ELSEVIER

Biochimica et Biophysica Acta 1324 (1997) 296–308

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Interaction of anionic cephalosporins with the intestinal and renal peptide transporters PEPT 1 and PEPT 2

Malliga E. Ganapathy [a,*], Puttur D. Prasad [b], Bryan Mackenzie [b,1], Vadivel Ganapathy [b], Frederick H. Leibach [b]

[a] *Department of Medicine, Medical College of Georgia, Augusta, GA 30912, USA*
[b] *Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA 30912, USA*

Received 11 October 1996; revised 8 November 1996; accepted 8 November 1996

Abstract

The present study was undertaken to investigate the interaction of anionic cephalosporins (cefixime, ceftibuten, and cefdinir) with the renal peptide transporter (PEPT 2) and the intestinal peptide transporter (PEPT 1) using four different experimental model systems. In the first approach, the human colon carcinoma cell line Caco-2 which expresses PEPT 1 and the SHR rat kidney cell line SKPT which expresses PEPT 2 were used. The uptake of the dipeptide Gly-Sar mediated by PEPT 1 or PEPT 2 in these cells was inhibited significantly by the anionic cephalosporins, with the following order of potency: ceftibuten > cefixime > cefdinir. The inhibition was competitive in nature. Even though the order of potency was the same for PEPT 1 and PEPT 2, PEPT 1 exhibited much lesser sensitivity to inhibition than PEPT 2. In the second approach, the cloned human PEPT 1 and PEPT 2 were functionally expressed in HeLa cells following which the cells were used to study the interaction of anionic cephalosporins with PEPT 1 and PEPT 2. Again, Gly-Sar uptake mediated by the human PEPT 1 and PEPT 2 in HeLa cells was found to be inhibited by the anionic cephalosporins with the same order potency as in Caco-2 and SKPT cells. In the third approach, brush border membrane vesicles isolated from rat kidneys were employed. In this approach also it was found that PEPT 2-mediated Gly-Sar uptake was inhibited by cefixime and ceftibuten. In the fourth approach, the human PEPT 1 was expressed in *Xenopus laevis* oocytes and PEPT 1-mediated transport of ceftibuten was investigated directly by electrophysiological methods. Ceftibuten evoked inward currents in PEPT 1-expressing oocytes but not in water-injected oocytes, showing that the transport of the anionic cephalosporin via PEPT 1 is associated with transfer of positive charge. The ceftibuten-evoked currents were saturable with respect to ceftibuten concentration and were markedly dependent on membrane potential. It is concluded that anionic cephalosporins interact with the peptide transporters expressed in the intestine (PEPT 1) as well as in the kidney (PEPT 2).

Keywords: Cephalosporin; Peptide transporter; Intestine; Kidney; Caco-2 cell; SKPT cell

1. Introduction

The peptide transporters expressed in the brush border membrane of the intestinal and renal epithelial cells are responsible for the absorption of small peptides which consist of two or three amino acids [1–5].

* Corresponding author. Fax: +1 (706) 7212000.
[1] Present address: Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1751, USA.

PII S0005-2736(96)00234-9

These transporters are energy-dependent, driven by a transmembrane H^+ gradient, and catalyze the co-transport of the peptide substrates with H^+ [6–8]. In addition to the endogenously occurring dipeptides and tripeptides, several pharmacologically active exogenous compounds which possess peptide-like chemical structures are also accepted as substrates by the intestinal and renal peptide transporters [9–11]. These compounds include the β-lactam antibiotics, cephalosporins and penicillins, angiotensin-converting enzyme inhibitors, renin inhibitors, and the antineoplastic agent bestatin. The peptide transporters are important determinants of the therapeutic efficacy of these peptidomimetic drugs by influencing their bioavailability and circulatory half-life.

Among the cephalosporin antibiotics, some are zwitterionic (e.g., cephalexin, cefadroxil, and cefaclor) while some are anionic (e.g., cefixime, ceftibuten, and cefdinir). It has been well established that zwitterionic cephalosporins are effectively transported by intestinal as well as renal peptide transporters [12–16]. However, it is not clear whether anionic cephalosporins are effective substrates for both of these peptide transporters. Studies from several laboratories have shown that the anionic cephalosporins cefixime, cefdinir and ceftibuten are transported by the intestinal peptide transport system [17–25]. But there is controversy regarding the interaction of these anionic cephalosporins with the renal peptide transport system. Tamai et al. [26] have demonstrated, with isolated renal brush border membrane vesicles, that cefixime is not recognized as a substrate by the renal system, whereas Naasani et al. [27–29] have shown, using the same experimental approach, that ceftibuten is a substrate for the renal system. Cefixime and ceftibuten are structurally very similar and both are dianionic. Therefore, it is surprising that the studies by Tamai et al. and by Naasani et al. have led to different conclusions with regard to whether or not anionic cephalosporins are substrates for the renal peptide transport system. It has to be pointed out here that the peptide transporters are not the only transport systems available for the absorption of anionic cephalosporins in the intestine and the kidney. The monocarboxylate transporter present in the intestine has been shown to participate in the absorption of the monoanionic cephalosporin cefdinir [20]. However, this particular system is most likely restricted to monoanionic cephalosprins. Dianionic cephalosporins such as cefixime and ceftibuten are not likely to be effective substrates for the monocarboxylate transporter.

The purpose of the present investigation was to study the interaction of anionic cephalosporins with the intestinal and renal peptide transporters using four different approaches. In the first approach, cultured cell lines of intestinal and renal origin which constitutively express H^+-coupled peptide transport activity were used. Caco-2 cells are of human intestinal origin and are known to possess a low affinity H^+/peptide cotransport system (PEPT 1) [30]. SKPT cells are of rat renal origin and are known to possess a high affinity H^+/peptide cotransport system (PEPT 2) [31]. In the second approach, cloned human intestinal and renal H^+/peptide cotransporters, namely PEPT 1 [32] and PEPT 2 [33] respectively, were functionally expressed in HeLa cells which were then used in the described studies. In the third approach, brush border membrane vesicles isolated from rat kidneys were employed. In each of these approaches, peptide transport activity was measured by the uptake of the dipeptide glycylsarcosine (Gly-Sar) in the presence of an inwardly directed H^+ gradient and the interaction of anionic cephalosporins with the peptide transporters was investigated by analyzing the ability of these compounds to compete with Gly-Sar for the uptake process. In the fourth approach, the cloned human PEPT 1 was functionally expressed in *Xenopus laevis* oocytes and the PEPT 1-mediated transport of the anionic cephalosporin ceftibuten was directly investigated by electrophysiological approaches. The results of the investigation clearly show that anionic cephalosporins are transported by intestinal as well as renal peptide transport systems.

2. Materials and methods

2.1. Materials

[2-^{14}C]Glycyl [1-^{14}C]sarcosine (specific radioactivity, 109 mCi/mmol) was custom synthesized by Cambridge Research Biochemicals (Cleveland, UK). Cell culture media were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). Fetal bovine serum, dexamethasone and apotransferrin were ob-

tained from Sigma (St. Louis, MO, USA). Cefixime, ceftibuten and cefdinir were generous gifts from Dr. Akira Tsuji (Kanazawa University, Kanazawa, Japan). The rat renal proximal tabular cell line SKPT, originating from SHR rat kidney, was provided by Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH, USA). The human colon carcinoma cell line Caco-2 and the human cervical carcinoma cell line HeLa were obtained from the American type Culture Collection. All other chemicals were of analytical grade.

