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The pharmacokinetic interaction between JBP485 and Cefalexin in rats

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Abbreviations: PAH, *p*-aminohippuate; PCG, benzylpenicillin; Gly-Sar, glycylsarcosine; PEPT1, H^+/peptide transporter 1; PEPT2, H^+/peptide transporter 2; OAT, organic anion transporter; AUC, area under the plasma concentration-time curve; $t_{1/2}$, half-life; CL, clearance; ANOVA, analysis of variance.

Abstract

The purpose of this study was to investigate the pharmacokinetic mechanism of interaction between JBP485 (a dipeptide) and cefalexin when they were co-administered in rats. The plasma concentrations of JBP485 and cefalexin were both decreased significantly after oral combination. But little differences were observed after simultaneous intravenous administration of the two agents, suggesting that the interaction target localized in the intestine during the absorption process. The uptake in everted intestinal sacs and absorption in jejunal perfusions of JBP485 and cefalexin were dramatically reduced after drug combination. When co-administered, both the decrease in accumulative renal excretion (81.9% to 68.1% of JBP485; 91.8% to 74.5% of cefalexin) and in renal clearance (2.89 to 1.87 ml/min/kg of JBP485; 2.23 to 1.58 ml/min/kg of cefalexin) indicated that other transporter(s) are involved in the process of excretion except PEPT2. Probenecid could reduce renal excretion of JBP485 and cefalexin. Moreover, the decreased uptake of JBP485 with probenecid, PAH or PCG in kidney slices could be explained by an inhibition in kidney via OATs, at least in part. The accumulation of JBP485 in hOAT1- or hOAT3-HEK293 cells was greater than that in vector-HEK293 cells, and the uptake could be inhibited by probenecid. These findings further confirmed that the pharmacokinetic mechanism of drug-drug interaction between JBP485 and cefalexin could be explained by their inhibition of the same transporters in the intestinal mucosa (PEPT1) and kidneys (PEPT2 and OATs). We provide the first evidence that JBP485 is not only a substrate of PEPTs but also is excreted through OATs.

Introduction

Drug-drug interactions (DDIs) arising from inhibition of the same transporters may lead to adverse effects. Some drugs with harmful DDIs have been withdrawn from the market. Numerous amino acid transporter systems are responsible for the uptake of free amino acids, while the uptake of di- and tripeptides is achieved by the intestinal low affinity/high capacity peptide transporter (PEPT1). PEPT1 is primarily expressed in the brush border membrane of the small intestine (Leibach F H and Ganapathy V, 1996). PEPT2 is expressed specifically on the apical (luminal) membrane of epithelial cells of the proximal tubules of the kidney. They are responsible not only for nutrient transport across absorptive cell membranes, but also the absorption (in the small intestines) and reabsorption (in the kidneys) of peptides, or peptide-like compounds, such as angiotensin-converting enzyme inhibitor (ACEI), cefadroxil and valacyclovir (Ganapathy V. et al., 1994).

JBP485 (cyclo-trans-4-L-hydroxyprolyl-L-serine) is a dipeptide (Kexin L et al., 2000) (Fig.1A) first isolated from Laennec (Yang T et al.,2009), which is a trade name for the hydrolyzate of human placenta (Annaert P and Brouwer K.R., 2005) in Japan, and has been synthesized by chemical means. Animal experiments have indicated that JBP485 exhibits obvious liver protective effects after oral administration (Ke-Xin Liu et al., 1998; Wu J et al., 2008; Yang T et al., 2009). JBP485 was well absorbed through the gastrointestinal tract. Our previous studies have shown that its absorption can be inhibited by glycylsarcosine (Gly-sar), which is a dipeptide model drug and the substrate of PEPTs. These studies have suggested that JBP485 is recognized by the peptide transporter system in the gastrointestinal tract. Cefalexin, as the first-generation oral cephalosporin (Fig.1B), has a broad spectrum of antibacterial activity (Ogawa M et al., 1994). It has already been demonstrated that dipeptides and cefalexin share certain structural features, such as a peptide bond with an α-amino group and a terminal carboxylic acid group (Bretschneider B et al., 1999). This structural similarity is apparently the basis for the molecular mimicry, enabling the peptide transporters to accept cefalexin as a substrate (Amidon G L and Lee H J, 1994). The oral absorption of cefalexin is rapid and safe. Moreover, the renal secretion of most cephalosporin is believed to be mediated by organic anion transporters

(OATs) located on the basolateral membrane of proximal tubule epithelia (Takeda M et al., 2002; Shitara Y et al., 2005).

Strategies have been used to modify drugs that are absorbed poorly by targeting them toward receptors/transporters for improved bioavailability (Sakaeda T et al., 2001; Anand B S et al., 2002a; Manfredini S et al., 2002). To observe whether the combination of JBP485 and cefalexin affects each of the components, to expound the pharmacokinetic mechanism(s) of interaction, as well as to provide a rationale for the clinical use of the drug combination, we have established an LC-MS/MS method for the determination of JBP485 and cefalexin. We have utilized *in vivo* oral administration, *in situ* intestinal perfusions, *in vitro* everted small intestinal sac preparations, *in vivo* urinary excretion, *in vitro* kidney slices, and transfected-cell uptake studies to investigate the change in pharmacokinetics when JBP485 and cefalexin were used together. Our results of renal excretion first indicated that JBP485 might be a substrate of OATs. These findings demonstrate the importance of recognizing that, when JBP485 and cefalexin are co-administered, the target transporters are not only PEPTs but also OATs.

Materials and methods

Chemicals. JBP485 was provided by Japan Bioproducts Industry Co. Ltd (Tokyo, Japan). Reference standards of cefalexin and paracetamol (internal standard, IS), with purities of >99.0%, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Probenecid, PAH (*p*-aminohippua), PCG (benzylpenicillin) and Gly-Sar (glycylsarcosine) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol was of high performance liquid chromatography (HPLC) grade (Tedia, USA). The stable transfectants expressing hOAT1- or hOAT3-HEK293 and vector cells (mock) were a generous gift from Professor Yuichi Sugiyama, Graduate School of Pharmaceutical Sciences, University of Tokyo (Tokyo, Japan) and Li-kun Gong (Shanghai Institute of Materia Medicable, Chinese Academy of Science, China). All other reagents and solvents were of analytical grade, and were commercially available.

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). They were allowed free access to water and chow diet, but were fasted for 12 h with water *ad libitum* before the pharmacokinetic experiments. All the animal experiments were performed according to local institutional guidelines for the care and use of laboratory animals.

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

In vivo absorption experiment in rats. Rats were divided randomly into three groups: (1) JBP485 alone (25 mg/kg) as control; (2) cefalexin alone (50 mg/kg) as control; (3) JBP485 (25 mg/kg) + cefalexin (50 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 and 600 min for JBP485 and cefalexin determination, as described below.

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 (Wilson T H, Wiseman G., 1954) 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 to check for perforation. The empty sac was filled with 1 ml Krebs–Ringer buffer (pH 7.4, KRB) containing 0.5 mM $MgCl_2$, 4.5 mM KCl, 120 mM NaCl, 0.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 1.2 mM $CaCl_2$, 15 mM $NaHCO_3$, 10 mM glucose, and 20 mg/l of phenolsulfonphthalein as a nonabsorbable marker. The distended sac was placed in incubation medium (mucosal solution) containing (1) 0.5 mM JBP485; (2) 1 mM cefalexin; (3) 0.5 mM JBP485+1 mM cefalexin. The incubation medium was surrounded by a water jacket maintained at 37 °C. A gas mixture of 95% O_2 and 5% CO_2 was bubbled through the external incubation 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 were collected for JBP485 and cefalexin determination as described below.

