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Comparison of the transport characteristics of ceftibuten in rat renal and intestinal brush-border membranes

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Abstract

The transport characteristics of ceftibuten, a dianionic cephem antibiotic, in rat renal and intestinal brush-border membranes were compared. Ceftibuten transport was mediated by two transport systems in the renal brush-border membrane and by one transport system in the intestinal brush-border membrane. The apparent kinetic parameters for the uptake of ceftibuten by the renal brush-border membrane vesicles, respectively, were: K_{m_1}, K_{m_2} values of 26 and 1946 μM and V_{max_1}, V_{max_2} values of 105 and 1400 pmol/mg protein per 30 s. The apparent kinetic parameters for the uptake by the intestinal brush-border membrane vesicles were: K_m of 425 μM and V_{max} of 1701 pmol/mg protein per 30 s. In the renal brush-border membrane, L-Ala-L-Pro was partially competitive and competitive inhibitor for the uptake by the high and low affinity systems, respectively. However, L-Ala-L-Pro was a non-competitive inhibitor for the uptake by the intestinal brush-border membrane vesicles. L-Carnosine was a specific and competitive inhibitor for the high affinity system in the renal brush-border membrane, while it had no effect on the low affinity system of the kidney or on the transport system of the intestine. It was concluded that the transport characteristics of ceftibuten in the renal and intestinal brush-border membranes are similar in some aspects but they are not identical.

Keywords: Brush-border membrane; Ceftibuten; Membrane transport; Oligopeptide; Renal proximal tubule; Small intestine

1. Introduction

Ceftibuten, a water-soluble dianionic cephem antibiotic, possesses good pharmacokinetic properties which include a high oral bioavailability and a relatively long elimination half-life when compared with other cephem antibiotics [1]. These favorable properties have attracted several researchers to investigate the transport mechanism of this compound across biomembranes at the molecular level.

Previous studies [2–5] on the transport mechanism of ceftibuten across intestinal brush-border membrane have shown that ceftibuten, in spite of being a strong dianionic compound, does share a common transport mechanism with oligopeptides. Moreover, we have recently examined the transport mechanism of this compound in the renal proximal tubular brush-border membrane [6] and, similarly, we have found that the reabsorption of this compound from the lumenal side is mediated by the oligopeptide transporter(s) without any interference from the anion transporters. However, during our investigations we have noticed that the transport mechanisms of ceftibuten in the renal brush-border membrane were not always identical to those in the intestinal brush-border membrane and some differences (such as substrate specificity) could be found [6,11]. Therefore, in order to gain additional details on the differences of the transport systems of ceftibuten in these two types of biological membranes, we further investigated and compared the transport characteristics of this compound using brush-border membrane vesicles isolated from renal proximal tubules and small intestine of rat. This study will increase the knowledge about the transport mechanisms of not only ceftibuten but also other peptide-like compounds across the brush-border membranes.

2. Materials and methods

2.1. Materials

Ceftibuten was kindly donated by Shionogi (Osaka, Japan). L-Ala-L-Pro and L-carnosine (β-alanyl-L-histidine)

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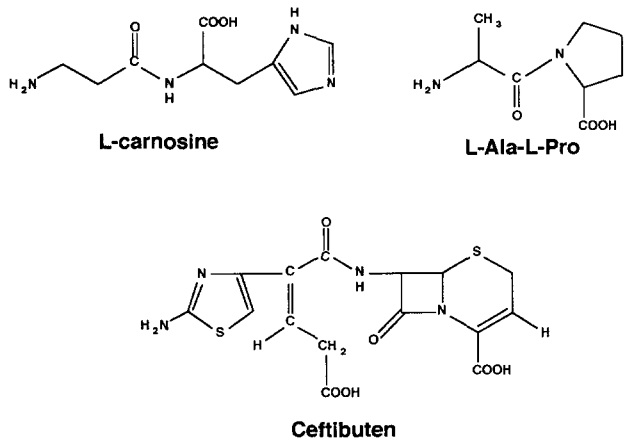

Fig. 1. Structures of ceftibuten, L-Ala-L-Pro and L-carnosine.

were purchased from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland), respectively. All other chemicals were of the highest grade available and used without further purification. The structures of ceftibuten, L-Ala-L-Pro and L-carnosine are shown in Fig. 1.

