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Nateglinide uptake by a ceftibuten transporter in the rat kidney brush-border membrane

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Abstract

Nateglinide, a novel oral hypoglycemic agent, possesses a carbonyl group and a peptide-type bond in its structure. We previously reported that nateglinide transport occurs via a single system that may be identical to the ceftibuten/H^+ cotransport system by the rat small intestine. We speculated that the absorption system present on the intestinal epithelium may be similar to that found on the renal tubular epithelium. The aim of this study was to characterize the transporters on the apical side of the kidney that may contribute to the reabsorption of ceftibuten and nateglinide. The uptake of nateglinide by rat renal brush-border membranes is associated with an H^+-coupled transport system. Ceftibuten competitively inhibited H^+-dependent nateglinide uptake. In contrast, Gly-Sar, cephradine and cephalexin had no effect on nateglinide uptake. Nateglinide competitively inhibited H^+-driven transporter-mediated ceftibuten uptake. We conclude that nateglinide transport occurs via a single system that is H^+-dependent and may be identical to the ceftibuten/H^+ cotransport system.

Keywords: Nateglinide; Kidney; Ceftibuten; Brush-border membrane vesicle

1. Introduction

Although nateglinide, an orally active hypoglycemic agent, quickly reaches the maximal serum concentration after oral administration, it has been reported that nateglinide itself is not transported by PepT1 or MCT1 [1,2]. We previously found that the uptake of nateglinide by rat intestinal brush-border membrane vesicles (BBMV) was associated with an H^+-coupled transport system and that Gly-Sar, cephradine and cephalexin, which are substrates of PepTs, did not significantly inhibit the uptake of nateglinide despite the fact that ceftibuten competitively inhibited H^+-dependent nateglinide uptake [3]. We speculated that the absorption system present on the intestinal epithelium may be similar to that found on the renal tubular epithelium. This transport system has not yet been elucidated at the molecular level. Transporter function offers the possibility of delivering a drug to the target organ, avoiding distribution to other organs (thereby reducing the chance of toxic effects), controlling the elimination process and/or improving oral bioavailability. Uwai et al. studied the interactions of sulfonylureas and nateglinide with a rat renal organic anion transporter (rOAT1), located on basolateral membranes, expressed in *Xenopus laevis* oocytes and they reported that these drugs were not translocated via the transporter but that they interact with rOAT1 [4]. Ceftibuten possesses a relatively long elimination half-life when compared with other cephem antibiotics. To clarify this reabsorption system, nateglinide is used as model compound.

The present study was undertaken to elucidate the mechanism of nateglinide transport in brush-border membranes and we examined the transporters from the viewpoint of function on renal BBMV that may contribute to the reabsorption of ceftibuten and nateglinide in the kidney.

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2. Materials and methods

2.1. Chemicals

Nateglinide was kindly donated by Yamanouchi (Tokyo, Japan). Cephradine and ceftibuten were kindly supplied by Sankyo (Tokyo, Japan). Gly-Sar was purchased from ICN Biomedicals, Ltd. (CA, USA). Cephalexin was purchased from Wako Pure Chemical (Osaka, Japan). All other reagents were of the highest grade available and used without further purification.

2.2. Animals

Male Wistar rats, aged 6 to 7 weeks (300–350 g in weight), were obtained from NRC Haruna (Gunma, Japan). The housing conditions were described previously [5]. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals".

2.3. Preparation of rat renal brush border membrane vesicles

BBMV from the rat kidney cortex were prepared by Ca^{2+} precipitation as described previously [6]. In most cases, the suspending buffer was solution B (100 mM D-mannitol, 100 mM KCl and 20 mM HEPES/Tris, pH 7.5). Enrichment of the brush-border membrane fraction was determined by alkaline phosphatase. The activity level of the brush-border membrane was routinely more than 10-fold higher than that of the initial homogenate. No increase in specific Na^+–K^+ATPase activity as a marker for the basolateral membrane was observed. Protein concentration in preparations of BBMV was adjusted to 6~10 mg/ml.

