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Saturable uptake of cefixime, a new oral cephalosporin without an α-amino group, by the rat intestine

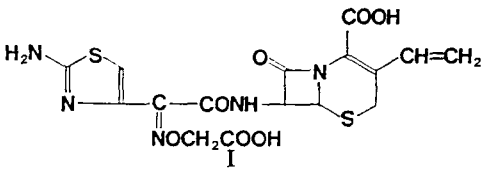
AKIRA TSUJI*, HIDEKI HIROOKA, TETSUYA TERASAKI, IKUMI TAMAI AND EMI NAKASHIMA†

Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan

The mechanism of intestinal uptake of cefixime, a new oral cephalosporin antibiotic, has been examined using the everted jejunum of rats. The initial uptake rates were apparently pH-dependent with the maximum rate at pH 5·0 and a 3-fold reduction at pH 7·0. The uptake at pH 5·0 followed mixed-type kinetics involving saturable and non-saturable processes in a manner similar to that for several amino-β-lactam antibiotics. Cefixime uptake was inhibited significantly by 20 mM permeants such as cyclacillin, cephradine, benzylpenicillin, propicillin, glycyl-L-proline and glycyl-glycine. Replacement of Na^+ in the medium with choline produced a slight but significant inhibition of cefixime uptake. In spite of the absence of significant inhibition by the amino acids glycine and proline, the dipeptide, glycyl-L-proline in Na^+-free medium showed a marked inhibitory effect. The inhibition kinetics of cefixime uptake by glycyl-L-proline and cyclacillin were consistent with competitive-type inhibition. This study provides the first evidence of saturable intestinal uptake of a cephem antibiotic without an α-amino group in the side chain, suggesting transport through the dipeptide carrier system(s).

Evidence of carrier-mediated transport of α-amino-β-lactam antibiotics such as amoxycillin and cephalexin across the animal small intestine has been well documented in our laboratory (Nakashima et al 1984a, b; Nakashima & Tsuji 1985; Tsuji et al 1981a, b). Skilful pharmacokinetic studies in humans have succeeded in showing the existence of carrier-mediated absorption of ampicillin and amoxycillin by the human intestine (Sjövall et al 1985a, b). The uptake system available for these amino-β-lactam antibiotics having a free amino group at the 6-position in penicillin and the 7-position in cephalosporin nuclei is closely related to the system(s) of transport of the structurally similar di- or tri-peptides (Addison et al 1975: Kimura et al 1978; Nakashima et al 1984a, b; Nakashima & Tsuji 1985; Okano et al 1986).

(6*R*,7*R*)-7-[(Z)-2-(2-Amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo-(4,2,0)-oct-2-ene-2-carboxylic acid (cefixime, I), a new cephalosporin antibiotic analogous to poorly absorbed ceftizoxime, was developed for oral use against Gram-negative bacilli (Kamimura et al 1984; Sakamoto et al 1985). The structure of cefixime is completely different from those of the cephalexin-analogue from the viewpoint of the lack of an α-amino group and the expectation of low lipophilicity at neutral pH. However, little is known of cefixime transport across the intestinal brush-border membrane. Cefixime, an α-amino group-deficient cephalosporin, is active orally and it is of interest to know whether it is taken up via the same carrier-mediated system as that for α-amino-β-lactam antibiotics, or via a previously unknown system, or by passive diffusion.



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The aim of the present work has been to determine the mechanism for absorption of cefixime. The results suggest that cefixime also can be transported by a dipeptide carrier across the intestinal membrane.

MATERIALS AND METHODS

Materials

Cefixime (FK-027, Fujisawa Pharmaceutical Co., Osaka, Japan) cyclacillin (Takeda Chemical Industries, Osaka, Japan), cephradine (Sankyo Co., Tokyo, Japan), benzylpenicillin and propicillin (Meiji Seika Kaisha, Tokyo, Japan) were gifts from the cited companies. [^{14}C]Inulin (2·1 mCi g^{-1}) was

* Correspondence.
† Present address: Hospital Pharmacy, Kanazawa University, Takara-machi, Kanazawa 920, Japan.

purchased from Amersham International Ltd, Bucks, UK.

All other chemicals were of the highest grade available commercially.

Animals

Male Sprague-Dawley rats (200–250 g) were fasted for about 20 h before the experiment, but water was freely available.