2.2. Preparation of the brush-border membrane vesicles

Male Wistar rats (200–250 g) were used for the isolation of membrane vesicles and all the procedures were carried out at 4°C. The small intestinal brush-border membrane vesicles were prepared by the Ca^{2+} precipitation method according to the method of Kessler et al. [7] with minor modifications as described previously [8]. The renal brush-border membrane vesicles were isolated from the renal cortex by the Mg^{2+}/EGTA precipitation method of Biber et al. [9] with several modifications. Briefly, cortex slices were homogenized with a Waring blender (Nihon Seiki, Japan) at 16500 rpm for 4.5 min in an appropriate volume of a homogenizing buffer composed of 300 mM mannitol, 12 mM Tris-HCl (pH 7.1) and 5 mM EGTA. Then, the homogenate was diluted with distilled water (1:1) and $MgCl_2$ was added to a final concentration of 10 mM. After 15 min, the mixture was centrifuged at 19000 $\times g$ for 15 min. The supernatant was then centrifuged at 27000 $\times g$ for 30 min. An appropriate volume of the homogenizing buffer was diluted with distilled water (1:1) and used to resuspend the resulting pellets. Then, the pellet suspension was Homogenized in a Dounce-type homogenizer (10 strokes). $MgCl_2$ was added to a final concentration of 10 mM and the first two steps of centrifugation were repeated once more. The resulting pellets were washed once with the experimental buffer. The washing was done by homogenizing in a Dounce-type homogenizer (10 strokes) and then centrifugation at 27000 $\times g$ for 30 min. The final pellets were resuspended in the experimental buffer using a Dounce-type homogenizer to give a final protein concentration of 10–15 mg/ml. Similar protein concentration was adopted for the intestinal brush-border membrane vesicles. Unless otherwise specified, the experimental buffer was comprised of 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris (pH 7.5). Enrichment of the brush-border membrane fraction, was 10-fold compared to the homogenate as revealed from the assessment of specific activity of the membrane enzyme marker, alkaline phosphatase. Uptake experiments of tetraethylammonium (TEA^+), (a typical substrate for the cation transport system) and of D-[C^{14}]glucose, revealed a functional integrity of the renal and the intestinal membrane preparations, respectively.

2.3. Uptake experiments

The uptake of ceftibuten into the freshly isolated membrane vesicles was performed at 25°C by the rapid filtration technique according to the method of Sugawara et al., [10] with minor modification. The reaction was initiated by mixing of 40 μl of membrane vesicle suspension with 200 μl of the transport buffer (unless stated otherwise, the transport buffer was composed of 100 mM D-mannitol 100 mM KCl, 20 mM Mes-Tris, pH 5.5) containing substrates. Then, at the stated times, the reaction was terminated by diluting the reaction mixture with 4 ml of the ice-cold stop buffer (150 mM NaCl, 20 mM Hepes-Tris, pH 7.5) followed by filtration through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diam.). The filter was then washed once with 8 ml of the ice-cold stop buffer. Ceftibuten trapped on the filter was extracted with 300 μl of the stop buffer.

2.4. Analytical procedures

The detection of ceftibuten was carried out by the use of high-performance liquid chromatography as described previously [4,5,10,11]. Separation of ceftibuten was achieved on a reverse-phase column (ODS Hitachi #3053, 5 μm, 4 mm i.d. $\times$ 250 mm) using a mobile phase consisting of acetonitrile/0.05 M citric acid-0.1 M KCl buffer, pH 2.5 (1:9). Samples were eluted at a flow rate of 0.7 ml/min. and the detection was set at 262 nm. Protein was measured by the method of Lowry et al. [12] with bovine serum albumin as standard.