2.2. Cell culture and uptake measurements

SKPT cells and Caco-2 cells were cultured in Dulbecco's Modified Eagles's/F-12 (1:1) medium and in minimal essential medium, respectively, as described previously [30,31]. Uptake of [^{14}C]Gly-Sar in cells was measured with the uptake buffer whose composition was 25 mM 4-morpholineethanesulfonic acid (Mes)/Tris (pH 6.0), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose [30,31]. The culture medium was first aspirated from the dish and the cell monolayer was washed once with 1 ml of the uptake buffer. Uptake was initiated by the addition of 1 ml of the uptake buffer containing radiolabeled substrate. Incubation was continued for 10 min, after which uptake was terminated by the removal of the medium followed by three times washing with ice-cold uptake buffer. The cells were then solubilized with 1 ml of 0.2 M NaOH, 1% SDS and the contents were transferred to a counting vial for determination of radioactivity.

2.3. Vaccinia virus expression of PEPT 1 and PEPT 2

This was done using the procedure described earlier [32,33]. Subconfluent HeLa cells in 24-well culture plates were first infected with a recombinant vaccinia virus $VTF_{7\text{-}3}$ which carries the gene for T7 RNA polymerase as a part of its genome. This enables the HeLa cells to express T7 RNA polymerase. Following the infection, the cells were transfected with pBluescript-PEPT 1 cDNA construct or with pBluescript-PEPT 2 cDNA construct. In these constructs, the cDNAs were under control of T7 promoter in the plasmid. Cells transfected with empty plasmid served as control. Transfection was mediated by lipofection. The virus-encoded T7 RNA polymerase catalyzes the transcription of the cDNA, allowing transient expression of the PEPT 1 or PEPT 2 protein in the HeLa cell plasma membrane. After 12 h post-infection, transport measurements were made at room temperature with [^{14}C]Gly-Sar. The uptake medium was 25 mM Mes/Tris (pH 6.0) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. The time of incubation for uptake measurements was 3 min. At the end of the incubation, the uptake was terminated by removal of the uptake medium by aspiration followed by washing two times with ice-cold uptake medium which did not contain radiolabeled substrate. The cells were then solubilized with 0.5 ml of 1% SDS, transferred to counting vials, and used for determination of radioactivity.

2.4. Preparation of renal brush-border membrane vesicles

Brush-border membrane vesicles were prepared from kidneys by the Mg^{2+}-aggregation method in the presence of [ethylene *bis*(oxyethylenenitrilo)] tetraacetic acid (EGTA) as described previously [34]. Kidneys were obtained from two different rat strains, Sprague-Dawley and SHR. The renal tissue comprising the cortical and outer medullary regions was used for the isolation of membrane vesicles because we have shown earlier that both regions possess peptide transport activity [35]. The tissue was homogenized in 10 volumes of homogenizing buffer (12 mM Tris/NaOH, 5 mM EGTA, 300 mM mannitol, pH 7.5) for 90 s using an Ultra Turrax, with the speed set at 50. The resulting homogenate was diluted with an equal volume of ice-cold water. A stock solution of 1 M $MgCl_2$ was added to the above suspension to give a final concentration of 10 mM $MgCl_2$. The mixture was stirred for 1 min and allowed to stand for 15 min. The suspension was then centrifuged at $3000 \times g$ for 10 min. The pellet was discarded, and the supernatant was centrifuged again at $60\,000 \times g$ for 30 min. The pellets containing brush-border membranes were washed twice with the preloading buffer by dilution and centrifugation. The preloading buffer was 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 75 mM Tris (pH 8.3), contain-

ing 100 mM K_2SO_4. A 25-gauge needle was used to suspend the pellets. The protein concentration of the final membrane preparation was adjusted to 6 mg/ml, and it was stored in liquid N_2 in small aliquots until use.

2.5. Uptake measurements in membrane vesicles

Uptake of [^{14}C]Gly-Sar was measured at room temperature (22–23°C) as described earlier [36] using a rapid filtration technique. Millipore membrane filters (DAWP type; 0.65 μm pore size) were used. Membrane vesicles were incubated with 50 μM valinomycin prior to use in uptake measurements. Uptake was initiated by mixing 40 μl of the membrane suspension with 160 μl of uptake buffer (50 mM Hepes, 50 mM Mes, 25 mM Tris, 300 mM mannitol, pH 6.0) containing radiolabeled peptide substrate. All uptake measurements were carried out with 10-s incubations. Uptake was terminated by the addition of 3 ml of ice-cold stop buffer (2 mM Hepes/Tris, 210 mM KCl, pH 7.5). The mixture was filtered and the filter retaining the membrane vesicles was washed with 3×5 ml of the stop buffer. The washed filter was transferred to a counting vial, and the radioactivity associated with the filter was counted by liquid scintillation spectrometry.

2.6. Oocyte expression and electrophysiological measurements

The procedures for preparation of oocytes from *Xenopus laevis*, synthesis of human PEPT 1 cRNA, and microinjection of cRNA into the oocytes have been described previously [37,38]. A two-microelectrode voltage-clamp system [39] was used to measure steady-state currents associated with PEPT 1-mediated transport at pH 6.0. Step changes in membrane potential were applied, each for a duration of 100 ms ($+50$ mV to -130 mV in 20 mV increments). The value for $K_{0.5}$, the substrate concentration at which current is half-maximal, was determined by measuring the currents over a ceftibuten concentration range of 0.05–5 mM and fitting the data to Michaelis-Menten equation describing a single transport system.

2.7. Data analysis

Experiments were done in duplicate or triplicate and each experiment was repeated two to three times. Results are given as means $\pm$ S.E. The kinetic parameters, the Michaelis-Menten constant, K_t, and the maximal velocity, V_{max}, were calculated by linear regression of the Eadie-Hofstee plot and confirmed by nonlinear regression methods using the *Fig. P*, version 6.0, computer program.