Preparations and uptake experiments

The preparation of everted intestine and the method of influx measurement were the same as those described by Nakashima et al (1984b). Briefly, the rats were anaesthetized with ether, and the proximal portion of the jejunum, about 30 cm long, excised, everted, divided into segments of 4 cm length, and fixed on a polyethylene tube of 4 mm outer diameter. Each tube had been marked with two lines to define a constant mucosal surface area. The preparations were incubated in a 0·9% NaCl (saline) solution for 1 min, and then incubated (100 strokes min^{-1}) in 25 mL of buffer solution containing cefixime and/or an inhibitor at various concentrations under O_2 at 37 °C. The volume of the extracellular fluid adhering to the mucosal surface (inulin space) was routinely measured using [^{14}C]inulin and the uptake amount of cefixime was corrected by subtracting the inulin space obtained in each experiment.

The incubation medium was a modified Krebs-Ringer Tris buffer solution of pH 5·0 containing (mM) NaCl 118, Tris 25, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·2, KH_2PO_4 1·2 and citric acid 9·2 as used by Matthews et al (1979) for the similar in-vitro intestinal uptake of dipeptides at pH 5·0. In the uptake experiments at various pH values, varying proportions of Tris and citric acid were used to achieve and maintain the desired pH.

Determination of the partition coefficient

For the distribution study in the 2-methylpropanol–water system, the aqueous phase was buffered with citrate to maintain the desired pH. In each aqueous phase, the Na^+ concentration was held constant at 0·1 M by the addition of sodium chloride. β-Lactam antibiotics were dissolved in the prepared buffer solution to make a final concentration of 0·05 mM. To minimize the volume change due to mutual miscibility, the aqueous phase and organic phase were saturated previously with each solvent. The detailed procedures for the determination and the calculation of the partition coefficient were described previously (Tsuji et al 1981a).

Analytical procedures

The intestinal tissue was cut off between the marks on the polyethylene tube and was homogenized (Ultra Turrax, Ika-Werk, Janke & Kunkel) with an ice cold 1/15 M phosphate buffer (pH 7·4) to give a 20% (w/v wet weight) homogenate. After centrifugation at 15 600*g* for 15 min, the amount of cefixime in the supernatant fraction was determined after deproteinization with acetonitrile (at a volume ratio of 1:1) by high performance liquid chromatographic (HPLC) assay.

The liquid chromatograph (model BIP-I, Japan Spectroscopic Co., Tokyo, Japan) was equipped with a UV detector (model UVIDEC 100-V, Japan Spectroscopic Co.) set at 290 nm. A chromatograph equipped with a reversed phase column (3·9 mm i.d. × 30 cm, μ-Bondapak C_{18}, Waters Associates, Milford, Mass.) packed in this laboratory was used. A guard column, C_{18}/Corasil (Waters Associate) was used between the analytical column and the injector. The mobile phase was 25% acetonitrile–75% water containing 10 mM tetrabutylammonium bromide, 10 mM ammonium acetate, and 167 mM acetic acid. The injection volume was 20 μL. The peak areas recorded with an integrator (model Chromatopack C-R3A, Shimadzu Co., Kyoto, Japan) were used for quantification. The calibration curves were generated after similar deproteinization with acetonitrile by the use of blank tissue samples containing known amounts of cefixime and were linear between the range of 10 to 1000 nmol g^{-1} of wet tissue weight with a coefficient of variation of <4·5%.

In a study for determination of the partition coefficient, the mobile phase was 5% acetonitrile–95% water containing 10 mM ammonium acetate for cefixime and ceftizoxime. The eluents were monitored at 290 nm for cefixime and at 254 nm for ceftizoxime.

Radioactivity of [^{14}C]inulin was determined by direct liquid scintillation counting in vials containing 10 mL of toluene-based scintillation fluid (500 mL toluene, 500 mL Triton X-100, 6·0 g 2,5-diphenyloxazole and 75 mg 1,4-bis-[2-(phenyloxazolyl)]-benzene). Quenching was corrected by the external standard method. Precisely weighed tissue samples were oxidized with a sample oxidizer (model ASC-113, Aloka Co., Tokyo, Japan) to $^{14}CO_2$ and radioactivity was determined by liquid scintillation counting (model LSC-700 liquid scintillation counter, Aloka Co.).