3. Results

3.1. Comparison of the concentration dependency and the kinetics of ceftibuten transport in the renal and intestinal brush-border membrane vesicles

The effect of increasing concentration of ceftibuten on its uptake in the presence of an inwardly directed H^+ gradient was investigated in both of the renal and the intestinal brush-border membrane preparations. The 30-s uptake rates were determined by measuring ceftibuten uptake with a range of concentrations from 50 μM to 20

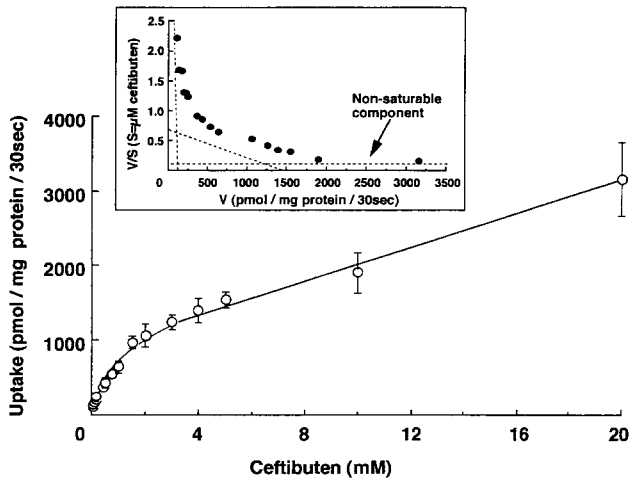

Fig. 2. Concentration dependency of ceftibuten initial (30 s) uptake by rat renal brush-border membrane vesicles in the presence of an inwardly directed H^+ gradient (pH_{in} 7.5, pH_{out} 5.5). Ceftibuten concentration was varied over the range of 0.05–20 mM. Values are the mean ± S.D. of eight determinations from two different preparations. Inset represents the Eadie–Hofstee plot with its components (dotted lines).

mM. Fig. 2 and Fig. 3 illustrate that the uptake was saturable as a function of concentration. The Eadie–Hofstee plot of the uptake into the renal bush-border membrane vesicles (inset of Fig. 2) reveals the presence of at least two transport components characterized as the high affinity low capacity system and the low affinity high capacity system. On the other hand, the Eadie–Hofstee plot of the uptake into the intestinal brush-border membrane vesicles (inset of Fig. 3) reveals a monocomponent transport system. The presence of a different number of transport systems between the renal and the intestinal brush-border

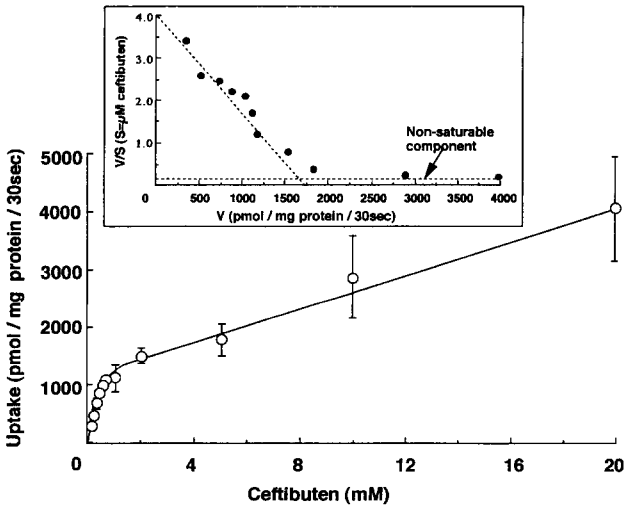

Fig. 3. Concentration dependency of ceftibuten initial (30 s) uptake by rat intestinal brush-border membrane vesicles in the presence of an inwardly directed H^+ gradient (pH_{in} 7.5, pH_{out} 5.5). Ceftibuten concentration was varied over the range of 0.05–20 mM. Values are the mean ± S.D. of seven determinations from two different preparations. Inset represents the Eadie–Hofstee plot with its components (dotted lines).