2.4. Uptake experiments

The uptake of nateglinide into BBMV was measured by the rapid filtration technique described previously [7]. In a routine assay, 40 μl of a suspension of membrane vesicles (0.2–0.3 mg protein) was added to 200 μl of incubation medium kept at 25 °C. The compositions on the media are described in the figure legend. At selected time intervals, the uptake was stopped by diluting the incubation medium with 5 ml of ice-cold 10 mM MES buffer (pH 5.5) containing 150 mM KCl. The mixture was immediately filtered through a Millipore filter (0.45 μm, 2.5 cm in diameter; HAWP). The filter was rinsed once with 10 ml of the same buffer. Substrate trapped on the filter was extracted with 300 μl of water, and the concentration of substrate was determined.

2.5. Analytical procedures

Nateglinide and ceftibuten were determined using an HPLC system equipped with a JASCO 880-PU pump and 870-UV UV/VIS detector described previously [8]. The column was a Hitachi ODS Gel #3053 (4 mm i.d. × 250 mm). In the assay for nateglinide, a mobile phase containing of 50 mM H_3PO_4 (pH 2.5): acetonitrile (55 : 45, v/v) was used. Column temperature and flow rate were 55 °C and 0.7 ml/min, respectively. The wavelength for detection of nateglinide was 210 nm. In the assay for ceftibuten, a mobile phase containing acetonitrile /0.05 M citric acid buffer with pH adjusted to 2.5 by NaOH (1:9) was used. Column temperature and flow rate were 25 °C and 0.7 ml/min, respectively. The wavelength for detection of ceftibuten was 262 nm. Protein was measured by the method of Lowry et al. with bovine serum albumin as a standard [9]. Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA). Nonlinear regression analysis and least-squares fitting for an Eadie–Hofstee plot of substrate uptake were performed by using Origin® (version 6.1J).

3. Results

3.1. Inhibitory effect of nateglinide on H^+-driven ceftibuten transporter-mediated ceftibuten uptake

Naasani et al. previously showed that ceftibuten is transported in the rat renal BBMV via at least two H^+-driven transport systems: PepT- and H^+-driven transporter [10]. In order to determine whether the nateglinide transport system is identical to the ceftibuten/H^+ cotransport system, the inhibitory effect of nateglinide on the uptake of ceftibuten was investigated. Gly-Sar was used as an inhibitor of PepT. As shown in Fig. 1, Gly-Sar significantly reduced the uptake of ceftibuten and this inhibitory effect was saturable at a concentration of more than 30 mM (data not shown). And nateglinide significantly reduced the uptake of ceftibuten. Moreover, the combination of Gly-Sar and nateglinide greatly reduced the uptake of cefutibuten. The effect of the combined treatment was significantly greater than that of Gly-Sar alone. The inhibitory effect of nateglinide on the H^+-driven ceftibuten transporter-mediated ceftibuten uptake was saturable at a concentration of more than 250 μM. We then investigated the kinetics of the inhibitory effect of nateglinide on the H^+-driven ceftibuten transporter-mediated ceftibuten uptake. Fig. 2 shows the concentration-dependence of H^+-driven ceftibuten transporter-mediated ceftibuten uptake in the presence or absence of nateglinide. Eadie–Hofstee plots suggested that nateglinide inhibits H^+-driven ceftibuten transporter-mediated ceftibuten uptake in a competitive manner.