In situ jejunal perfusion technique. 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 (Qinghao Zhang et al., 2009). 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. Oxygenated perfusion solution 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. The solution for jejunal perfusion was the same as the KRB above. After a 30-min equilibration period, 0.5 mM JBP485 alone, 1 mM cefalexin alone, or 0.5 mM JBP485+1 mM cefalexin dissolved in KRB were administered. Portal vein blood was collected using a vein detained needle purchased from clinic at certain times for JBP485 and cefalexin determination, as described below.

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In vivo plasma concentration and renal excretion. Rats were anesthetized with ether and grouped according to the following method and were administered intravenously via the jugular vein: (1) JBP485 (25 mg/kg); (2) cefalexin (50 mg/kg); (3) JBP485 (25 mg/kg) +cefalexin (50 mg/kg). Blood samples were collected at 1, 5, 10, 30, 60, 120, 240, 360, 480 and 600 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 **(**Qinghao Zhang et al., 2009**).** Urine was collected directly from the bladder at 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h. In addition, the two drugs were administered together with probenecid (100 mg/kg). The concentrations of JBP485 and cefalexin 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 Y (Nozaki Y et al., 2007a). We investigated the viability of kidney slices by the uptake of PAH (a classical substrate of OAT1) in a concentration-dependent manner. In brief, kidneys were cut into slices accurately using a ZQP-86 tissue slicer (Zhixin Co., Ltd, China) (thickness 300 μm, surface area about 0.15 cm^2) and prepared in buffer. After a pre-incubation for 3 min under a carbogen atmosphere at 37 °C in 6-well culture plates, with gentle shaking, kidney slices were transferred to 24-well culture plates containing fresh carbogen-saturated JBP485 and/or cefalexin for further incubation. In addition, JBP485 in the presence or absence of Gly-sar (20 mM), probenecid (2 mM), PAH (0.5 mM) or PCG (0.5 mM) was used. The uptake was measured at 0, 1, 5, 10 and 15 min. At the end of the incubation period, kidney slices were washed with ice-cold Hanks' balanced salt solution (HBSS; pH 7.5). Accumulated concentrations of JBP485 and cefalexin were determined by LC/MS/MS after the kidney slices were homogenized. Krebs-bicarbonate slicing buffer consisted of 120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM NaH_2PO_4/Na_2HPO_4, adjusted to pH 7.4.

Uptake experiments using transporter expression systems. Uptake experiments with hOAT1- or hOAT3-HEK and mock cells were performed at 37 °C in HBSS adjusted to pH 7.4. Cultured cells were washed and pre-incubated in the transport buffer for 15 min at 37 °C. The uptake was initiated by adding transport buffer (1 ml) containing drugs

including JBP485 (0.5 mM), with or without cefalexin (1 mM). After incubation for the designated times at 37 °C, the experiment was terminated by removing the medium, followed by washing three times with 1 ml of ice-cold HBSS. The inhibition of probenecid (2 mM), PAH (0.5 mM) and PCG (0.5 mM) were also investigated. Samples were then analyzed by LC/MS/MS after homogenization.

Determination of JBP485 and cefalexin by LC-MS/MS. Plasma samples (50 μl) were added to 50 μl of the internal standard solution (paracetamol, 200 ng/ml) and 400 μl methyl alcohol for de-proteinization. After centrifugation at 12000 g for 10 min, the upper organic layer was transferred into a polythene tube and dried with nitrogen at 37 °C. The dried residue was dissolved in 200 μl of the mobile phase. 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 (IKA-T10 model, Germany) in an ice-bath environment. The other preparations were carried out the same as the plasma samples. To KRB and cell lysate samples (25 μl), 25 μl IS (paracetamol, 200 ng/ml), 50 μl methyl cyanide were added, followed by centrifugation for 10 min at 12000 g. Ten microliters of each sample were injected into the LC-MS/MS (API 3200; Applied Biosystems, Foster City, CA, USA) for analysis.

Data analysis

Pharmacokinetics of JBP485 and cefalexin plasma concentrations. The main pharmacokinetic parameters were calculated according to 3P97 program. The quality of the fit was judged by evaluating the standard error of parameter estimates and the coefficient of determination (r^2), and by the visual inspection of residual plots. The main pharmacokinetic parameters were calculated following the equations below. The plasma clearance (CL_p) was calculated by:

$$CL_p = Dose/AUC_{i.v.} \quad (1)$$

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

$$AUC_{i.v.} = A/\alpha + B/\beta \quad (2)$$

The oral availability (F) was calculated as:

$$F = AUC_{p.o.}/AUC_{i.v.} \quad (3)$$

where $AUC_{p.o}$ is the AUC after oral administration. It was calculated by the trapezoidal

rule. The renal clearance of JBP485 or cefalexin (CL_R) were calculated as

$$CL_R = A_{total} / AUC_{i.v.} \quad (4)$$

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

$$CL_{uptake} = A_{total} / AUC_{(0 \rightarrow 60)} \quad (5)$$

where A_{total} is the cumulative uptake amount of JBP485 or cefalexin in kidney slices over 60 min. AUC ($_{0 \rightarrow 60}$) is the area under the JBP485 or cefalexin plasma 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. If $P < 0.05$ or $P < 0.01$, differences were considered statistically significant.

Results

The pharmacokinetic interaction between JBP485 and cefalexin in intravenous and oral administration *in vivo*. To understand the target of interaction between JBP485 and cefalexin, the drugs were administered simultaneously by the intravenous or oral routes. As shown in Fig. 2A and 2B, when the two drugs were administered orally in combination, their plasma concentrations and AUCs were decreased significantly compared to the control groups. The AUCs of JBP485 and cefalexin were only 35.2% and 23.4% of the respective control groups, and the Ka values of JBP485 and cefalexin were about 70% and 50% of their control groups, respectively. In addition, the other kinetic data of JBP485 such as $T_{1/2\beta}$ (2 folds), Tmax (delayed), Cmax (reduced to nearly 10%), MRT (increased about 2.4 folds) and F (dropped to 35%) were all changed after drug combination compared to single administration. These parameters for cefalexin showed the similar changes when co-administration with JBP485 (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. 2C and 2D, and Table 1).

Drug interaction in intestinal uptake studies in vitro. To exclude the impact of the changes in the physiological conditions, and to further confirm that the target of the interaction between JBP485 and cefalexin was in the small intestine, we applied the rat everted gut sac model (Cummins C L et al., 2003) to investigate the interaction between JBP485 and cefalexin *in vitro* (Fig. 3). The serosal side concentrations of JBP485 and cefalexin in the experimental groups were both decreased significantly compared to the corresponding control group (Fig. 3A, 3B). The AUCs of the experimental group were 52.9% (for JBP485) and 42.5% (for cefalexin) of that of the respective controls. This suggested that JBP485 and cefalexin could inhibit the intestinal uptake of the other *in vitro*.

I*n situ* single-pass intestinal perfusion studies of drug interactions. Because the uptake in everted small intestinal sac *in vitro* is limited by their lack of an intact blood supply, we also performed *in situ* jejunal perfusions to clarify the mechanism of the interaction between JBP485 and cefalexin. When the two drugs were perfused

simultaneously, the plasma concentrations of JBP485 and cefalexin in the portal vein were significantly lower than that of control groups (Fig. 4A, 4B). AUCs of the experimental group were 43.6% and 47.9% of controls, respectively.

Drug interactions in renal excretion *in vivo*. The cumulative urine excretion during 24 hours were 81.9% (for JBP485) and 91.8% (for cefalexin) when JBP485 (25 mg/kg) or cefalexin (50 mg/kg) was intravenously administered alone (Fig. 5A, 5B). This indicated that renal excretion is the major way for the two drugs. When JBP485 and cefalexin were intravenously administered at the same time the cumulative urine excretion decreased to 68.1% (for JBP485) and 74.5% (for cefalexin) of the corresponding controls, respectively (Fig. 5A and 5B). The renal clearance rate of JBP485 or cefalexin was decreased to 70.9% (Fig. 5C) or 64.7% (Fig. 5D) of the control group.