Table 1
Apparent kinetic parameters of ceftibuten initial (30 s) uptake by rat renal and intestinal brush-border membrane vesicles

Parameter	Kidney [a]		Intestine
	HA	LA	
V_{max} (pmol/mg protein per 30 s)	105	1400	1701
K_m (μM)	26	1950	425
K_d (pmol/mg protein per 30 s per mM)	85		95

[a] HA and LA refer to the high and low affinity transport systems, respectively.

membranes is also evident from the non-linear regression analysis and least-squares fitting, where, the best fitting for the uptake by the renal brush-border membrane vesicles was attainable when a double Michaelis–Menten model with non-saturable component (simple diffusion) was applied (see Eq. (1)). On the other hand, the best fitting for the uptake by the intestinal brush-border membrane vesicles was achieved with a normal Michaelis-Menten model with non-saturable component (see Eq. (2)). The kinetic constants of the transport systems are summarized in Table 1.

$$V = \frac{V_{max_1}[s]}{K_{m_1}[s]} + \frac{V_{max_2}[s]}{K_{m_2}[s]} + K_d[s] \quad (1)$$

$$V = \frac{V_{max}}{K_m[s]} + K_d[s] \quad (2)$$

where: V is the initial uptake rate, [s] is the initial substrate concentration, K_d is the constant of the non-saturable component, K_m is the affinity constant, V_{max} is the maximum uptake rate

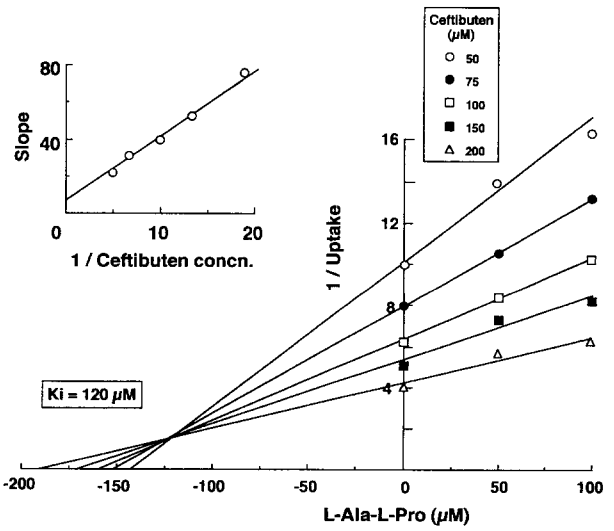

Fig. 4. Dixon plot analysis of ceftibuten initial (30 s) uptake by rat renal brush-border membrane vesicles at a low range of substrate concentrations in the presence of L-Ala-L-Pro and an inwardly directed H^+ gradient (pH_{in} 7.5, pH_{out} 5.5). The inset shows a replot of the slopes of Dixon plot. Values are the mean of four measurements.

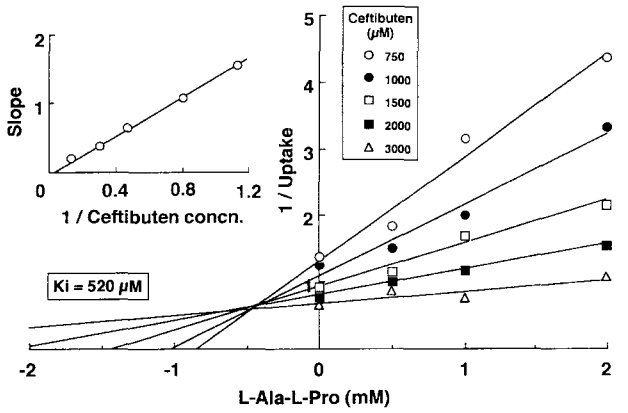

Fig. 5. Dixon plot analysis of ceftibuten initial (30 s) uptake by rat renal brush-border membrane vesicles at a high range of substrate concentrations in the presence of L-Ala-L-Pro and an inwardly directed H^+ gradient (pH_{in} 7.5, pH_{out} 5.5). The inset shows a replot of the slopes of Dixon plot. Values are the mean of four measurements.