3. Results and discussion

3.1. Studies with cultured cells which differentially express PEPT 1 or PEPT 2

Recent molecular cloning studies have established that the H^+-coupled peptide transporters expressed in the intestine and kidney are structurally different, encoded by different genes [32,33,37,40–43]. The cloned human intestinal peptide transporter (PEPT 1) and the human kidney peptide transporter (PEPT 2) exhibit only 50% homology in amino acid sequence. PEPT 1 is expressed primarily in the intestine and, to a much lesser extent, in the kidney. PEPT 2 is expressed in the kidney and other tissues such as the brain, lung, liver and heart but not in the intestine [33,43]. Even though both of these transporters catalyze the transport of small peptides in a H^+ gradient-dependent manner, there are important differences between these transporters in substrate affinity [40,43,44] as well as in substrate specificity [44]. In particular, β-lactam antibiotics are differentially recognized by PEPT 1 and PEPT 2 [44]. We have identified recently two cultured cell lines, one expressing PEPT 1 and not PEPT 2 and the other expressing PEPT 2 and not PEPT 1 [44]. These are the human colon carcinoma cell line Caco-2 (PEPT 1) and the SHR rat kidney proximal tabular cell line SKPT (PEPT 2). These cell lines provide excellent model systems to differentially study the substrate selectivity of PEPT 1 and PEPT 2. In the present study, we have used these two cell lines to investigate the interaction of anionic cephalosporins with PEPT 1 and PEPT 2. These studies were done with two dianionic cephalosporins (cefixime and ceftibuten) and one monoanionic cephalosporin (cefdinir). The cells were cultured as confluent monolayers and the activity of the H^+-coupled peptide transporters was measured by determining the uptake of the dipeptide Gly-Sar in the presence of an inwardly directed H^+

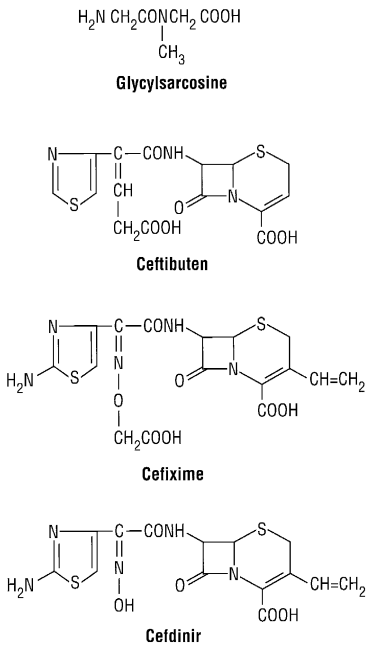

Fig. 1. Chemical structures of Gly-Sar and the anionic cephalosporins ceftibuten, cefixime, and cefdinir.

gradient. This was done by measuring the uptake from an extracellular medium of pH 6.0. Gly-Sar was chosen as the peptide substrate because of its resistance to hydrolysis by membrane-bound peptidases. The chemical structures of these antibiotics are given in Fig. 1 for comparison with the chemical structure of Gly-Sar. Measurements of Gly-Sar uptake in Caco-2 cells and in SKPT cells were made using an incubation time of 10 min. The uptake was found to be linear over this incubation period (Fig. 2). We first investigated the influence of increasing concentrations of the anionic cephalosporins on the uptake of Gly-Sar in these cells (Fig. 3). The activity of the peptide transporters measured at 5 μM Gly-Sar in the absence of cephalosporins was 187.4 ± 6.1 pmol/mg of protein/10 min in Caco-2 cells (PEPT 1) and 121.4 ± 1.2 pmol/mg of protein/10 min in SKPT cells (PEPT 2). All three anionic cephalosporins inhibited the peptide transporter activity in both cell lines to a significant extent. In the case of PEPT 1, the inhibitory potency was in the following order: ceftibuten > cefixime > cefdinir. The IC_{50} values (i.e., the concentration necessary to cause 50% inhibition) for these cephalosporins were 0.9 ± 0.1 mM, ~ 10 mM, and > 10 mM respectively. In the case of

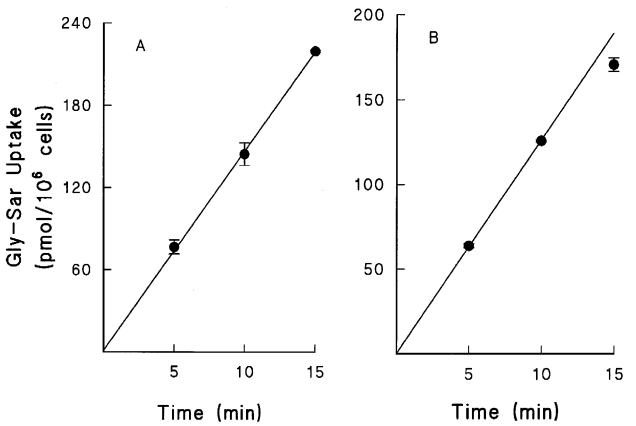

Fig. 2. Time course of Gly-Sar uptake in Caco-2 cells (A) and in SKPT cells (B) at a Gly-Sar concentration of 5 μM.

PEPT 2, the order of the inhibitory potency was the same as in the case of PEPT 1, i.e., ceftibuten > cefixime > cefdinir. The IC_{50} values were 0.48 ± 0.03 mM, 3.2 ± 0.1 mM, and ~ 10 mM respectively. Even though the order of the inhibitory potency was the same, PEPT 2 was comparatively more sensitive to inhibition by the anionic cephalosporins than PEPT 1. These findings are in agreement with the earlier observations that PEPT 1 is a low-affinity transporter, whereas PEPT 2 is a high-affinity transporter [32,40,43]. The most important finding from these

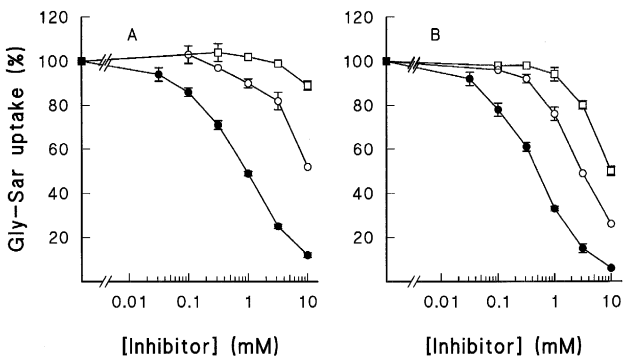

Fig. 3. Inhibition of [^{14}C]Gly-Sar uptake by anionic cephalosporins in Caco-2 cells (A) and SKPT cells (B). Uptake of 5 μM [^{14}C]Gly-Sar was measured for 10 min in monolayer cultures of Caco-2 cells and SKPT cells at pH 6.0 in the absence and presence of increasing concentrations of anionic cephalosporins. Uptake of Gly-Sar measured in the absence of the inhibitors was taken as 100%. This value was 187.4 ± 6.1 pmol/mg of protein/10 min for Caco-2 cells and 121.4 ± 1.2 pmol/mg of protein/10 min for SKPT cells. Key: ●, ceftibuten; ○, cefixime; □, cefdinir.

experiments is, however, that the anionic cephalosporins interact with the kidney-specific peptide transporter PEPT 2. This is in contrast to the report by Tamai et al. [26] but in agreement with the reports by Naasani et al. [27–29].

