $V_c$ and $k_{21}$ were the bioavailability, the dosage of administration, the apparent volume of distribution in the central compartment and the rate constant of drug transport from peripheral compartment to central compartment, respectively.

$$\text{AUC} = \int_0^{\infty} C \, dt = \int_0^{\infty} (N \cdot e^{-k_a t} + L \cdot e^{-\alpha t} + M \cdot e^{-\beta t}) \, dt$$

$$= \frac{N}{\alpha} + \frac{L}{\beta} + \frac{M}{k_a}$$

The half-life of the elimination phase ($t_{1/2\beta}$) was calculated as follows:

$$t_{1/2\beta} = \frac{0.693}{\beta}$$

Statistical analysis

Statistical analysis was carried out using SPSS version 11.5 software. Test results were expressed as the mean ± S.D. of three experiments. To test for statistically significant differences among multiple treatments for a given parameter, one-way analysis of variance (ANOVA) was performed. Differences were considered statistically significant if $P < 0.05$ or $P < 0.01$.

Results

The pharmacokinetic interaction between JBP485 and zinc after oral administration in vivo: To understand the target of interaction between JBP485 and zinc, JBP485 was orally administered 15 min after oral administration of ZnSO$_4$. The plasma concentrations of JBP485 in the experimental groups decreased significantly compared to the control groups (Fig. 1). The AUC of the experimental group was only 56.2% of that of the control group. In addition, other pharmacokinetic parameters of JBP485 such as $t_{1/2\beta}$ (increased to 2.3 fold of the control group), $T_{\text{max}}$ (delayed) and $C_{\text{max}}$ (reduced to 32.2% of the control group) changed in the presence of zinc when compared to the administration of JBP485 alone (Table 1). These results indicated that the gastrointestinal tract was the site of interaction between JBP485 and zinc.

Effects of zinc on the uptake of JBP485 in everted gut sac of rats in vitro: To exclude the effect of changes in physiological conditions and to further confirm that the site of interaction between JBP485 and zinc was in the small intestine, we used the rat everted gut sac model to investigate the interaction between JBP485 and zinc in vitro. After pre-treatment of the intestinal sags with ZnSO$_4$ for 15 min, the concentrations of JBP485 in the serosal side of the experimental groups decreased significantly when compared to the control groups (Fig. 2). The AUCs of the experimental groups were 62.7% of the controls. This indicates that zinc can inhibit the intestinal uptake of JBP485 in vitro.
Effects of zinc on absorption of JBP485 by single-pass intestinal perfusion in vivo: Because uptake in the everted small intestinal sac in vitro model is limited by the lack of an intact blood supply, we performed in situ jejunal perfusions to clarify the mechanism of interaction between JBP485 and zinc. After pre-perfusion of the intestine with ZnSO₄ for 15 min, the plasma concentrations of JBP485 in the portal vein were significantly lower than that of the control groups (Fig. 3). The AUCs of the experimental groups were 52.5% of the controls. This result further indicated that the site of interaction between JBP485 and zinc was the intestine.

Effect of zinc on uptake of JBP485 in HeLa-hPEPT1 cells: To investigate the mechanism of interaction between JBP485 and zinc, the specific uptake of JBP485 by PEPT1 in HeLa-hPEPT1 cells with or without pre-treatment with zinc was determined. Results indicate that the specific uptake of JBP485 by PEPT1 is significantly decreased by adding zinc (Fig. 4A). In addition, pre-treatment with zinc decreases the affinity of JBP485 for PEPT1 [Kₘ values 1.30 (control) versus 2.12 (zinc treatment) mM] without affecting Vₘₐₓ values [1.73 (control) versus 1.71 (zinc treatment) nmol/mg protein/3 min] (Fig. 4B).

Effect of pH on interaction of JBP485 and zinc in HeLa-hPEPT1 cells: To further investigate the mechanism of interaction between JBP485 and zinc, the pH-dependent uptake of JBP485 by PEPT1 in HeLa-hPEPT1...
cells with or without zinc pre-treatment was determined. The uptake of JBP485 was not affected by changes in pH values after zinc treatment (*P < 0.05, **P < 0.01). Each point represents mean ± S.D. (n = 3).

**Effect of Zn²⁺ and DEPC on uptake of JBP485 in HeLa-hPEPT1 cells**: To understand whether Zn²⁺ or zinc gluconate can affect the uptake of JBP485, we compared the inhibitory effect of ZnSO₄ with that of DEPC and zinc gluconate on the uptake of JBP485 by PEPT1. Both DEPC and ZnSO₄ inhibited the uptake of JBP485 in HeLa-hPEPT1 cells. However, zinc gluconate did not inhibit the uptake of JBP485 in this cell line (Fig. 6). These results show that Zn²⁺, but not zinc gluconate, which is a complex compound, can inhibit the transport activity of PEPT1.