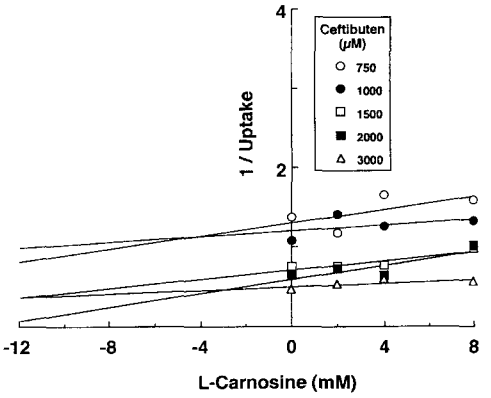

Fig. 7. The ineffectiveness of L-carnosine in inhibiting the uptake of ceftibuten by rat renal brush-border membrane vesicles at a high range of substrate concentrations and in the presence of an inwardly directed H^+ gradient (pH_{in} 7.5, pH_{out} 5.5). The data are plotted as Dixon plot. Values are the mean of four measurements.

3.2. Dixon plot analysis in the presence of dipeptides and an inwardly directed H^+ gradient

In the presence of L-Ala-L-Pro

In a previous study [11], the inhibitory effect of L-Ala-L-Pro on the uptake of ceftibuten by rat intestinal brush-border membrane vesicles has been investigated by Dixon plot analysis. The results of this analysis have demonstrated that the uptake of ceftibuten was inhibited by L-Ala-L-Pro in a non-competitive manner with an apparent K_i value of 1.21 mM. In the case of the renal brush-border membrane, the presence of two transport systems with different affinities and capacities addresses the requirement of carrying out the Dixon plot analysis at two different ranges of substrate concentrations in order to accommodate the operational capacity of each transporter. Accordingly, the 30-s uptake values of ceftibuten at a range of concentrations from 50 μM to 200 μM or from 750 μM to 3 mM were plotted as Dixon analysis in the presence of increasing concentrations of L-Ala-L-Pro (Fig. 4 and Fig. 5). The kinetics of the inhibition at low substrate concentrations (Fig. 4) are consistent with a partially competitive inhibition. Meanwhile, the kinetics of the inhibition at a high range of substrate concentrations (Fig. 5) are clearly in agreement with a competitive type of inhibition because the replot of the slope of Dixon plot almost coincided with the origin in contrast to that at low substrate concentrations.

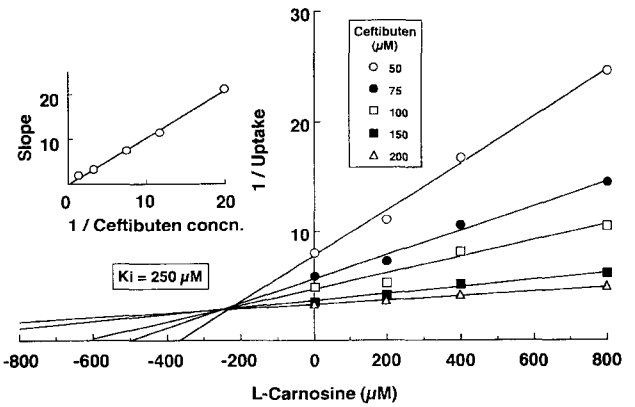

Fig. 6. Dixon plot analysis of ceftibuten initial (30 s) uptake by rat renal brush-border membrane vesicles at a low range of substrate concentrations in the presence of L-carnosine and an inwardly directed H^+ gradient (pH_{in} 7.5, pH_{out} 5.5). The inset shows a replot of the slopes of Dixon plot. Values are the mean of four measurements.

In the presence of L-carnosine

Based on an earlier study [10], it was reported that L-carnosine does not affect the uptake of ceftibuten by rat intestinal brush-border membrane vesicles in anyway. On the contrary, we have recently reported that L-carnosine is effective in inhibiting the uptake of ceftibuten by rat renal brush-border membrane vesicles [6]. To further examine these effects, as in the above studies with L-Ala-L-Pro, Dixon analysis of the 30 s uptake of ceftibuten in the presence of L-carnosine was carried out at two different levels of substrate concentrations. Fig. 6 illustrates that at low substrate concentrations, L-carnosine exhibited a typical competitive inhibition on the uptake of ceftibuten. Surprisingly, as shown in Fig. 7, L-carnosine did not exhibit any inhibitory effect when a high level of substrate concentrations was used.