There is no controversy with regard to the recognition of the anionic cephalosporins as substrates by the intestinal peptide transport system. Our results with Caco-2 cells also clearly show that cefixime and ceftibuten interact with PEPT 1. Several earlier studies, with intestinal brush border membrane vesicles as well as with Caco-2 cells, have already demonstrated that the anionic cephalosporins interact with the intestinal peptide transporter in a competitive manner with respect to dipeptide substrates [17,18,21,22]. In the present study, we investigated the kinetic nature of the inhibition of Gly-Sar transport mediated by the kidney-specific peptide transporter PEPT 2 by two of the anionic cephalosporins, namely cefixime and ceftibuten. Gly-Sar uptake was measured over the concentration range of 25–500 μM in the absence or in the presence of the cephalosporins. The concentrations of the cephalosporins chosen for these studies were approximately equal to the IC_{50} values (ceftibuten, 0.5 mM; cefixime, 3 mM). Fig. 4A describes the relationship between the uptake rate and Gly-Sar concentration (v versus [S]). The values for v given in Fig. 4A represent the rates of total uptake and have not been corrected for the nonsaturable diffusional component. Analysis of the kinetic parameters of the uptake process was, however, done only for the saturable component. For this purpose, the diffusional component of Gly-Sar uptake was determined by measuring the uptake in the presence of excess Gly-Pro (10 mM), a competing substrate for the peptide transport process. The diffusional component represented 4% of total uptake at 25 μM Gly-Sar and 18% of total uptake at 500 μM Gly-Sar. Fig. 4B describes the data for saturable component given as Eadie-Hofstee plots. For control Gly-Sar uptake as well as for Gly-Sar uptake measured in the presence of ceftibuten or cefixime, the Eadie-Hofstee plots were linear ($r^2 > 0.98$), indicating participation of a single transport system in Gly-Sar uptake. In the absence of the cephalosporins, the Michaelis-Menten constant, K_t, for Gly-Sar was 74.5 ± 2.9 μM and the maximal velocity, V_{max}, was 1.77 ± 0.02 nmol/mg of protein/10 min. These values agree well with the

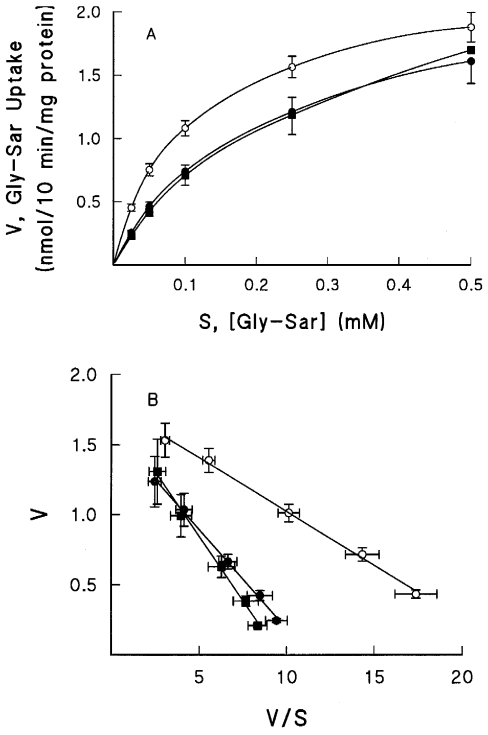

Fig. 4. Kinetics of inhibition of Gly-Sar uptake by cefixime and ceftibuten in SKPT cells. Uptake of Gly-Sar was measured in monolayer cultures of SKPT cells in the absence (○) or presence of 3 mM cefixime (●) or 0.5 mM ceftibuten (■). The uptake was measured at pH 6.0 with a 10-min incubation period. Concentration of Gly-Sar was varied between 25 and 500 μM, keeping the concentration of [^{14}C]Gly-Sar constant at 5 μM and adding unlabeled Gly-Sar to desired concentrations. Non-mediated component was determined from the uptake of radiolabel measured in the presence of 10 mM Gly-Pro. This component was subtracted from total uptake to calculate mediated uptake which was used in kinetic analysis. Results are given as uptake rate versus Gly-Sar concentration (A) and as Eadie-Hofstee plots (B) For v versus [s] plots, uptake rates without correction for the diffusional component were used and for Eadie-Hofstee plots, uptake rates after correction for the diffusional component were used.

previously reported values [31,44]. The corresponding values in the presence of the anionic cephalosporins were 135.3 ± 3.7 μM and 1.58 ± 0.02 nmol/mg of protein/10 min in the case of cefixime and 188.0 ± 11.6 μM and 1.78 ± 0.05 nmol/mg of protein/10 min in the case of ceftibuten. In both cases, the presence of the cephalosporins increased the K_t value for Gly-Sar by about 2-fold. The V_{max} remained unaffected. These results show that cefixime and ceftibuten inhibit the PEPT 2-mediated

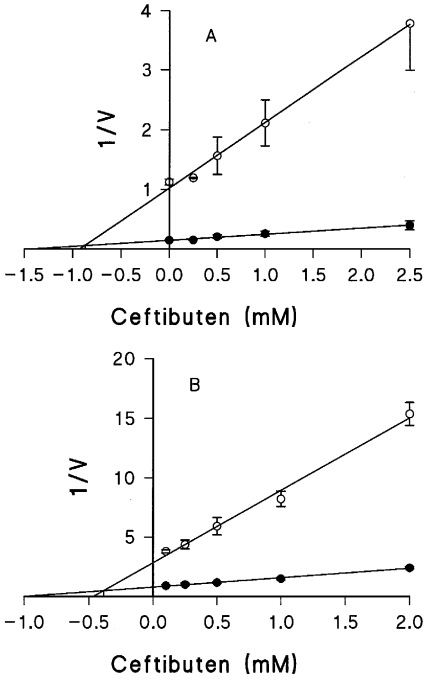

Fig. 5. Determination of inhibition constants by Dixon plots. Uptake of Gly-Sar was measured in monolayer cultures of Caco-2 cells (A) and SKPT cells (B) with a 10 min incubation. For Caco-2 cells, uptake was measured at Gly-Sar concentration of 50 μM (○) and 500 μM (●) in the presence of increasing concentrations of ceftibuten (0–2.5 mM). For SKPT cells, uptake was measured at Gly-Sar concentrations of 10 μM (○) and 70 μM (●) in the presence of increasing concentrations of ceftibuten (0.1–2 mM). The diffusional component, measured in the presence of excess amount of Gly-Pro (50 mM for Caco-2 cells and 10 mM for SKPT cells), was subtracted from total uptake to calculate mediated uptake. V, (nmol/mg of protein/10 min).

Gly-Sar uptake competitively. Thus, the interaction of the anionic cephalosporins with the renal peptide transporter (PEPT 2) is kinetically similar to that with the intestinal peptide transporter (PEPT 1).

We have also determined the inhibition constants (K_i) for ceftibuten, the most potent anionic cephalosporin to inhibit Gly-Sar uptake by PEPT1 and by PEPT2. This was done by measuring mediated uptake of Gly-Sar in Caco-2 cells and in SKPT cells at two different Gly-Sar concentrations (50 μM and 500 μM for Caco-2 cells; 10 μM and 70 μM for SKPT cells) in the presence of increasing concentrations of ceftibuten (0–2.5 mM). The results are given as Dixon plots (1/uptake rate versus inhibitor concentration). The plots were linear at both Gly-Sar concentrations in Caco-2 cells (Fig. 5A) as well as in SKPT cells (Fig. 5B), and the lines intersected above the abscissa in the fourth quadrant in both cells, as expected from the competitive nature of the inhibition of Gly-Sar uptake by ceftibuten. The K_i values were calculated from the points of intersection. The K_i value was 0.87 mM for Caco-2 cells and 0.38 mM for SKPT cells. These values are similar to be corresponding K_i values (0.89 mM and 0.45 mM, respectively) calculated from the IC_{50} data in Fig. 3 by the method of Cheng and Prusoff [45] using the K_t value of 1.1 mM for Caco-2 cells [30] and 75 μM for SKPT cells (present study).