**Effect of other divalent cations on uptake of JBP485 in HeLa-hPEPT1 cells**: To investigate if other divalent cations can also inhibit the uptake of JBP485, we examined the effects of ferrous ions and copper ions on the uptake of JBP485 by HeLa-hPEPT1 cells. FeSO₄ and CuSO₄ also inhibited the uptake of JBP485 by PEPT1, and the potency of inhibitory effects was: Cu²⁺ > Zn²⁺ > Fe²⁺ (Fig. 7).

**Effect of zinc on expression of Pept1 mRNA and Pept1 protein in intestinal mucosa**: To examine if zinc alters the mRNA or protein levels of Pept1, RT-PCR and Western blotting were performed to determine the relative levels of Pept1 mRNA and protein in the intestinal mucosa with or without ZnSO₄ pre-treatment. There was no significant difference in the expression of Pept1 mRNA or protein in the control and zinc-treated groups, which indicated that zinc had no effect on basal intestinal Pept1 expression. The β-actin expression was similar in the control and zinc-treated groups (Figs. 8A and 8B).

**Discussion**

Oligopeptides are absorbed through brush border membrane PEPT1 into intestinal epithelial cells. JBP485 is a dipeptide and a substrate of PEPT1 that exhibits liver protective effects after oral administration in animal experiments. Zinc is an essential metal ion for the body which is orally administered and absorbed through the intestines. There are increasing numbers of people who are taking zinc as a drug or a supplement for zinc deficiency and its clinical effects have been confirmed. A previous study confirmed that zinc inhibited Gly-Sar uptake in rat oocytes expressing Pept1 and Pept2. In addition, zinc inhibited the absorption of cefitubon, which is a substrate of PEPT1, by oral co-administration in vivo and jejunal perfusions in situ. PEPT1
and PEPT2 are mainly expressed in the intestinal mucosa and kidney. This study aimed to determine whether zinc could inhibit the intestinal absorption of JBP485 by PEPT1. However, zinc is mainly excreted in feces and is only minimally excreted in urine. Therefore, zinc is unlikely to interact with PEPT2 in the kidney.

When JBP485 was administered orally 15 min after the oral administration of ZnSO₄, the plasma concentrations of JBP485 were significantly decreased in comparison with the control group (Fig. 1). The AUC of the experimental group was only 56.2% of that of the control group (Table 1). These results indicate that zinc inhibits the intestinal absorption of JBP485. Based on these results, we speculate that the small intestine is the site of interaction between JBP485 and zinc. We also found that zinc increased the value of \( t_{1/2} \), which means that zinc can inhibit the elimination of JBP485. Our previous studies have indicated that JBP485 is excreted through organic anion transporters (OATs), which are transporters in the kidney. Thereby, we are going to examine whether zinc inhibits the secretion of JBP485 via OATs.

We focused on the small intestine to observe the interaction between JBP485 and zinc by using everted gut sacs in vitro. After pre-treatment with ZnSO₄, the concentrations of JBP485 in the serosal side were significantly reduced (Fig. 2), suggesting that zinc can inhibit the intestinal uptake of JBP485 in vitro. This result supports the observation of the in vivo experiment which suggested that the small intestine is the site of interaction between JBP485 and zinc. To further confirm this observation, we used jejunal perfusions in situ to investigate the interaction between the two drugs. In this case, the concentrations of JBP485 in the portal vein were significantly lower than those of the control groups after pre-perfusion with ZnSO₄ (Fig. 3). This observation indicates that zinc can inhibit the intestinal absorption of JBP485, and so further confirms that the site of interaction between JBP485 and zinc is the small intestine.

The specific uptake of JBP485 by PEPT1 was significantly decreased after pre-treatment with zinc (Fig. 4A). Zinc decreased the affinity of JBP485 to PEPT1, as indicated by the increased \( K_m \) value, without affecting the \( V_{max} \) value (Fig. 4B). These results suggest that zinc inhibits the transport activity of PEPT1 rather than acting to decrease the expression of PEPT1. To examine if zinc had an effect on the expression of Pept1, RT-PCR and Western blotting were used to analyze relative Pept1 mRNA and protein levels in the intestinal mucosa with or without the pre-treatment with zinc (Figs. 8A and 8B). No significant difference in the expression of Pept1 mRNA or protein between the control and zinc-treated groups was observed. This indicates that zinc has no effect on the relative expression of Pept1 in the basal intestine.