To further examine the mechanism for renal excretion of JBP485 and cefalexin, the drugs were administered intravenously with 200 mg/kg probenecid (a classic substrate of OATs) (Khamdang S et al., 2003). Probenecid inhibited further the renal excretion of JBP485 and cefalexin when the three drugs were administered together (Fig. 6A, 6B). Probenecid also reduced the cumulative renal excretion of JBP485 or cefalexin when given in conjunction with the drugs (Fig. 6C, 6D). These findings indicate that JBP485 and cefalexin are excreted via OATs in the kidneys.

Drug interaction in kidney slices. To further understand the target transporter related with renal excretion of JBP485 and cefalexin, we used rat fresh kidney slices to investigate the uptake of the two drugs. Cefalexin (1 mM) could significantly inhibit JBP485 (0.5 mM) uptake in renal slices (Fig. 7A). The rate of uptake (Ke-Xin Liu et al., 1992) calculated by equation (5) for JBP485 was 50.2% of the control group. Conversely, JBP485 (0.5 mM) could also inhibit the uptake of cefalexin (1 mM) in renal slices (Fig. 7B), and the rate of uptake was 74.4% of the control group. To clarify the excretion mechanism of JBP485, we investigated the effects of Gly-sar, probenecid, PAH (a specific substrate of OAT1) and PCG (a specific substrate of OAT3) on JBP485 uptake. The uptake of JBP485 was significantly inhibited by Gly-sar (Fig. 8A), probenecid (Fig. 8B), PAH (Fig. 8C) and PCG (Fig. 8D), suggesting that JBP485 is a substrate for both PEPTs and OATs.

The uptake interaction of JBP485 and cefalexin in hOAT1- or hOAT3-HEK Cells.

To examine whether JBP485 or cefalexin is the substrate of OAT1 or OAT3, the time profiles of JBP485 and cefalexin uptake in hOAT1- or hOAT3- and vector-HEK cells were investigated. The uptake of JBP485 by hOAT1 (Fig. 9A) and hOAT3 (Fig. 9B) was markedly greater than that in vector-HEK at all time points. A similar uptake by hOAT1 (Fig. 9C) and hOAT3 (Fig. 9D) was also found in the case of cefalexin**.** This observation suggests that JBP485 and cefalexin are the substrates of OAT1 and OAT3, respectively. The uptakes of JBP485 in OAT1 (Fig. 9E) and OAT3 (Fig.9F), and the uptakes of cefalexin in OAT1 (Fig. 9G) and OAT3 (Fig.9H), were inhibited significantly by adding JBP485 and cefalexin simultaneously in the transfected cell system. Furthermore, the uptake of JBP485 in hOAT1- or hOAT3-HEK cells could be markedly inhibited by the typical OATs substrate probenecid (Fig. 10A for OAT1 and 10B for OAT3), the OAT1 substrate PAH (Fig. 10C) and the OAT3 substrate PCG (Fig. 10D), suggesting that OAT1 and OAT3 are involved in the uptake of JBP485 .

Discussion

Clinical drug-drug interactions are expected to enhance the beneficial effect of a particular drug, or reduce its toxicity. Some drug combinations, however, will enhance the toxic effect or reduce the pharmacological effect; these are undesirable drug-drug interactions. Antibiotics are used widely, and it is important to pay attention to potentially adverse effects when the antibiotics are combined with other drugs. Due to the broad substrate specificity of drug transporters, drug-drug interactions involving these transporters are emerging frequently. Quinidine, which is a inhibitor of P-gp, could increase plasma concentrations of digoxin because quinidine blocks biliary and urinary excretion of digoxin via P-gp (Hedman A et al., 1991). Since the therapeutic range of digoxin is narrow, changes in its plasma concentration are potentially very serious. Research and development of peptide drugs is an area of much current interest. Oligopeptides are known to be absorbed through brush border membrane peptide transporters 1(PEPT1) into the intestinal epithelial cells (Kusuhara H et al., 2002). β-lactam antibiotics with a unique amide structure can also be recognized by PEPTs (Berggren S et al., 2004; Daniel H and Kottra G, 2004) in the intestines. Thus, when combined with each other, their pharmacological effect will be affected. With this in mind, in this study we set up a sensitive and efficient LC-MS/MS method for simultaneous detection of JBP485 and cefalexin to interpret the mehanism(s) of their interaction, providing a strong basis for clinical therapy.

When JBP485 (25 mg/kg) and cefalexin (50 mg/kg) were administered together orally, their plasma concentrations were significantly decreased in comparison with the control groups (Fig. 2A, 2B). The bioavailability of JBP485 was decreased to 35% of the control group and cefalexin was decreased to 23% of control group (Table 1). These results indicate that there is obvious mutual inhibition in intestinal absorption when the drugs are co-administered orally. Ka values were only approximately half of their corresponding control groups after oral combination (Table 1), indicating that the absorption velocity of the two drugs are delayed significantly. These results show clearly that when peptide drugs and β-lactam antibiotics are co-administered by the oral route, the extent and velocity of their absorption are both inhibited, obviously causing their therapeutic effect to be depressed. Nonetheless, there was no inhibition phenomenon when JBP485 and

cefalexin were administered intravenously at the same time (Fig. 2A, 2B); there were no statistically significant differences in CLp, AUCs and K_e (Table 1). The pharmacokinetic contrast between the two routes of administration indicated the fact that the small intestine is the location of the interaction between JBP485 and cefalexin.

We focused on the small intestine, using everted gut sacs to observe the interaction between JBP485 and cefalexin. Cefalexin is generally accepted as a substrate of PEPTs (Berggren S et al., 2004). When JBP485 and cefalexin were used simultaneously, the respective concentrations of serosa side were both reduced (Fig. 3A and 3B), suggesting that JBP485 is absorbed through PEPT1 in the gastrointestinal tract *in vitro*. To confirm this speculation, we also applied jejunal perfusions *in situ* to investigate the mechanism of the interaction between JBP485 and cefalexin. We found that the concentrations of the two drugs in the portal vein were significantly lower than the respective control group respectively (Fig. 4A, Fig. 4B). These observations indicate that JBP485 and cefalexin may compete for PEPT1 in the small intestine, and that the mechanism of drug-drug interaction involves the inhibition of the absorption of each other.

PEPT1 is expressed extensively in the small intestines and PEPT2 has a high expression level in the kidneys (Ganapathy M E et al., 1998). To understand the interaction between JBP485 and cefalexin in renal excretion, we used bladder catheterization to observe the cumulative urinary excretion of the two drugs during 24 h after JBP485 and cefalexin were given together via intravenous administration. The cumulative urinary excretion (Fig. 5A, 5B) and the renal clearance (Fig. 5C, 5D) were found to be significantly reduced in comparison to the control groups. As is well known, PEPT2 is responsible for tubular re-absorption of drugs (uptake transporters). Our previous study (Qinghao Zhang et al., 2009) showed that when cefditoren (cephalosporin) and Gly-Sar (oligopeptide) were used together, Gly-Sar significantly influences renal excretion of cefditoren by inhibiting renal tubule cefditoren reabsorption via PEPT2. The values of CL_r for cefditoren in the presence of Gly-Sar were significantly higher than that in the absence of Gly-Sar. If JBP485 and cefalexin compete for PEPT2 as well, the amounts of re-absorption would be decreased, and renal clearance should be increased. Our results, however, contradicted our hypothesis, suggesting that other transporters are also involved in the excretion of JBP485 and cefalexin.