3.2. Studies with the cloned human intestinal and renal peptide transporters, PEPT 1 and PEPT 2

The SKPT cell line which expresses PEPT 2 is of rat kidney origin. There is no human kidney cell line known to express PEPT 2. Even though the studies thus far described have shown that anionic cephalosporins interact with PEPT 2 present in the SKPT cells, it is important to know whether such interaction also occurs in the case of the human kidney peptide transporter. We have recently isolated the cDNA which codes for the human kidney-specific peptide transporter [33]. This cDNA can be functionally expressed in HeLa cells using the vaccinia virus expression system [33,40,44]. HeLa cells are ideal for these studies because there is no endogenous peptide transport activity in these cells. After functional expression of the human PEPT 2 cDNA in HeLa cells, the cells were used to study the interaction of anionic cephalosporins with PEPT 2 (Fig. 6). In control cells, transfected with pBluescript vector alone, the peptide transport activity measured as Gly-Sar uptake at a concentration of 50 μM was 17.3 ± 0.5 pmol/10^6 cells/3 min. This uptake was entirely due to nonsaturable diffusional process. The transport activity increased 4-fold to 65.4 ± 1.6 pmol/10^6 cells/3 min in cells transfected with pBluescript-PEPT 2 cDNA construct. The difference between these two values represented the PEPT 2-mediated activity. This PEPT 2-specific Gly-Sar uptake activity was found to be inhibited to a significant extent by anionic cephalosporins. At a concentration of 10 mM, the inhibition of Gly-Sar uptake caused by cefixime, ceftibuten, and cefdinir was 54%, 96% and 48% respectively (Fig. 6). These data clearly demonstrate

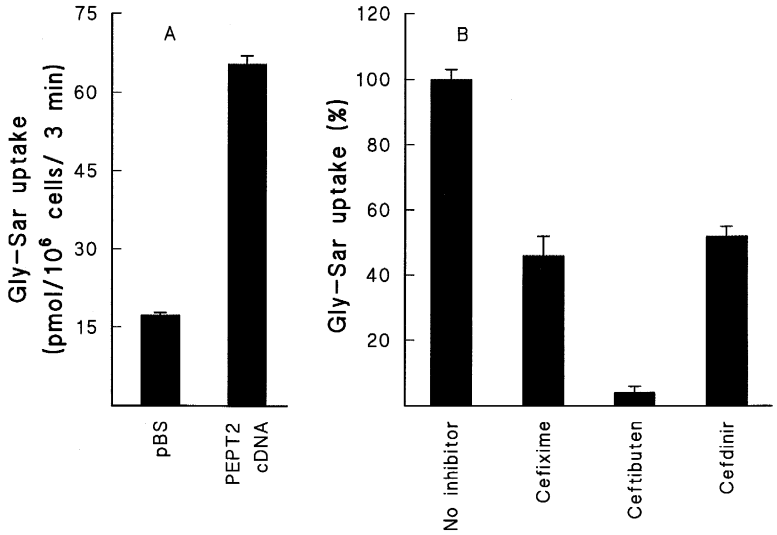

Fig. 6. Influence of anionic cephalosporins on Gly-Sar uptake mediated by the cloned human kidney peptide transporter PEPT 2. HeLa cells were transfected with either empty pBluescript alone (pBS) or with PEPT 2 cDNA. The cDNA was functionally expressed in these cells by the vaccinia virus expression system. Uptake of 50 μM Gly-Sar was measured in these cells at pH 6.0 with a 3-min incubation. (A) Gly-Sar uptake in control and in PEPT 2-expressing cells. (B) PEPT 2-mediated Gly-Sar uptake in the absence or presence of anionic cephalosporins (10 mM), given as percent of uptake measured in the absence of the inhibitors. The 100% value was 47.9 ± 1.3 pmol/10^6 cells/3 min.

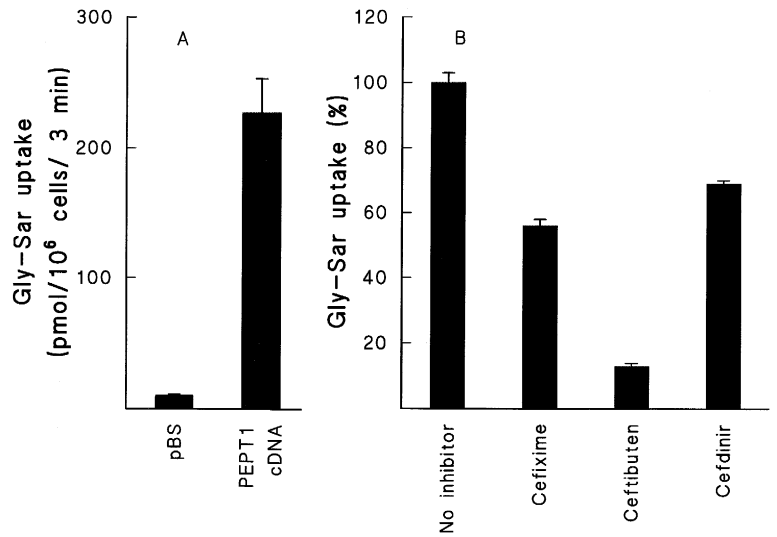

Fig. 7. Influence of anionic cephalosporins on Gly-Sar uptake mediated by the cloned human intestinal peptide transporter PEPT 1. HeLa cells were transfected with either empty pBluescript alone (pBS) or with PEPT 1 cDNA. The cDNA was functionally expressed in these cells by the vaccinia virus expression system. Uptake of 20 μM Gly-Sar was measured in these cells at pH 6.0 with a 3-min incubation. (A) Gly-Sar uptake in control and in PEPT 1-expressing cells. (B) PEPT 1-mediated Gly-Sar uptake in the absence or presence of anionic cephalosporins (10 mM), given as percent of uptake measured in the absence of the inhibitors. The 100% value was 217 ± 25 pmol/10^6 cells/3 min.