3.2. Effect of proton gradient on nateglinide uptake

In the next part of this study, we directly evaluated the transport mechanism of nateglinide. The effect of an

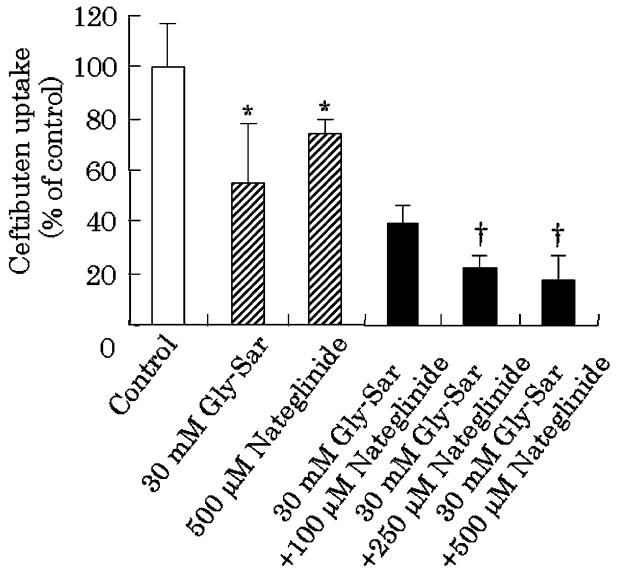

Fig. 1. Additive inhibitory effects of Gly-Sar and nateglinide on the uptake of ceftibuten by rat renal BBMV. Uptake of 100 μM ceftibuten by rat renal BBMV was measured for 20 s with or without (control) inhibitors. Each column represents the mean with S.D. of 3–6 determinations. *$P<0.05$, significantly different from that in the absence of Gly-Sar, †$P<0.05$, significantly different from that in the absence of nateglinide.

inwardly directed H^+ gradient on the uptake of nateglinide was investigated by measuring nateglinide uptake into rat renal BBMV in the presence ($pH_{in}=7.5$, $pH_{out}=5.5$) and absence ($pH_{in}=5.5$, $pH_{out}=5.5$) of an inwardly directed H^+ gradient. Fig. 3a shows that typical overshoot uptake of nateglinide was observed in the presence of an H^+

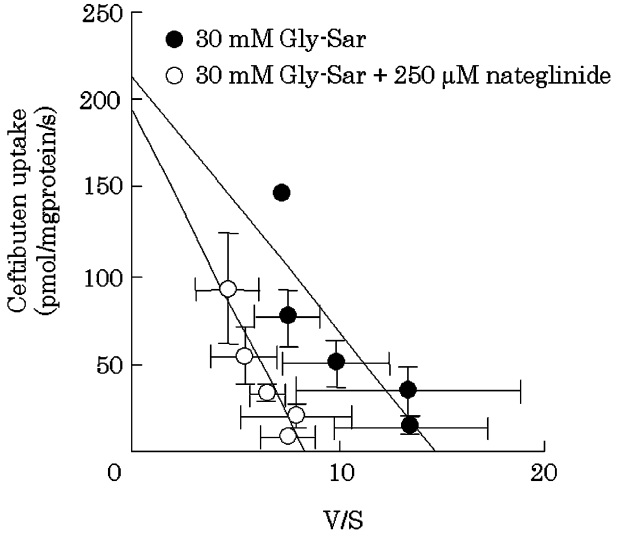

Fig. 2. Competitive inhibition by nateglinide of H^+-driven ceftibuten transporter-mediated ceftibuten uptake by rat renal BBMV. Uptake of increasing concentrations of ceftibuten by rat renal BBMV was measured for 20 s in the presence of 30 mM Gly-Sar alone or in the presence of 30 mM Gly-Sar and 250 μM nateglinide. Incubation conditions were identical to those described in the legend to Fig. 1. Each point represents the mean±S.D. of 3 determinations.