As reported previously (Kojima R et al., 2002; Rubio et al., 2002), cephalosporin antibiotics are not only the substrate of PEPTs, but also transported by organic anion transporters (OATs) (Takeda M et al., 1999). OATs are responsible for the renal tubular active secretion to urine. We consider that this transporter may be OATs which played an important role in the urinary excretion. We focused OATs to clarify the mechanism for change in CLr when JBP485 and cefalexin were co-administered. If JBP485 and cefalexin compete with OATs, the amounts of renal tubular secretion into urine would be reduced. To confirm if JBP485 is the substrate of OATs, we chose probenecid (a typical substrate of OATs) as an inhibitor, to investigate whether it influences the urinary excretion of JBP485 and cefalexin *in vivo*. The cumulative urinary excretion of JBP485 and cefalexin were further reduced to 82.9% (JBP485) and 72.2% (cefalexin), compared with the respective control groups (Fig. 6A and 6B). Taking into consideration the effect of PEPT2 on the uptake of JBP485 and cefalexin in urinary excretion *in vivo*, these findings substantiate our hypothesis that compared with PEPT2, OATs had an overwhelmingly greater role in the renal excretion of JBP485 and cefalexin.

In the experiment with kidney slices, the uptake of JBP485 (Fig. 7A) or cefalexin (Fig. 7B) was inhibited remarkably by the addition of the two drug simultaneously. In addition, Gly-sar (Fig. 8A), PAH (a special substrate of OAT1) (Yano I et al.,1998; Sweet DH et al.,1997) (Fig. 8B), PCG (a special substrate of OAT3) (Soichiro Matsushima et al., 2009) (Fig. 8C) and probenecid (Fig. 8D) could all inhibit the uptake of JBP485. These findings indicated that JBP485 is a substrate for both PEPT2 and OATs. To further demonstrate the fact we used the transfected hOAT1- or hOAT3-HEK cells to perform the same experiment as with kidney slices, and obtained results similar to that with the kidney slices. That is, JBP485 or cefalexin could inhibit the uptake of the other when added to the transfected hOAT1- or hOAT3-HEK cells simultaneously (Fig. 9E, 9F, 9G, 9H). Probenecid (Fig. 10A, 10B), PAH (Fig. 10C) and PCG (Fig. 10D) could inhibit the uptake of JBP485. Taken together, our results indicate that at least two kinds of transporters (PEPT2 and OATs) participate in the excretion of JBP485 and cefalexin in the kidneys.

In conclusion, there are drug-drug interactions between JBP485 and cefalexin when they are co-administered. The targets of interaction are PEPT1 in the intestines, and

PEPT2 as well as OATs in the kidneys. Our results are novel in demonstrating for the first time that the dipeptide drug JBP485 is not only the substrate of PEPTs, but also of OATs.

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Footnotes

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Fig. 1. Chemical structures of JBP485 (A) and cefalexin (B).

Fig. 2. Mean plasma concentration-time curves of JBP485 and cefalexin after oral and intravenous administration in rats. Panel A shows the plasma concentration of JBP485 after oral administration. Panel B presents the plasma concentration of cefalexin after oral administration. Panel C gives the plasma concentration of JBP485 after intravenous administration. Panel D shows the plasma concentration of JBP485 after intravenous administration. 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 (*, $p<0.05$; **, $p<0.01$). Data are expressed as mean ± S.D. (n=5).

Fig. 3. Time profile of JBP485 and cefalexin transported in everted small intestinal sac preparations. Panel A presents the serosal lateral concentration of JBP485; panel B shows the serosal lateral concentration of cefalexin. Statistical differences of each points were compared with the control groups by a two-tailed unpaired *t*-test, with $p< 0.05$ as the limit of significance (*, $p<0.05$; ** , $p<0.01$). Data are expressed as mean ± S.D. (n=5).

Fig. 4. Mean plasma concentration-time curves of JBP485 and cefalexin during intrajejunal perfusions. Panel A shows the plasma concentration of JBP485; panel B gives the plasma concentration of cefalexin. Statistical differences of each of the points were compared with the control groups by a two-tailed unpaired *t*-test, with $p< 0.05$ as the limit of significance (*, $p<0.05$; **, $p<0.01$). Data are expressed as mean ± S.D. (n=5).

Fig. 5. Urine excretion curves and renal clearances after intravenous injection of JBP485 and cefalexin. A: urine excretion of JBP485; B: urine

excretion of cefalexin; C: renal clearance of JBP485; D: renal clearance of cefalexin. Data are expressed as mean ± S.D. (* $p<0.05$ vs. control, ** $p<0.01$ vs. control; n=5)

Fig. 6. Effects of probenecid on urinary excretion of JBP485 and cefalexin in rats *in vivo*. 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 (*, $p<0.05$; **, $p<0.01$). Data are expressed as mean ± S.D. (n=5).

Fig. 7. Time course of JBP485 (A) and cefalexin (B) uptake into kidney slices (*, $p<0.05$; **, $p<0.01$; mean ± S.D., n=3)

Fig. 8. The inhibition effects of Gly-sar (A), probenecid (B), PAH (C) and PCG (D) on JBP485 uptake in kidney slices. 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 (*, $p<0.05$; **, $p<0.01$). Data are expressed as mean ± S.D. (n=5).

Fig. 9. Time profiles of the uptake of JBP485 and cefalexin by hOAT1- or hOAT3- and vector-HEK cells, and the mutual interaction between JBP485 and cefalexin. The time-dependent uptake of JBP485 (0.5 mM) (A and B) and cefalexin (1 mM) (C and D) by hOAT1- or hOAT3- and vector-HEK cells were examined at 37 °C. The mutual inhibition between JBP485 and cefalexin (E, F, G and H) in hOAT1-/hOAT3-HEK cells was also examined. (*, $p<$ 0.05; **, $p<0.01$). Each point represents the mean ± S.D. (n=5).

Fig. 10. The effect of probenecid, PAH and PCG on the uptake of JBP485 in hOAT1- or hOAT3-HEK293 cells. The uptake of JBP485 (0.5 mM) by hOATs-HEK for 5 min was determined in the absence or presence of 2 mM probenecid (A and B), 0.5 mM PAH (C) and 0.5 mM PCG (D). (*, $p<$ 0.05; **, $p<0.01$). Each point represents the mean ± S.D. (n=5).

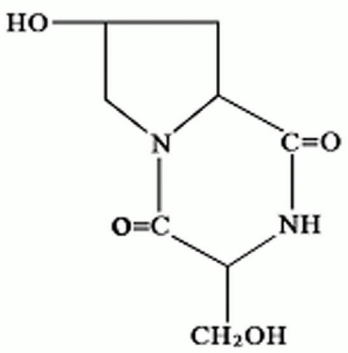
Table1 . Pharmacokinetic parameters of JBP485 and cefalexin following per os or i.v. administration