It is well established that PEPT1 is a secondary active transporter which is driven by a H⁺ gradient. This study finds that the uptake of JBP485 is not affected by changes in pH values after zinc treatment (Fig. 5). We suspect that zinc...
inhibits the protonation of PEPT1 from the results of the pH-dependent uptake of JBP485 by PEPT1. In other words, we suggest that zinc inhibits the binding of H\(^+\) to PEPT1. Kinetic analysis showed that zinc competitively inhibits the uptake of JBP485 by PEPT1 because the \(K_m\) value increased, without affecting the \(V_{\text{max}}\) value (Fig. 4B). From all these findings, it seems likely that zinc competitively inhibits the binding of H\(^+\) to PEPT1. Kinetic analysis also showed that zinc decreased the affinity of JBP485 for PEPT1 because the \(K_m\) value increased (Fig. 4B). However, we think that this may be just one of the potential mechanisms by which zinc inhibits the protonation of PEPT1, because previous studies have provided evidence that the four NH\(_2\)-terminal trans-membrane regions and domains 7–9 of PEPT1 play an important role in determining the substrate affinity.\(^{28,29}\) These important structural elements may be responsible for the substrate recognition of JBP485 to PEPT1. We could not exclude the possibility that zinc binds to the important structural elements of PEPT1 and interrupts the substrate recognition.

On the other hand, we quite agree with the viewpoint of Okamura et al.,\(^{7}\) who were probably the first research team to discover the inhibitory effect of zinc on PEPT1. Okamura et al. discussed the further mechanism that the interaction between zinc and PEPT1 may occur at the histidine residue that acts as the H\(^+\)-binding site. Their study reported that the uptake of Gly-Sar by PEPT1 was inhibited by DEPC, which is a histidine residue modifier. Okamura et al. confirmed that DEPC may interfere with the H\(^+\)-histidine interaction and Gly-Sar–histidine interaction, but zinc may only interfere with the H\(^+\)-histidine interaction. Moreover, Terada et al.’s findings suggested that only the \(\alpha\)-amino group of substrates could interact with the histidine residue of PEPTs and may be involved in the mechanism of substrate recognition by the peptide transporters.\(^{30}\) Unlike Gly-Sar, JBP485 is a dipeptide without an \(\alpha\)-amino group. We compared the inhibitory effect of ZnSO\(_4\) with that of DEPC and found that DEPC could also inhibit the uptake of JBP485 by PEPT1 (Fig. 6). We speculate that zinc and DEPC may interfere with the H\(^+\)-histidine interaction only, rather than with the JBP485–histidine interaction. A previous study proposed that H57 and H121 (two histidine residues of the PEPT1 protein) are intimately involved in the binding of the coupling ion H\(^+\) and the recognition of transportable peptide substrates, respectively.\(^{31}\) In future studies, we will perform mutagenesis of human PEPT1 directed at the two histidine residues (H57 and H121) to distinguish the functional roles of these histidine residues in the transport of JBP485 by PEPT1.

We also compared the inhibitory effect of ZnSO\(_4\) with that of zinc gluconate (Fig. 6). Our results are novel in demonstrating for the first time that Zn\(^{2+}\), but not zinc gluconate, can inhibit the transport activity of PEPT1. The reason is that zinc gluconate is a complex compound which is stable in aqueous solution and in the gastrointestinal tract.\(^{32,13}\) Clinically, this result indicates that the pharmacokinetics of JBP485 would not change in the presence of zinc gluconate. Therefore, we suggest that patients who take JBP485 orally should take zinc gluconate or chelated zinc, but not ZnSO\(_4\), as a supplement or treatment.

In addition, we examined the effects of other divalent cations (Fe\(^{3+}\) and Cu\(^{2+}\)) on the uptake of JBP485. Our results demonstrate that other divalent cations, including Fe\(^{3+}\) and Cu\(^{2+}\), can also inhibit the uptake of JBP485 by PEPT1 (Fig. 7). The mechanism of interaction between JBP485 and Fe\(^{3+}\) or Cu\(^{2+}\) may be similar: divalent cations competitively inhibit the H\(^+\) binding to PEPT1 and interrupt the driving force of PEPT1. In addition, the inhibitory effects were: Cu\(^{2+}\) > Zn\(^{2+}\) > Fe\(^{3+}\).

In conclusion, zinc decreases the intestinal absorption of JBP485 by inhibiting the transport activity of PEPT1 rather than by decreasing the expression of PEPT1. Zinc ions competitively inhibit H\(^+\) binding to PEPT1 and interrupt the driving force of PEPT1. In addition, our results are novel in demonstrating for the first time that zinc ions, but not zinc gluconate, can inhibit the transport activity of PEPT1.

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