4. Discussion

The results of the present study provided further details on the transport mechanism of ceftibuten in particular and oligopeptides in general across the brush-border membranes. The uptake of ceftibuten by the renal brush-border membrane vesicles appeared to be mediated by two carrier systems with different affinities and capacities. The possi-

bility that the high affinity low capacity system is nothing more than a binding artifact is excluded because the uptake rates at low temperature (4°C) were very small and unsaturable (data not shown). In our previous work [6], we have demonstrated that the transport of ceftibuten in rat renal brush-border membrane is mediated, at least in part, by a common transport mechanism with oligopeptides. Since the renal brush-border membrane is well furnished with several types of anion transporters [13], one of the transport systems for ceftibuten across this membrane could be merely a result of the interference from the anion transporters. However, based on our previous study [6], this is unlikely because the transport of ceftibuten in the renal brush-border membrane was found to be independent of Na^+ ions and all types of organic acids (mono-and dicarboxylate) have not had any inhibitory effect. The inhibitory effect of L-Ala-L-Pro on each of the transport systems of ceftibuten in the renal brush-border membrane provided an evident that both of the ceftibuten transporters were common transporters for oligopeptides. This is further supported by the results of Silbernagl et al. [14], Daniel et al. [15] and Skopicki et al. [16], where, it has been shown that the oligopeptides transport in the rat renal brush-border membrane is mediated by two transport systems with different affinities and capacities. Concerning the intestinal transport, the present study revealed that the transport of ceftibuten is mediated by one transport system. The specificity and the type of inhibition induced by dipeptides on this transport system were not always similar to those for the transport systems in the renal brush-border membrane. The differences are summarized in Table 2. The effect of L-Ala-L-Pro on the transport in the renal brush-border membrane was partially competitive and competitive for the high and the low affinity systems, respectively. Whereas, it has been found that this dipeptide is a non-competitive inhibitor for the transport in the intestinal brush-border membrane [11]. Interestingly, L-carnosine, which is in the cationic form at pH 5.5, had the potential to inhibit only the high affinity system in the renal brush-border membrane; however, it had no effect on the low affinity system of the kidney and on the transport system of the intestine as well. The failure of L-carnosine in inhibiting the low affinity transport system of the kidney and the transport system of the intestine indicates that the substrate binding sites of these systems are positively charged. This assumption is further supported by the studies with histidyl or thiol group-specific agents [17–19]. These studies have shown that histidyl groups are involved in the transport of dipeptides in both the renal and the intestinal brush-border membranes. However, when thiol group-specific agents were used, only the renal transport of dipeptides was affected. It is likely that the substrate binding site of the high affinity transport system in the kidney consists mainly of neutral groups (such as thiol groups) that permit the dual transport of positively or negatively charged substrates. The existence of the same carrier protein for oligopeptides in the intestine and kidney has been recently referred by Fel et al. [20]. This group has succeeded in cloning the expression of a putative carrier protein for oligopeptides in the small intestine, kidney, liver and brain of the rabbit. From this finding, collectively with the results of the present study, it seems that the low affinity transporter in the renal brush-border membrane and the transporter in the intestinal brush-border membrane are of similar features and the differences noticed between these two types of brush-border membranes are due to the presence of an extra high affinity transport system in the renal brush-border membrane. Finally, it is worth noting that the existence of such a high affinity system for the transport of ceftibuten in the renal brush-border membrane (in the reabsorptive direction across the proximal tubular cell) is of pharmacological importance because it provides an explanation for the relatively long elimination half-life and for the specific effect of this antibiotic on the renal infections.

Table 2
Types and constants [b] of inhibition induced by L-Ala-L-Pro and L-carnosine on the uptake of ceftibuten into rat renal and intestinal brush-border membrane vesicles

Transport system		L-Ala-L-Pro	L-Carnosine
Kidney	HA	Partially competitive, $K_i = 120$	Competitive, $K_i = 250$
	LA	Competitive, $K_i = 520$	No inhibition
Intestine [a]		Non-competitive, $K_i = 1210$	No inhibition

[a] Reported in Ref. [11]. [b] Units in μM.

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