	Parameters		*JBP485*	*JBP485+Cefalexin*	*Cefalexin*	*Cefalexin+JBP485*
p.o.	C_{max}	(μg/ml)	12.75±0.77	1.75±0.52[b]	53.97±4.09	6.41±1.43[b]
	T_{max}	(min)	60	240[b]	60	120[b]
	Ka	(1/min)	0.11±0.0042	0.077±0.0013	0.0047±0.0006	0.0024±0.0001
	Ke	(1/min)	0.0064±0.0004	0.0029±0.0011	0.0044±0.0022	0.0021±0.0016
	MRT	(min)	194.07±1.64	469.784±2.47[b]	208.87±1.59	361.52±1.94[a]
	$AUC_{0\to\infty}$	(min·μg/ml)	2724.63±83.81	958.97±76.32[b]	13657.48±95.10	3159.78±88.23[b]
	$T_{1/2\beta}$	(min)	135.19±4.06	247.96±2.06[a]	86.98±3.13	157.42±2.69[a]
	V_d/F	(L/kg)	0.30±0.02	0.67±0.04	0.77±0.03	1.74±0.01
	CL_p/F	(ml/kg/min)	1.91±0.32	1.93 ±0.13	3.39±0.32	3.74±0.41
	F	(%)	26.1±4.23	9.2±0.96[b]	81.2±9.72	18.8±1.81[b]
i.v.	C_{max}	(μg/ml)	131.24 ±3.21	114.36±2.79	482.84±5.08	476.51±2.68
	Ke	(1/min)	0.0085±0.0017	0.0098±0.0004	0.0079±0.0008	0.0080±0.0009
	MRT	(min)	145.53±1.84	169.83±1.47	101.22±1.09	102.33±1.56
	$AUC_{0\to\infty}$	(min·μg/ml)	10455.37±130.17	11119.61±109.61	16817.26±163.34	16716.96±128.52
	$T_{1/2\beta}$	(min)	134.24±1.02	132.86±1.31	86.80±2.01	87.48±1.36
	V_d	(L/kg)	0.28±0.04	0.23±0.01	0.42±0.03	0.42±0.05
	CL_p	(ml/kg/min)	2.39±0.19	2.25±0.15	3.32±0.32	3.36±0.18
	CL_R	(ml/kg/min)	2.23±0.16	1.58±0.09[a]	2.89±0.15	1.87±0.15[a]

[a] $p<0.01$, [b] $p<0.001$ compared with single administration, values represent the mean±S.D. (n= 5). Statistics were conducted using the two-sided unpaired Student's *t* test.

Fig. 1

A

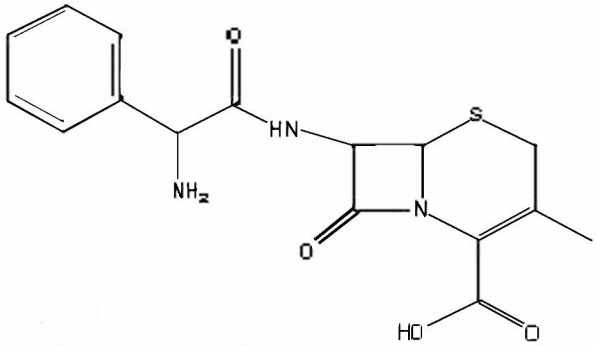


(MW=200.08)

$C_8H_{12}N_2O_4$

(Cyclo-trans-4-hydroxyprolyl-L-serine)

B



(MW=347.38)