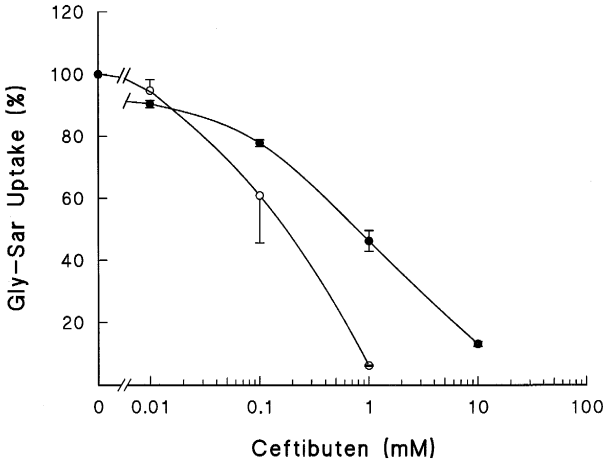

Fig. 8. Dose-response relationship for the inhibition of PEPT 1- and PEPT 2-mediated Gly-Sar uptake by ceftibuten. HeLa cells were transfected with either PEPT 1 cDNA (●) or PEPT 2 cDNA (○). The cDNAs were functionally expressed in these cells by the vaccinia virus expression system. Uptake of Gly-Sar was measured with a 3-min incubation. Concentration of Gly-Sar was 20 μM for PEPT 1 and 40 μM for PEPT 2. Concentration of ceftibuten was varied between 0 and 10 mM. Uptake measured in cells transfected with empty pBluescript was subtracted from uptake measured in cDNA-transfected cells to determine cDNA-specific uptake. Results are given as percent of uptake measured in the absence of ceftibuten. The 100% value was 144 ± 10 pmol/10^6 cells/3 min for PEPT 1 and 31 ± 4 pmol/10^6 cells/3 min for PEPT 2.

that anionic cephalosporins interact with the human kidney peptide transporter PEPT 2.

We also studied the interaction of anionic cephalosporins with the human intestinal peptide transporter under similar conditions. The human PEPT 1 cDNA was functionally expressed in HeLa cells using the vaccinia virus expression system. Gly-Sar uptake measured at a concentration of 20 μM was 9.8 ± 1.4 pmol/10^6 cells/3 min in HeLa cells transfected with pBluescript vector alone. This value increased 23-fold to 227.3 ± 26.0 pmol/10^6 cells/3 min in cells transfected with pBluescript-PEPT 1 cDNA construct (Fig. 7). The PEPT 1-specific Gly-Sar uptake was found to be inhibited by 44%, 87%, and 31% in the presence of cefixime, ceftibuten, and cefdinir (10 mM each) respectively. Thus, anionic cephalosporins are recognized by human PEPT 1 and human PEPT 2.

Fig. 8 describes the dose-response relationship for the inhibition of PEPT 1- and PEPT 2-mediated Gly-Sar uptake by ceftibuten, the most potent inhibitor among the anionic cephalosporins tested in this study. The concentration of Gly-Sar was 20 μM for PEPT 1 and 40 μM for PEPT 2. The IC_{50} value for the inhibition was found to be 0.76 ± 0.13 mM for PEPT 1 and 0.14 ± 0.01 mM for PEPT 2. The corresponding K_i values were calculated from these IC_{50} values according to the method of Cheng and Prusoff [45]. The K_t value used in this calculation was 0.29 mM for PEPT 1 [32] and 74 μM for PEPT 2 [40]. These K_t values were determined under experimental conditions similar to those used in the present study. The K_i value for the inhibition of Gly-Sar uptake by ceftibuten was 0.71 ± 0.12 mM for PEPT 1 and 0.09 ± 0.01 mM for PEPT 2.

3.3. Studies with brush border membrane vesicles isolated from rat kidney

The studies by Tamai et al. [26] which showed no interaction of cefixime with the renal peptide transporter were done with brush border membrane vesicles isolated from Sprague-Dawley rat kidneys. The

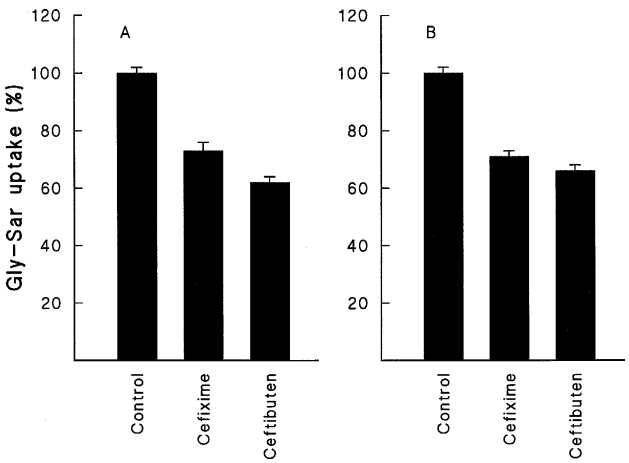

Fig. 9. Influence of cefixime and ceftibuten on Gly-Sar uptake in brush border membrane vesicles isolated from Sprague-Dawley (A) and SHR (B) rat kidneys. Uptake of Gly-Sar (30 μM) was measured in these vesicles in the presence of an inwardly directed H^+ gradient and an inside-negative K^+-diffusion potential with a 10-s incubation. When present, the concentration of cefixime was 10 mM and that of ceftibuten was 2.5 mM. Results are given as percent of uptake measured in the absence of inhibitors (control). The 100% value was 104.5 ± 1.6 pmol/mg of protein/10 s in Sprague-Dawley rats and 76.0 ± 1.3 pmol/mg of protein/10 s in SHR rats.

studies described thus far in this paper were done either with SKPT cells which originated from SHR rat kidney or with the cloned human PEPT 2. In order to find out whether species and/or animal strain differences might have contributed to the varying results obtained in these studies, we investigated the interaction of anionic cephalosporins with the renal peptide transporter using brush border membrane vesicles prepared from Sprague-Dawley rat kidneys and from SHR rat kidneys (Fig. 9). Gly-Sar uptake in these membrane vesicles were measured in the presence of an inwardly directed H^+ gradient and a valinomycin-induced inside-negative K^+-diffusion potential. The Gly-Sar uptake activity was slightly higher in Sprague-Dawley rat kidney membrane vesicles than in SHR rat kidney membrane vesicles. At a Gly-Sar concentration of 30 μM, the uptake activity was 104.5 ± 1.6 pmol/mg of protein/10 s in Sprague-Dawley rats and 76.0 ± 1.3 pmol/mg of protein/10 s in SHR rats. Cefixime and ceftibuten inhibited the Gly-Sar uptake activity significantly in both rat strains. There was no difference in the extent of inhibition between Sprague-Dawley and SHR rats. In both rat strains, the inhibition caused by 10 mM cefixime was about 30%. Ceftibuten was considerably more potent than cefixime in inhibiting Gly-Sar uptake. The inhibition caused by 2.5 mM ceftibuten was about 35%.

There was a significant quantitative difference in the inhibitory potency of the anionic cephalosporins between SKPT cells and SHR rat kidney brush border membrane vesicles. This difference is not explainable on the basis of animal species or animal strain differences because SKPT cells were originally derived from SHR rat kidney. The data show that the anionic cephalosporins are more potent in inhibiting Gly-Sar uptake in SKPT cells than in isolated renal brush border membrane vesicles. Intact cells are metabolically active and maintain stable intracellular pH (hence transmembrane H^+ gradient) and membrane potential. Furthermore, Gly-Sar uptake is linear in intact cells over the time period of uptake measurement. In contrast, isolated brush border membrane vesicles cannot maintain the experimentally imposed transmembrane H^+ gradients and membrane potential. Even with an incubation period as short as 10 s, Gly-Sar uptake is not linear. Another factor is the concentration of Gly-Sar used in these studies. In SKPT cells, Gly-Sar concentration was 5 μM which is almost 15-fold less than the Michaelis-Menten constant. On the other hand, Gly-Sar concentration was 30 μM when renal brush border membrane vesicles were employed. These factors are most likely responsible for the relatively lesser potency of the anionic cephalosporins in inhibiting Gly-Sar uptake in membrane vesicles than in intact cells.