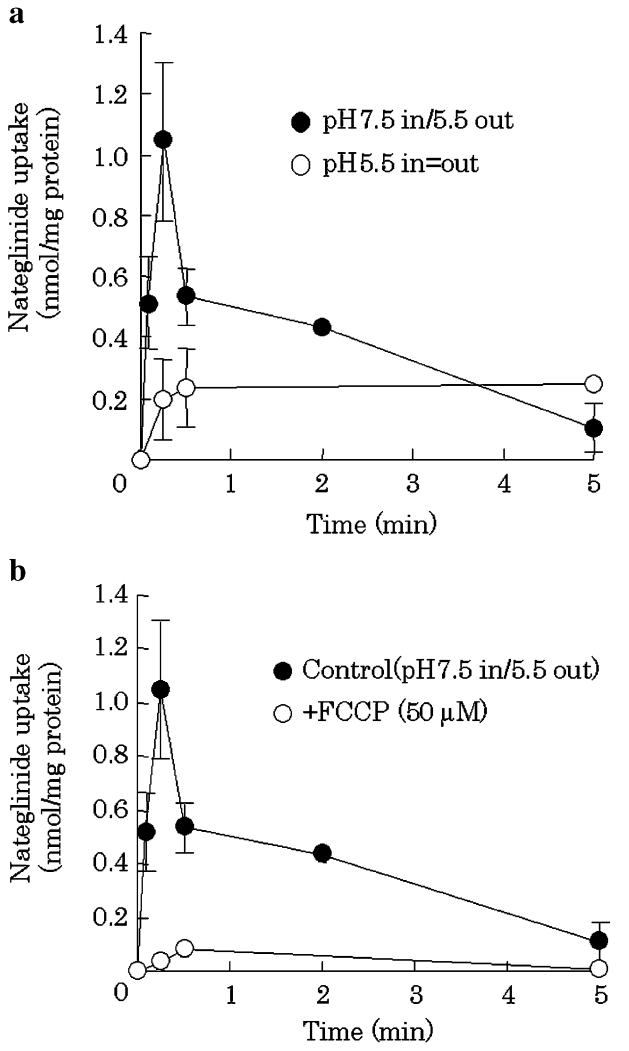

Fig. 3. (a) Stimulating effects of inward H^+-gradient on the uptake of nateglinide by rat renal BBMV. Uptake of 50 μM nateglinide by rat renal BBMV was measured. Each point represents the mean±S.D. of 3–6 determinations. (b) Effect of FCCP on the uptake of nateglinide by rat renal BBMV. Uptake of 50 μM nateglinide by rat renal BBMV was measured with or without (control) FCCP. Each point represents the mean±S.D. of 3–6 determinations.

gradient. Fig. 3b shows the effect of carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore, on the uptake of nateglinide by rat renal BBMV. The demonstration that abolishing the transmembrane H^+ gradient by FCCP caused the overshoot to disappear entirely.

3.3. Concentration dependence of nateglinide uptake

To characterize the uptake of nateglinide, the concentration dependence of the uptake of nateglinide was examined. The uptake of nateglinide was saturated at a higher nateglinide concentration (Fig. 4). The K_m and V_{max} values were determined by kinetic analysis to be 94.2 μM and 61.9 pmol/mg protein/s, respectively.