$C_{16}H_{17}N_3O_4S$

Cefalexin

Fig. 2

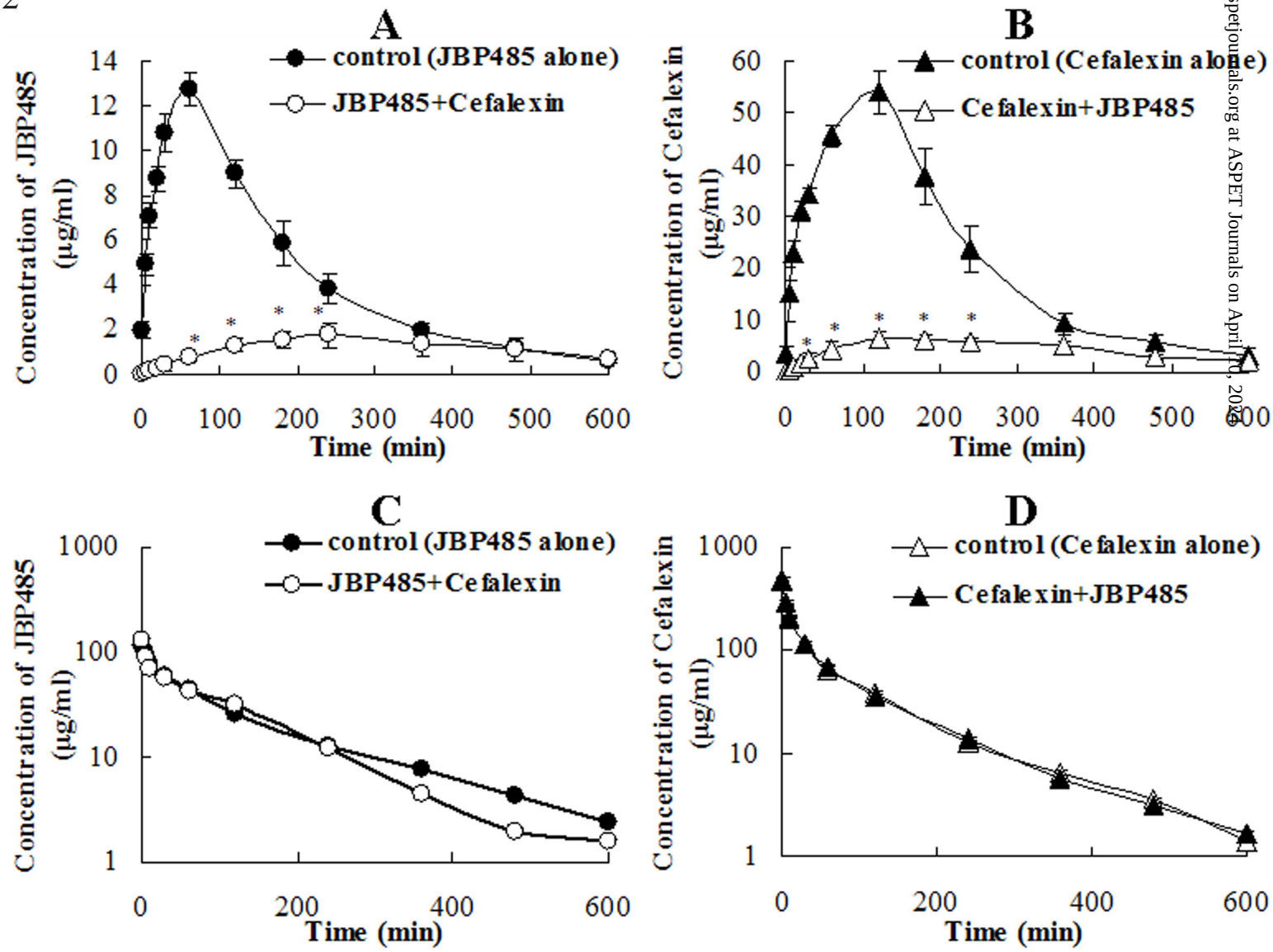


Fig. 3

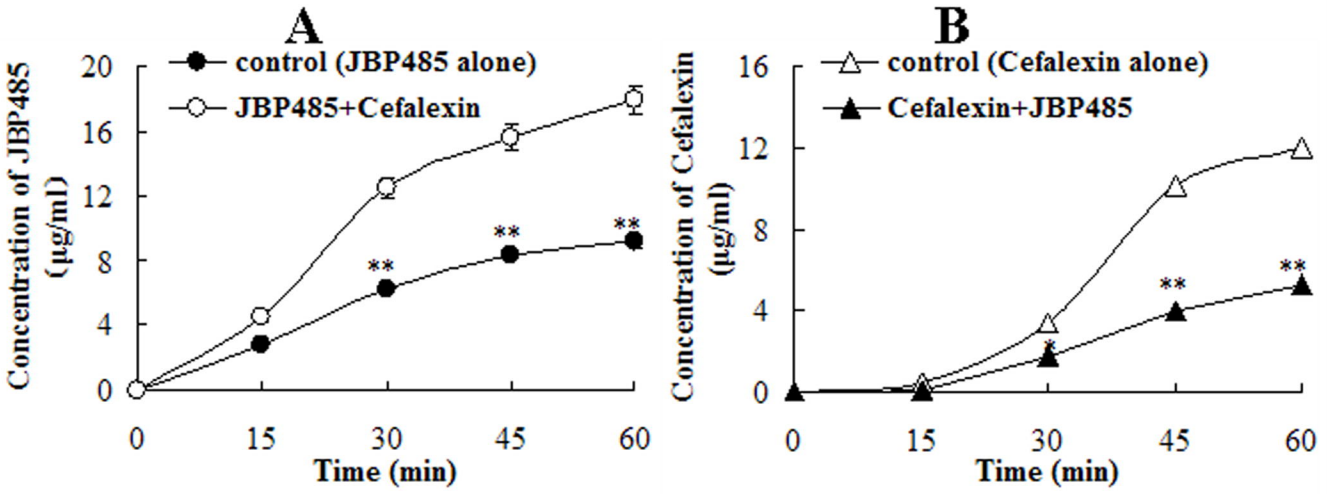

A
control (JBP485 alone)
JBP485+Cefalexin
Concentration of JBP485 (μg/ml)
Time (min)
B
control (Cefalexin alone)
Cefalexin+JBP485
Concentration of Cefalexin (μg/ml)
Time (min)

Fig. 4

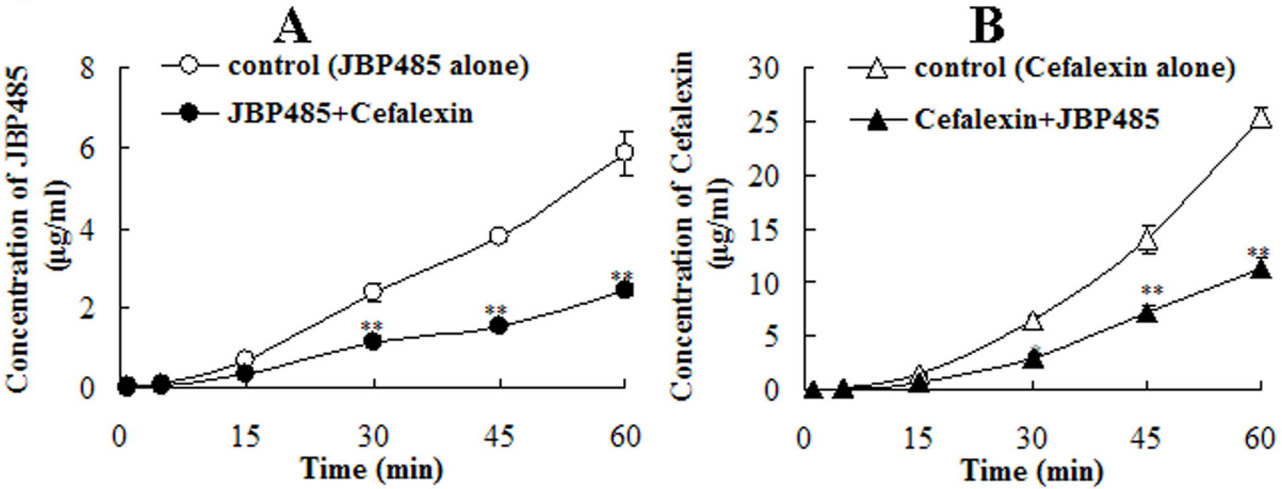

A
control (JBP485 alone)
JBP485+Cefalexin
Concentration of JBP485 (μg/ml)
Time (min)
B
control (Cefalexin alone)
Cefalexin+JBP485
Concentration of Cefalexin (μg/ml)
Time (min)

Fig. 5

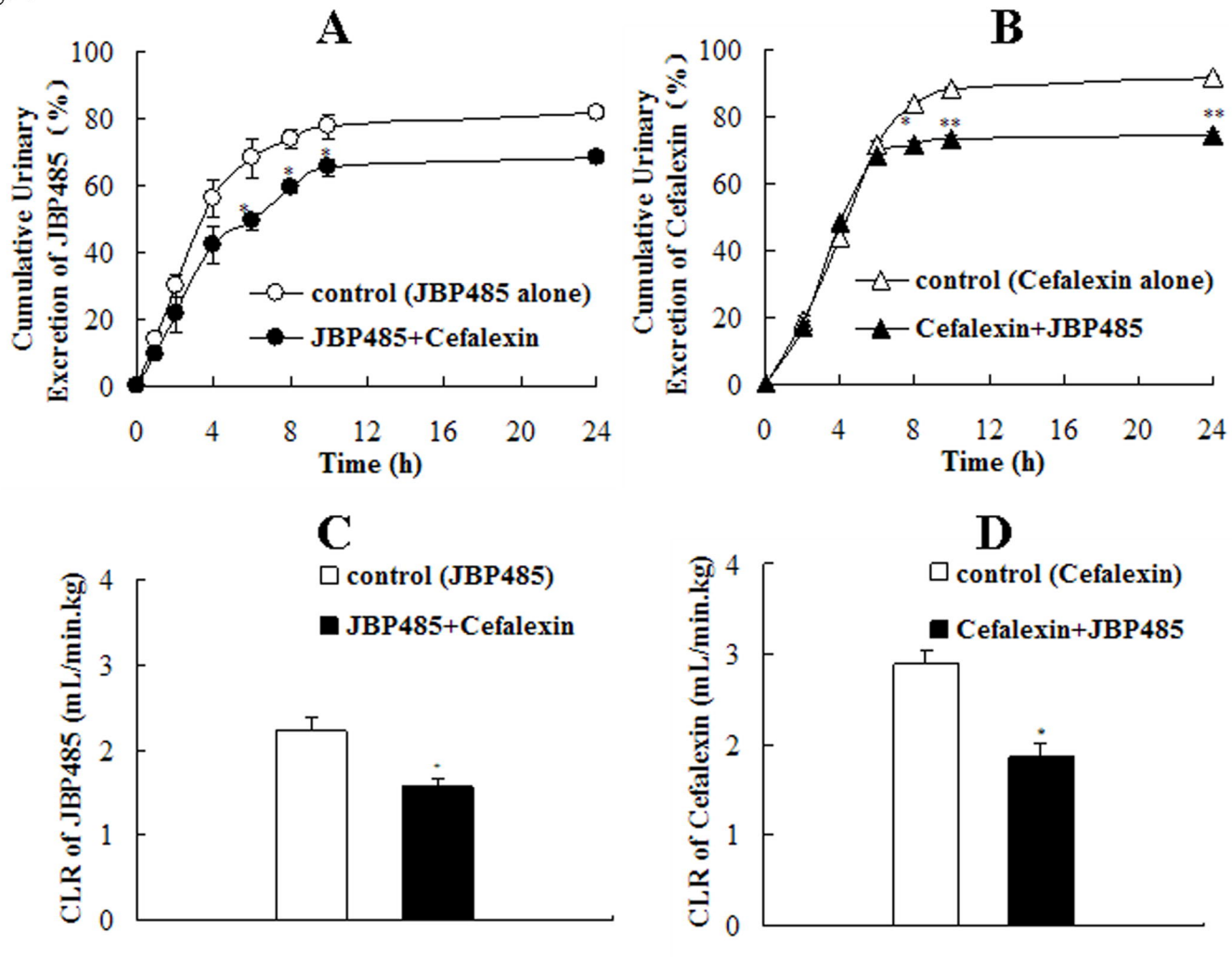

A
Cumulative Urinary Excretion of JBP485 (%)
control (JBP485 alone)
JBP485+Cefalexin
Time (h)
B
Cumulative Urinary Excretion of Cefalexin (%)
control (Cefalexin alone)
Cefalexin+JBP485
Time (h)
C
control (JBP485)
JBP485+Cefalexin
CLR of JBP485 (mL/min.kg)
D
control (Cefalexin)
Cefalexin+JBP485
CLR of Cefalexin (mL/min.kg)