3.4. Studies of ceftibuten transport in Xenopus oocytes expressing human PEPT 1

Several studies have investigated the transport of the anionic cephalosporin cefixime via the peptide transport system in intestinal brush border membrane vesicles [17,19,21]. These investigations have provided unequivocal evidence for the involvement of the intestinal peptide transporter (PEPT 1) in the transport of cefixime. However, in contrast to several peptide substrates whose transport via PEPT 1 is known to be associated with the transfer of positive charge across the membrane, the transport of cefixime was interestingly found to be associated with the transfer of negative charge [17]. Such a transport mechanism would render the PEPT 1-mediated transport of anionic cephalosporins unfavorable in the intestine because of the presence of an inside-negative membrane potential across the brush border membrane. Therefore, we investigated the issue of charge transfer associated with PEPT 1-mediated transport of the anionic cephalosporin ceftibuten in *Xenopus* oocytes expressing human PEPT 1. These oocytes are readily amenable for direct measurements of transport-associated charge transfer using electrophysiological means. In this approach, the oocyte is voltage-clamped and transport-associated changes in membrane potential are compensated by passing currents of appropriate polarity and magnitude to maintain the membrane potential at the clamped level. This current is the measured parameter of transport. This experimental approach provides direct information on the nature of charge transfer associated with a transport process. In control (water-injected) oocytes, ceftibuten-evoked currents were not detectable, indicating the absence of endogenous electrogenic ceftibuten transport pathways in *Xenopus* oocytes. However, in oocytes expressing human PEPT 1, pH-dependent, ceftibuten-evoked currents were readily

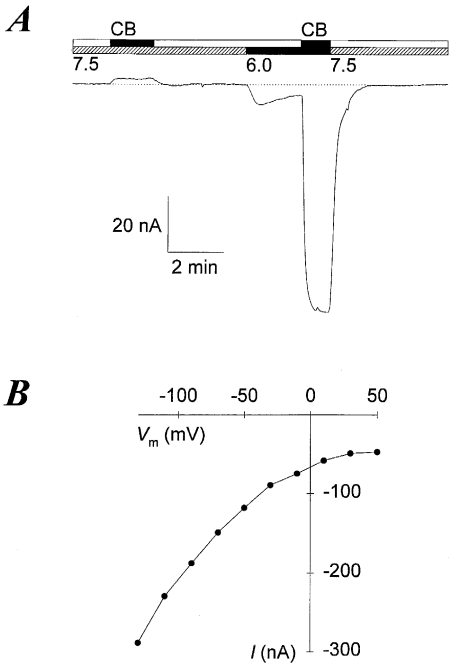

Fig. 10. Ceftibuten-evoked currents in an oocyte expressing hPEPT 1. (A) pH-dependence of the ceftibuten-evoked current. The oocyte was clamped at a membrane potential (V_m) of −50 mV and current continuously monitored while superfusing test solutions for the periods indicated in the *top panel*. Ceftibuten (CB, *solid bars*) was superfused at 5 mM in 100 mM NaCl medium at pH 7.5 (diagonal hatch) and at pH 6.0 (shaded bar). Each time, ceftibuten was washed out with substrate-free medium at pH 7.5 (the current trace has been corrected for drift). (B) current/voltage relationship of the 5 mM ceftibuten-evoked currents (I).

observed (Fig. 10A). At pH 7.5, 5 mM ceftibuten evoked a tiny outward current, showing that PEPT 1-mediated ceftibuten transport is indeed associated with the transfer of negative charge under these conditions. But, at pH 6.0, 5 mM ceftibuten evoked marked inward current which shows that PEPT 1-mediated ceftibuten transport in the presence of a H^+ gradient is associated with the transfer of positive charge. It appears that, in the absence of a H^+ gradient, transport of the anionic substrate uncoupled from H^+ may occur via PEPT 1 to some extent. The ceftibuten-evoked currents at pH 6.0 were concentration-dependent and obeyed Michaelis-Menten kinetics. The $K_{0.5}$ (substrate concentration at which current was half-maximal) for ceftibuten was 0.4 ± 0.1 mM at −50 mV (data not shown). The current-voltage relationship for 5 mM (saturating) ceftibuten (Fig. 10B) was qualitatively similar to that for the zwitterionic peptide Gly-Sar [38] or for the zwitterionic cephalosporin cefadroxil [41]. The ceftibuten-evoked currents displayed a non-linear dependence upon membrane potential, with no reversal of currents at depolarized membrane potential (up to +50 mV) and no indication of saturating with hyperpolarization (up to − 130 mV).

We were unable to perform similar electrophysiological studies with PEPT 2 because injection of human PEPT 2 cRNA into oocytes failed to generate detectable currents. The human PEPT 2 cDNA is functional as evident from the cDNA-induced Gly-Sar uptake in HeLa cells. The reasons for the inability of the PEPT 2 cRNA to be functionally expressed in oocytes measured either by tracer uptake or by the electrophysiological approach are not known. The human PEPT 2 cDNA has a truncated 3′ non-coding region and lacks a poly(A) tail and it is possible that this compromises the stability of the cRNA in the oocytes. The recently cloned rabbit PEPT 2 cDNA possesses a poly(A) tail and the injection of the cRNA derived from this cDNA has been shown to lead to the functional expression of the peptide transporter as detected by the electrophysiological approach [43].

In conclusion, the present study has provided unequivocal evidence for the interaction of anionic cephalosporins with the intestinal and renal H^+/peptide cotransporters. We have documented this interaction using four different approaches. This investigation has produced several important findings. The results of this investigation demonstrate for the first time that the human PEPT 1 cloned from normal intestine can transport anionic cephalosporins, thus corroborating the observations in the human colon carcinoma cell line Caco-2. This study also establishes definitively that the renal peptide transporter interacts with anionic cephalosporins. The interaction is documented with cultured rat kidney cells which exclusively express PEPT 2, with the human PEPT 2 cloned from kidney, and with rat renal brush border membrane vesicles. The present study also shows using direct electrophysiological approaches that ceftibuten transport mediated by human intestinal peptide transporter PEPT 1 in the presence of an inwardly directed H^+ gradient is associated with

transfer of positive charge. Further experiments aimed at determining the H^+/ceftibuten coupling ratio and the possible involvement of other ions (e.g., OH^- and K^+) as additional cotransported substrates are needed to elucidate the molecular mechanism responsible for this charge transfer.

Acknowledgements

This work was supported by NIH Grant GM 54122. The authors thank Sarah A. Taylor and Joyce Hobson for excellent secretarial assistance.