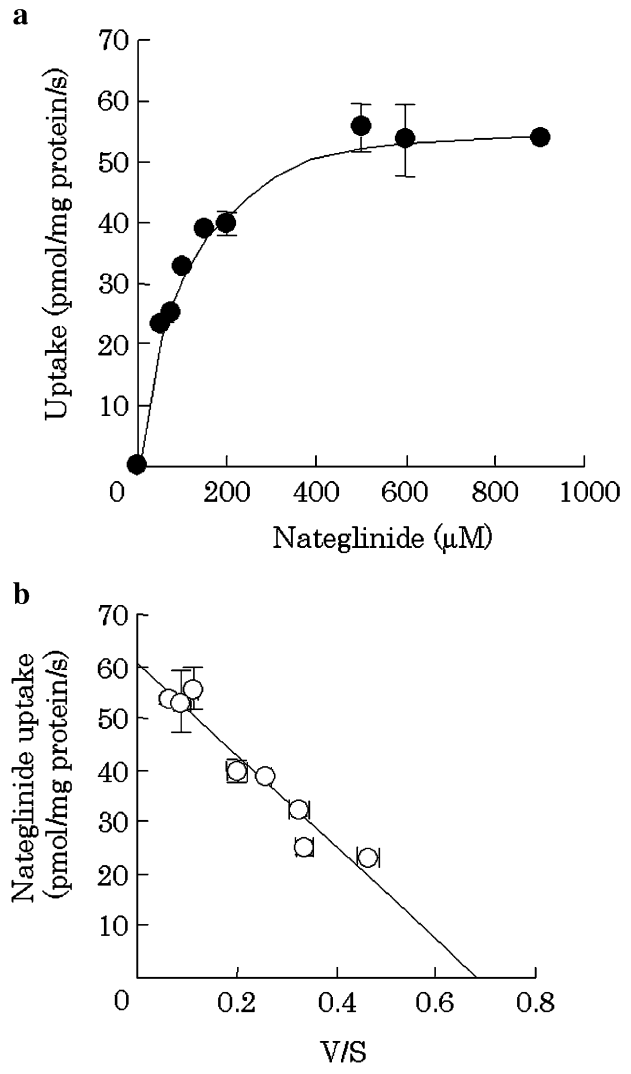

Fig. 4. (a) Concentration dependence of nateglinide initial uptake by rat renal BBMV. Uptake of increasing concentrations of nateglinide by rat renal BBMV was measured for 15 s. Uptake values in the absence of an inwardly directed H^+-gradient were subtracted from those in the presence of an inwardly directed H^+-gradient. Each point represents the mean±S.D. of 3 determinations. (b) Eadie–Hofstee plot analysis of nateglinide initial uptake by rat renal BBMV.

3.4. Inhibitory effect of ceftibuten on nateglinide uptake

To characterize the carrier that is responsible for the uptake of nateglinide in renal BBMV, we investigated the effect of ceftibuten on the uptake of nateglinide. As shown in Fig. 5, ceftibuten significantly reduced the uptake of nateglinide in a concentration dependent manner. On the other hand, Gly-Sar, a typical substrate of PepT [11,12], did not significantly inhibit the uptake of nateglinide. Cephradine and cephalexin, which are also substrates of PepT1, did not significantly inhibit the uptake of nateglinide. Results of Eadie–Hofstee plot analysis of nateglinide uptake in the presence of ceftibuten and an inwardly directed H^+ gradient are

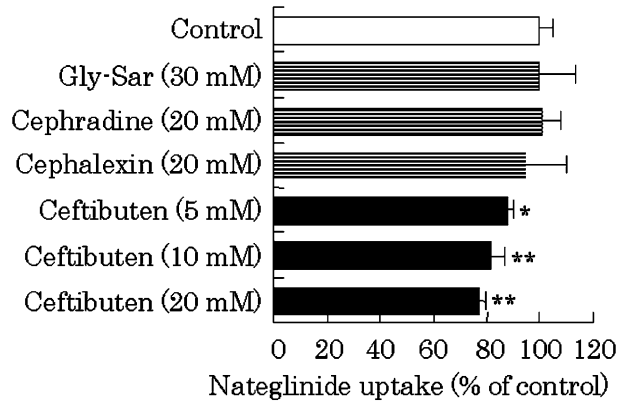

Fig. 5. Inhibitory effects of PepT substrates on the uptake of nateglinide by rat renal BBMV. Uptake of 50 μM nateglinide in the presence of an inwardly directed H^+-gradient by rat renal BBMV was measured for 15 s with or without (control) inhibitors. Each point represents the mean with S.D. of 3 determinations. *Significantly different from control at $P<0.05$, **$P<0.01$.

shown in Fig. 6. Ceftibuten was demonstrated to inhibit the uptake of nateglinide competitively.