Fig. 6

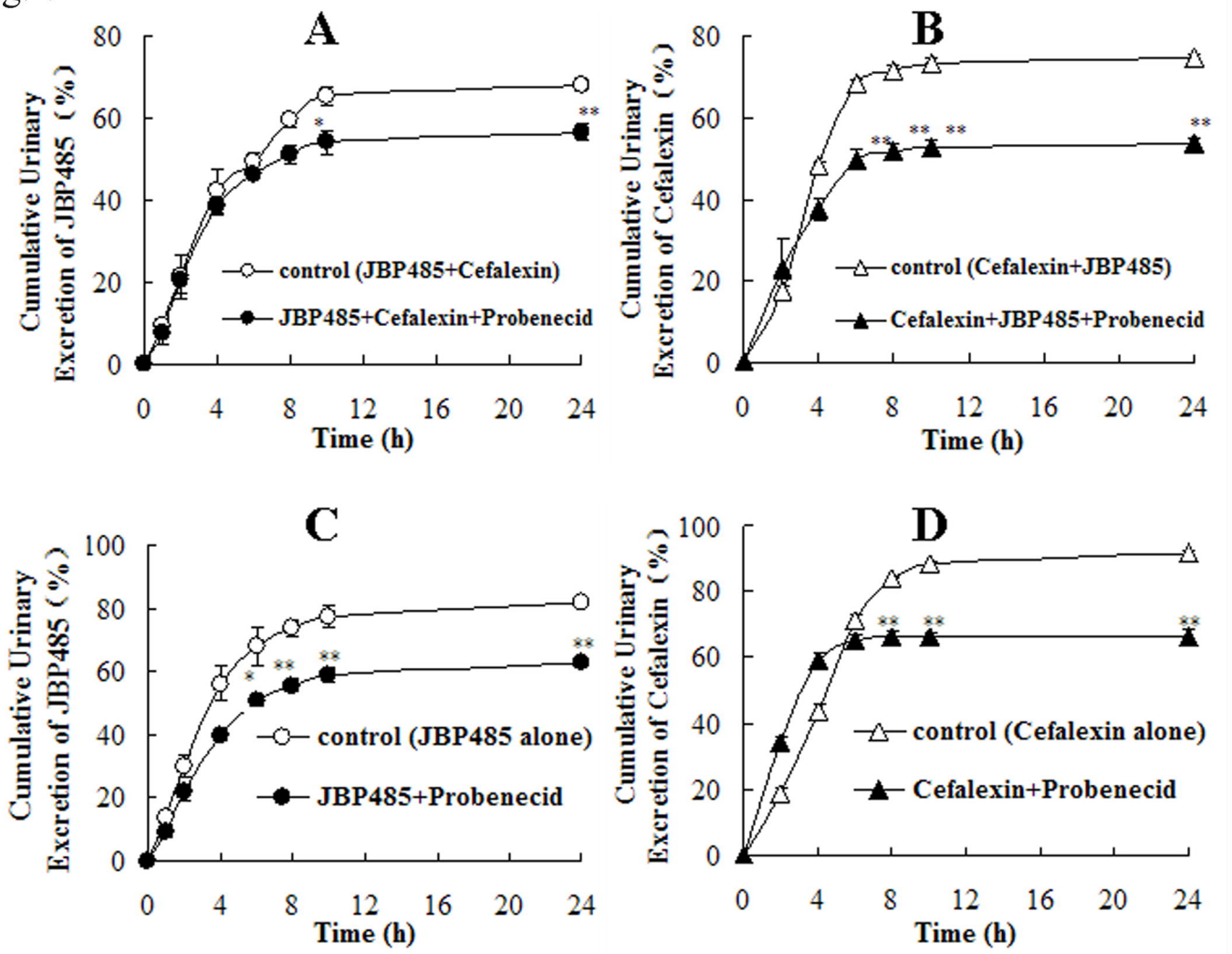
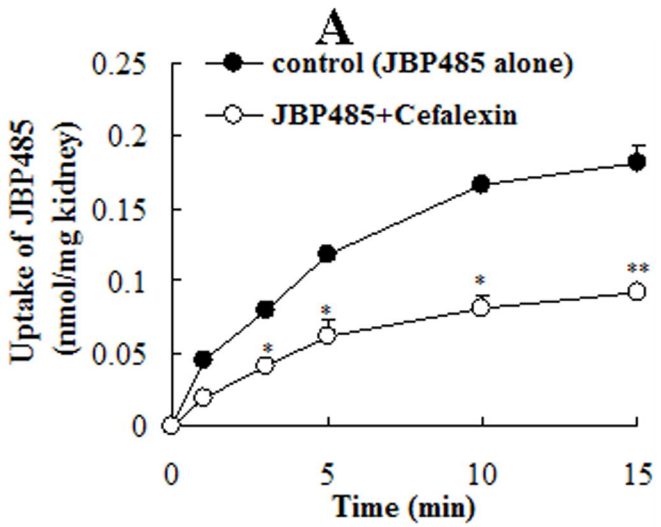

A
Cumulative Urinary Excretion of JBP485 (%)
control (JBP485+Cefalexin)
JBP485+Cefalexin+Probenecid
Time (h)
B
Cumulative Urinary Excretion of Cefalexin (%)
control (Cefalexin+JBP485)
Cefalexin+JBP485+Probenecid
Time (h)
C
Cumulative Urinary Excretion of JBP485 (%)
control (JBP485 alone)
JBP485+Probenecid
Time (h)
D
Cumulative Urinary Excretion of Cefalexin (%)
control (Cefalexin alone)
Cefalexin+Probenecid
Time (h)

Fig. 7


A
control (JBP485 alone)
JBP485+Cefalexin
Uptake of JBP485 (nmol/mg kidney)
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
Time (min)
*
*
*
**

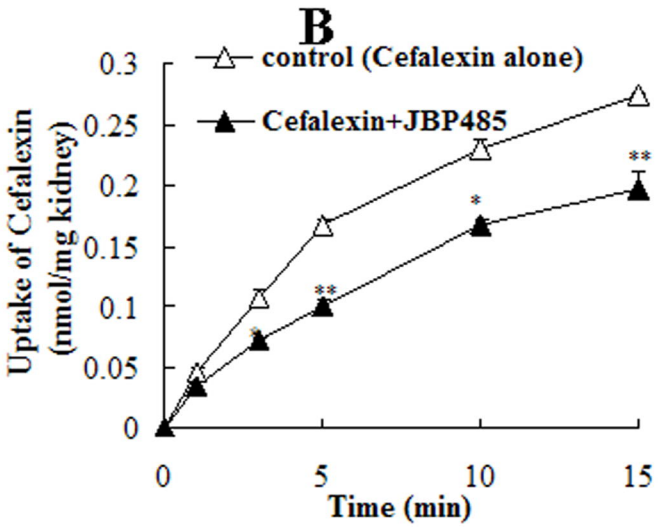

B
control (Cefalexin alone)
Cefalexin+JBP485
Uptake of Cefalexin (nmol/mg kidney)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
Time (min)
*
**
*
**