References

[1] Ganapathy, V. and Leibach, F.H. (1986) Am. J. Physiol. 251, F945–F953.
[2] Ganapathy, V. and Leibach, F.H. (1991) Curr. Opin. Cell Biol. 3, 695–701.
[3] Ganapathy, V., Bransch, M. and Leibach, F.H. (1994) in Physiology of the Gastrointestinal Tract (Johnson, L.R., ed.), 3rd edn., pp. 1773–1794. Raven Press, New York.
[4] Meredith, D. and Boyd, C.A.R. (1995) J. Membr. Biol. 145, 1–12.
[5] Leibach, F.H. and Ganapathy, V. (1996) Annu. Rev. Nutr. 16, 99–119.
[6] Ganapathy, V. and Leibach, F.H. (1985) Am. J. Physiol. 249, G153–G160.
[7] Hoshi, T. (1985) Jpn. J. Physiol. 35, 179–191.
[8] Ganapathy, V., Miyamoto, Y. and Leibach, F.H. (1987) Contr. Infusion. Ther. Clin. Nutr. 17, 54–68.
[9] Amidon, G.L. and Lee, H.J. (1994) Annu. Rev. Pharmacol. Toxicol. 34, 321–341.
[10] Tsuji, A. (1995) in Peptide-Based Drug Design (Taylor, M.D. and Amidon, G.L.), pp. 101–134. American Chemical Society, Washington, DC, 1995.
[11] Tsuji, A. and Tamai, I. (1996) Pharm. Res. 13, 963–977.
[12] Nakashima, E., Tsuji, A., Mizuo, H. and Yamana, T. (1984) Biochem. Pharmacol. 33, 3345–3352.
[13] Inui, K., Okano, T., Takano, M., Saito, H. and Hori, R (1984) Biochim. Biophys. Acta 769, 449–454.
[14] Dantzig, A.H. and Bergin, L. (1990) Biochim. Biophys. Acta 1027, 211–217.
[15] Inui, K.I., Yamamoto, M. and Saito, H. (1992) J. Pharmacol. Exp. Ther. 261, 195–201.
[16] Daniel, H. and Adibi, S.A. (1993) J. Clin. Invest. 92, 2215–2223.
[17] Tsuji, A., Terasaki T., Tamai, I. and Hirooka, H. (1987) J. Pharmacol. Exp. Ther. 241, 594–601.
[18] Tsuji, A., Tamai, I., Hirooka, H. and Terasaki, T. (1987) Biochem. Pharmacol. 36, 565–567.
[19] Inui, K., Okano, T., Maegawa, H., Kato, M., Takano, M. and Hori, R. (1988) J. Pharmacol. Exp. Ther. 247, 235–241.
[20] Tsuji, A., Tamai, I., Nakanishi, M., Terasaki, T. and Hamano, S. (1993) J. Pharm. Pharmacol. 45, 996–998.
[21] Kramer, W., Gutjahr,U., Kowalewski, S. and Girbig, F. (1993) Biochem. Pharmacol. 46, 542–546.
[22] Dantzig, A.H., Duckworth, D.C. and Tabas, L.B. (1994) Biochim. Biophys. Acta 1191, 7–3.
[23] Muranushi, N., Horie, K., Masuda, K. and Hirano, K. (1994) Pharm. Res. 11, 1761–1765.
[24] Sugawara, M., Iseki, K., Miyazaki, K., Shiroto, H., Kondo, Y. and Uchino, J. (1991) J. Pharm. Pharmacol. 43, 882–884.
[25] Matsumoto, S., Saito, H. and Inui, K.I. (1994) J. Pharmacol. Exp. Ther. 270, 498–504.
[26] Tamai, I., Tsuji, A. and Kin, Y. (1988) J. Pharmacol. Exp. Ther. 246, 338–344.
[27] Naasani, I., Sugawara, M., Kobayashi, M., Iseki, K. and Miyazaki, K. (1995) Pharm. Res. 12, 605–608.
[28] Naasani, I., Sato, K., Iseki, K., Sugawara, M., Kobayashi, M. and Miyazaki, K. (1995) Biochim. Biophys. Acta 1231, 163–168.
[29] Naasani, I., Kikuchi, T., Sugawara, M., Kobayashi, M., Iseki, K. and Miyazaki, K. (1996) Biochim. Biophys. Acta 1283, 185–191.
[30] Brandsch, M., Miyamoto, Y., Ganapathy, V. and Leibach, F.H. (1994) Biochem. J. 299, 253–260.
[31] Brandsch, M., Brandsch, C., Prasad, P.D., Ganapathy, V., Hopfer, U. and Leibach, F.H. (1995) FASEB J. 9, 1489–1496.
[32] Liang, R., Fei, Y.J., Prasad, P.D., Ramamoorthy, S., Han, H., Yang-Feng, T.L., Hediger, M.A., Ganapathy, V. and Leibach, F.H. (1995) J. Biol. Chem. 270, 6456–6463.
[33] Liu, W., Liang, R., Ramamoorthy, S., Fei, Y.J., Ganapathy, M.E., Hediger, M.A., Ganapathy, V. and Leibach, F.H. (1995) Biochim. Biophys. Acta 1235, 461–466.
[34] Tiruppathi, C., Ganapathy, V. and Leibach, F.H. (1987) Pediatr. Res. 22, 641–646.
[35] Miyamoto, Y., Coone, J.L., Ganapathy, V. and Leibach, F.H. (1988) Biochem. J. 249, 247–253.
[36] Ganapathy, V., Mendicino, J.F. and Leibach, F.H. (1981) J. Biol. Chem. 256, 118–124.
[37] Fei, Y.J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F.H., Romero, M.F., Singh, S.K., Boron, W.F. and Hediger, M.A. (1994) Nature 368, 563–566.
[38] Mackenzie, B., Loo, D.D.F., Fei, Y.J., Liu, W., Ganapathy, V., Leibach, F.H. and Wright, E.M. (1996) J. Biol. Chem. 271, 5430–5437.
[39] Loo, D.D.F., Hazama, A., Supplisson, S., Turk, E. and Wright, E.M. (1993) Proc. Natl. Acad. Sci. USA 90, 5767–5771.
[40] Ramamoorthy, S., Liu, W., Ma, Y.Y., Yang-Feng, T.L., Ganapathy, V. and Leibach, F.H. (1995) Biochim. Biophys. Acta 1240, 1–4.
[41] Boll, M., Markovich, D., Weber, W.M., Korte, H., Daniel, H. and Murer, H. (1994) Pflugers Arch. Eur. J. Physiol. 429, 146–149.

[42] Saito, H., Okuda, M., Terada, T., Sasaki, S. and Inui, K.I. (1995) J. Pharmacol. Exp. Ther. 275, 1631–1637.
[43] Boll, M., Herget, M., Wagner, M., Weber, W.M., Markovich, D., Biber, J., Clauss, W., Murer, H. and Daniel, H. (1996) Proc. Natl. Acad. Sci. USA 93, 284–289.
[44] Ganapathy, M.E., Brandsch, M., Prasad, P.D., Ganapathy, V. and Leibach, F.H. (1995) J. Biol. Chem. 270, 25672–25677.
[45] Cheng, Y.C. and Prusoff, W.H. (1993) Biochem. Pharmacol. 22, 3099–3108.