4. Discussion

Since nateglinide is absorbed rapidly from the intestine, it is likely to be absorbed via a specific transporter [13,14]. It has been reported that nateglinide itself is not transported by PEPT1 or MCT1 [1,2]. Recently, we reported that nateglinide transport occurs via a single system that may be identical to the ceftibuten/H^+ cotransport system by the rat small intestine [3]. Ceftibuten is a third generation cephalosporin that shows good oral bioavailability [15] and Ceftibuten transport across Caco-2 cells is thus

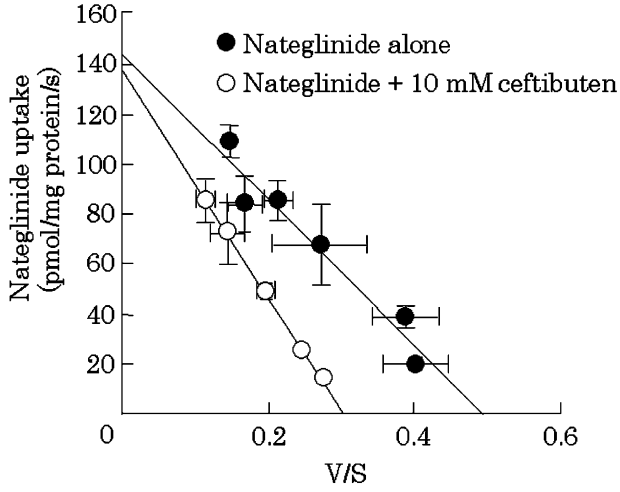

Fig. 6. Competitive inhibition by ceftibuten of the uptake of nateglinide by rat renal BBMV. Uptake of increasing concentrations of nateglinide in the presence of an inwardly directed H^+-gradient by rat renal BBMV was measured for 15 s in the presence or absence (control) of 10 mM ceftibuten. Each point represents the mean±S.D. of 3 determinations.

mediated by the pH and energy dependent PEPT1 [16]. Naasani et al. previously showed that ceftibuten is transported in the rat renal BBMV via at least two H^+-driven transport systems: PepT and H^+-driven transporter [10]. In the present study, we examined the contribution of a ceftibuten transporter to the uptake of nateglinide by rat renal BBMV. In the first part of this study, we investigated the kinetics of the inhibitory effect of Gly-Sar, a substrate of PepT, on ceftibuten uptake. Gly-Sar inhibited concentration-dependence of ceftibuten uptake in a competitive manner and revealed monocomponent: ceftibuten/H^+ cotransport system (data not shown). To clarify whether nateglinide is transported via the H^+-driven ceftibuten transporter, we examined the inhibitory effect of nateglinide on the H^+-driven ceftibuten transporter-mediated ceftibuten uptake. Nateglinide significantly reduced the uptake of ceftibuten via the H^+-driven ceftibuten transporter. This result suggests that a ceftibuten transporter may contribute to the uptake of nateglinide. Moreover, the results shown Fig. 2 suggest that ceftibuten and nateglinide share the same transport system at renal BBMV.

To confirm the function of the nateglinide transport system in rat renal BBMV, we investigated the effect of an inwardly directed H^+ gradient on the uptake of nateglinide. The nateglinide uptake exhibited an overshoot in the presence of an H^+ gradient. The initial uptake of nateglinide was saturable at higher concentrations. Moreover, FCCP, a protonophore, inhibited the H^+-dependent uptake of nateglinide by rat renal BBMV. These results suggest that nateglinide is transported across the rat renal brush-border membrane via an H^+-coupled transport system.