Fig. 8

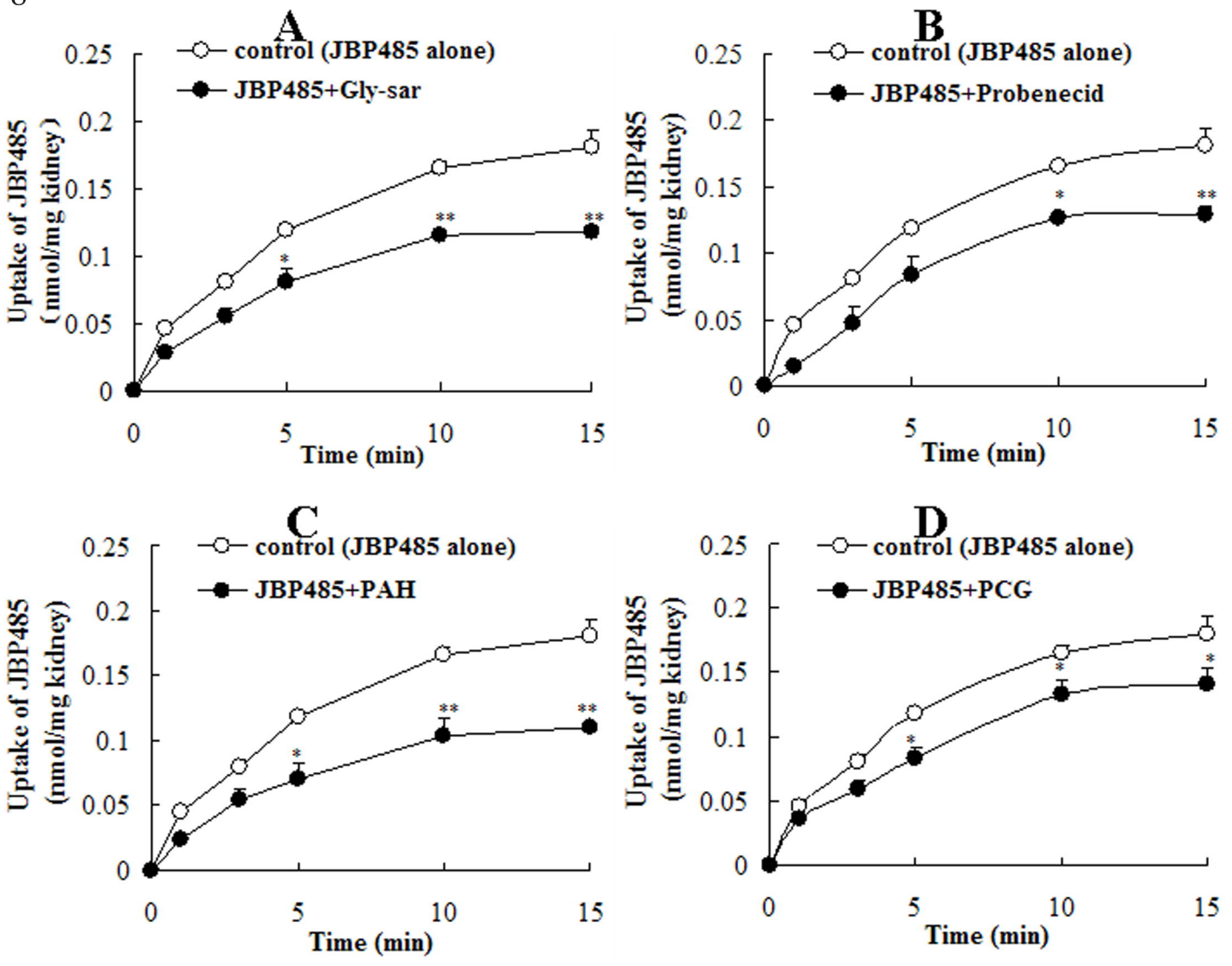

A
control (JBP485 alone)
JBP485+Gly-sar
Uptake of JBP485 (nmol/mg kidney)
Time (min)
B
control (JBP485 alone)
JBP485+Probenecid
Uptake of JBP485 (nmol/mg kidney)
Time (min)
C
control (JBP485 alone)
JBP485+PAH
Uptake of JBP485 (nmol/mg kidney)
Time (min)
D
control (JBP485 alone)
JBP485+PCG
Uptake of JBP485 (nmol/mg kidney)
Time (min)

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Fig. 9

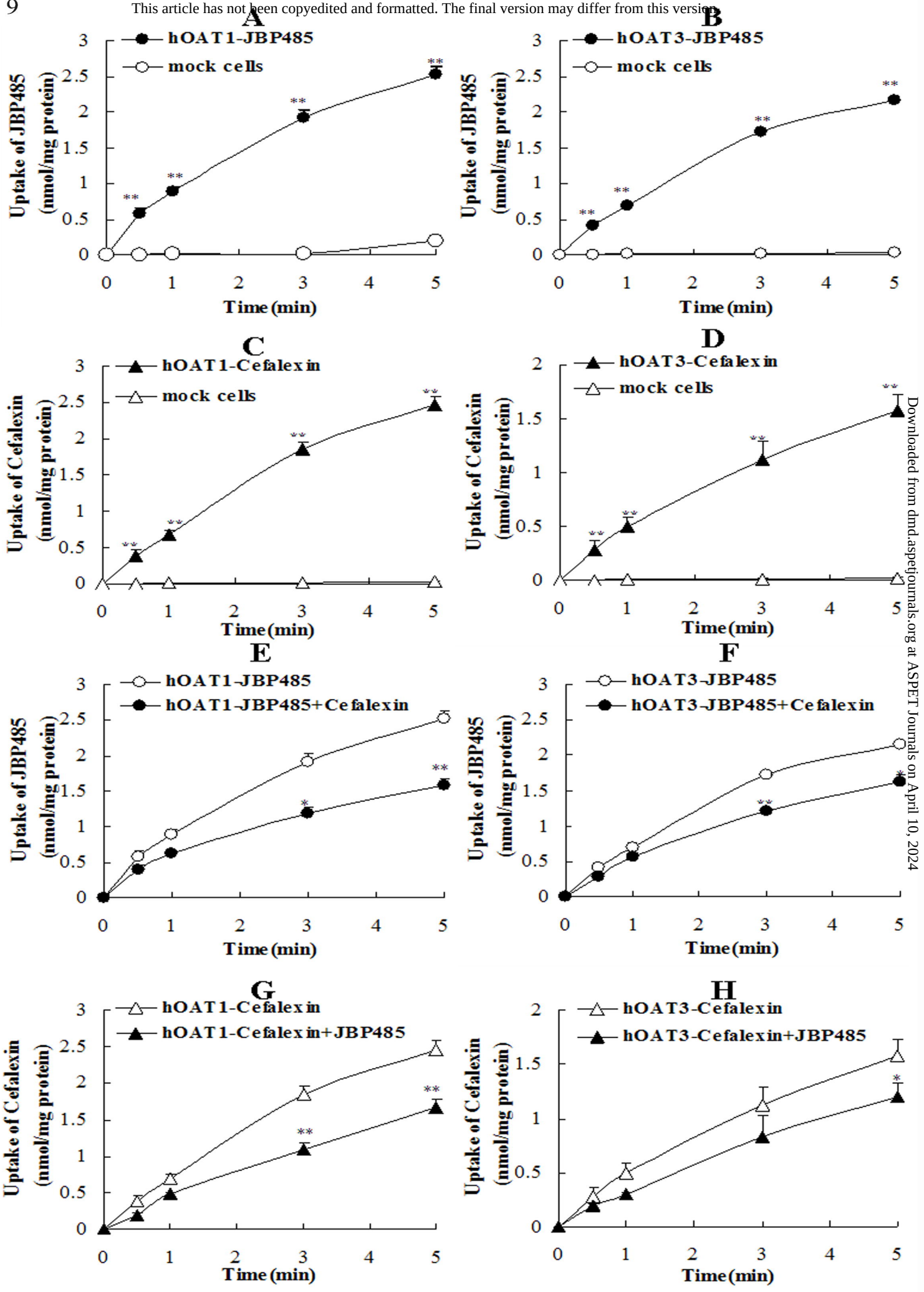

Fig. 10