To confirm the contribution of the ceftibuten/H^+ cotransport system to the uptake of nateglinide in the renal brush-border membrane, we examined the opposite inhibitory effect of ceftibuten on the uptake of nateglinide. Ceftibuten significantly reduced the uptake of nateglinide in a competitive manner. These results further support the hypothesis that ceftibuten and nateglinide share the same transport system at the renal brush-border membrane. Although the estimated K_i value of ceftibuten for nateglinide uptake (17.9 mM) is not similar to the estimated the Km value for saturable uptake of nateglinide (94.2 μM), we think that the difference of affinity may derive from the contribution of H^+/ceftibuten transporter. On the other hand, although we examined trans-stimulation effect of ceftibuten on the uptake of nateglinide and vice versa, trans-stimulation was not observed. These results may indicate that yet unknown transporter or H^+-dependent transporter (i. e. monocarboxylate transporter) other than ceftibuten transporter is involved in nateglinide uptake. This transport system has not yet been elucidated at the molecular level. Further investigations in progress are to elucidate at the molecular level and to clarify the difference of the affinity.

In contrast, Gly-Sar, cephradine and cephalexin, which are also substrates of PepT [11,12], did not significantly inhibit the uptake of nateglinide. Moreover, p-aminohippurate (PAH), a substrate of OAT [17], had no effect on nateglinide uptake (data not shown). These findings indicate that nateglinide is not transported via PepT or OAT in the renal brush-border membrane.

Transporters present in the renal brush-border membranes mediate the reabsorption of many compounds and thereby influence the plasma levels of their substrates [18]. Classic and well-characterized drug substrates of PEPT1 and PEPT2 are the numerous amino beta-lactam antibiotics of the cephalosporin and penicillin classes [19], selected angiotensin-converting enzyme inhibitors such as captopril, and ester prodrugs such as enalapril and fosinopril [20,21]. Their interaction with PEPT1 in the gut epithelium provides very good oral availability (generally between 40% and 90% of a dose) of these drugs. The renal tubular peptide transporters may, by their ability for efficient reabsorption, contribute to the renal clearance of the drugs and thereby affect the pharmacokinetics. The present findings provide important information that will enable improvements in drug reabsorption or drug design by targeting the nateglinide/H^+ cotransport system. By utilizing the nateglinide/H^+ cotransport system as the target for drug delivery, it will be possible to avoid drug–drug interactions involving PepT.

In conclusion, ceftibuten transport occurs via at least two H^+-dependent transport systems: one is PepT and the other is the ceftibuten /H^+ transport system in the rat renal brush-border membrane. On the other hand, we have demonstrated that nateglinide transport occurs via a single system that is H^+-dependent but is distinct from PepT and may be identical to the ceftibuten/H^+ cotransport system in the rat renal brush-border membrane.

References

[1] T. Terada, K. Sawada, H. Saito, Y. Hashimoto, K. Inui, Inhibitory effect of novel oral hypoglycemic agent nateglinide (AY4166) on peptide transporters PEPT1 and PEPT2, Eur. J. Pharmacol. 392 (2000) 11–17.

[2] A. Okamura, A. Emoto, N. Koyabu, H. Ohtani, Y. Sawada, Transport and uptake of nateglinide in Caco-2 cells and its inhibitory effect on human monocarboxylate transporter MCT1, Br. J. Pharmacol. 137 (2002) 391–399.

[3] S. Itagaki, Y. Saito, S. Kubo, Y. Otsuka, Y. Yamamoto, M. Kobayashi, T. Hirano, K. Iseki, H+-dependent transport mechanism of nateglinide in the brush-border membrane of the rat intestine, J. Pharmacol. Exp. Ther. 312 (2005) 77–82.

[4] Y. Uwai, H. Saito, Y. Hashimoto, K. Inui, Inhibitory effect of anti-diabetic agents on rat organic anion transporter rOAT1, Eur. J. Pharmacol. 398 (2000) 193–197.

[5] T. Itoh, S. Itagaki, K. Sasaki, T. Hirano, I. Takemoto, K. Iseki, Pharmacokinetic modulation of irinotecan metabolites by sulphobromophthaleinx in rats, J. Pharm. Pharmacol. 56 (2004) 809–812.

[6] C. Evers, W. Haase, H. Murer, R. Kinne, Properties of brush border vesicles isolated from rat kidney cortex by calcium precipitation, Membr. Biochem. 1 (1978) 203–219.

[7] S. Itagaki, S. Shimamoto, T. Hirano, K. Iseki, M. Sugawara, S. Nishimura, M. Fujimoto, M. Kobayashi, K. Miyazaki, Comparison of urinary excretion of phenolsulfonphthalein in an animal model for Wilson's disease (Long-Evans Cinnamon rats) with that in normal

Wistar rats: involvement of primary active organic anion transporter, J. Pharm. Pharm. Sci. 7 (2004) 227–234.
[8] S. Itagaki, Y. Otsuka, S. Kubo, H. Okumura, Y. Saito, M. Kobayashi, T. Hirano, K. Iseki, Intestinal uptake of nateglinide by an intestinal fluorescein transporter, Biochim. Biophys. Acta 1668 (2005) 190–194.
[9] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[10] I. Naasani, K. Sato, K. Iseki, M. Sugawara, M. Kobayashi, K. Miyazaki, Comparison of the transport characteristics of ceftibuten in rat renal and intestinal brush-border membranes, Biochim. Biophys. Acta 1231 (1995) 163–168.
[11] M.E. Ganapathy, M. Brandsch, P.D. Prasad, V. Ganapathy, F.H. Leibach, Differential recognition of beta-lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2, J. Biol. Chem. 270 (1995) 25672–25677.
[12] X.Y. Chu, G.P. Sanchez-Castano, K. Higaki, D.M. Oh, C.P. Hsu, G.L. Amidon, Correlation between epithelial cell permeability of cephalexin and expression of intestinal oligopeptide transporter, J. Pharmacol. Exp. Ther. 299 (2001) 575–582.
[13] Y. Sato, M. Nishikawa, H. Shinkai, E. Sukegawa, Possibility of ideal blood glucose control by a new oral hypoglycemic agent, N-[(trans-4-isopropylcyclohexyl)-carbonyl]-D-phenylalanine (A-4166), and its stimulatory effect on insulin secretion in animals, Diabetes Res. Clin. Pract. 12 (1991) 53–59.
[14] T. Ikenoue, M. Akiyoshi, S. Fujitani, K. Okazaki, N. Kondo, T. Maki, Hypoglycaemic and insulinotropic effects of a novel oral antidiabetic agent, (−)-N-(trans-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (A-4166), Br. J. Pharmacol. 120 (1997) 137–145.
[15] W.H. Barr, C.C. Lin, E. Radwanski, J. Lim, S. Symchowicz, M. Affrime, The pharmacokinetics of ceftibuten in humans, Diagn. Microbiol. Infect. Dis. 14 (1991) 93–100.
[16] R.M. Menon, W.H. Barr, Transporters involved in apical and basolateral uptake of ceftibuten into Caco-2 cells, Biopharm. Drug Dispos. 23 (2002) 317–326.
[17] T. Sekine, S.H. Cha, H. Endou, The multispecific organic anion transporter (OAT) family, Pflugers Arch. 440 (2000) 337–350.
[18] M.J. Dresser, M.K. Leabman, K.M. Giacomini, Transporters involved in the elimination of drugs in the kidney: organic anion transporters and organic cation transporters, J. Pharm. Sci. 90 (2001) 397–421.
[19] A.H. Dantzig, Oral absorption of beta-lactams by intestinal peptide transport proteins, Adv. Drug Deliv. Rev. 23 (1997) 63–76.
[20] C. Shu, H. Shen, U. Hopfer, D.E. Smith, Mechanism of intestinal absorption and renal reabsorption of an orally active ace inhibitor: uptake and transport of fosinopril in cell cultures, Drug Metab. Dispos. 29 (2001) 1307–1315.
[21] T. Zhu, X.Z. Chen, A. Steel, M.A. Hediger, D.E. Smith, Differential recognition of ACE inhibitors in *Xenopus laevis* oocytes expressing rat PEPT1 and PEPT2, Pharm. Res. 17 (2000) 526–532.