RESULTS

pH-dependency of the uptake rate

The amount of cefixime taken up by the rat everted intestine after correction for cefixime adhering to the mucosal surface on the basis of the inulin space increased linearly with time up to at least 5 min (data not shown), as observed previously for the other β-lactam antibiotics and glycyl-glycine (Nakashima et al 1984b). Thereafter, the uptake experiment was carried out for exactly 2 min and the uptake rate was expressed as the value of nmol min^{-1} g^{-1} of wet tissue weight. The degradation of cefixime in buffer solution used in this study was determined to be negligible during the uptake experiments (the maximum 1·75% h^{-1} at 37 °C).

The effect of pH on cefixime uptake over the pH range between 3·5 and 7·0 is shown in Fig. 1. The rate of uptake of 1 mM cefixime was apparently pH-dependent. The intestinal uptake of cefixime was slow at neutral pH, but increased with decrease in pH and reached the maximum rate below pH 5·0. The differences between the uptake rate for cefixime below pH 5·5 and that at pH 7·0 were significant ($P < 0·05$).

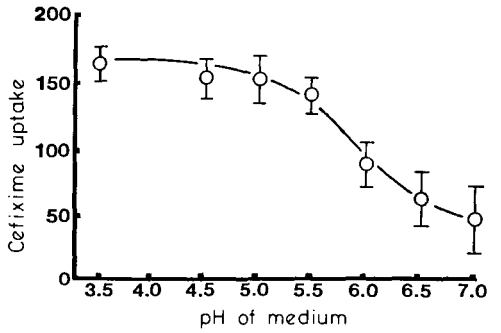

FIG. 1. Effect of pH of incubation medium on the rate of 1 mM cefixime uptake (nmol min^{-1} per g wet tissue) by isolated jejunum of rats. Each point shows the mean ± s.e.m. of four determinations.

The uptake rate for cefixime was compared with those for the other antibiotics determined previously in similar experiments (Nakashima et al 1984b). At pH 7·0, the rate for 1 mM cefixime was 48 ± 24 nmol min^{-1} g^{-1} of wet tissue, about 5 times faster than that of 1 mM cefazolin (Nakashima et al 1984b) which is a poorly absorbable cephalosporin, and very close to that of 1 mM cephalexin. At pH 5·0, the rate of uptake of 1 mM cefixime was 152 ± 18 nmol min^{-1} g^{-1} of wet tissue and was equivalent to that of 1 mM cyclacillin which is a rapidly absorbed amino-β-lactam antibiotic at pH 7·0.

Partition coefficient between 2-methylpropanol and water

The apparent partition coefficients (P_{app}) between 2-methyl propanol and water of cefixime and ceftizoxime at acidic pH in the range of 2–4 are shown in Table 1. P_{app} of cefixime at the pH above 4 was so small that it could not be determined. The value of P_{app} of cefixime is comparable with that of the poorly absorbable ceftizoxime (Table 1) and there was no appreciable correlation in the pH-dependences between P_{app} (Table 1) and cefixime uptake rate (Fig. 2). Since the pK_a values of the two kinds of carboxylic acid of cefixime are 2·10 (at 2-position) and 3·73 (at 7-position) and that of the amino group at the thiazoyl moiety is 2·69 (Takaya, personal communication), the apparent increase of lipophilicity of cefixime with decrease of pH is in accordance with increases of the un-ionized forms of the carboxylic acid groups on cefixime. Similarly, the lipophilicity of ceftizoxime increases with the decrease of pH.

Table 1. Apparent partition coefficient, P_{app}, of cefixime and ceftizoxime between 2-methylpropanol and water as a function of the pH of the aqueous phase at 37 °C.

	P_{app}[c]	
pH[a]	Cefixime[b]	Ceftizoxime[b]
2·0	1·322 ± 0·051	0·415 ± 0·004
2·5	0·849 ± 0·055	0·511 ± 0·017
3·0	0·352 ± 0·011	0·459 ± 0·012
3·5	0·061 ± 0·001	0·249 ± 0·006
4·0	0·010 ± 0·0001	0·122 ± 0·002

[a] Determined at equilibrium.
[b] Initial concentration of the antibiotics was 0·05 mM.
[c] Each value represents the mean ± s.e.m. of 3–6 experiments.

Concentration-dependency of initial uptake

As observed previously for amino-β-lactam antibiotics (Nakashima et al 1984b), the initial uptake rate for cefixime at pH 5·0 as a function of the initial concentration of cefixime was curvilinear over the range of 0·05–5 mM (Fig. 2). The initial uptake rates, therefore, can be expressed by the following equation;

$$J = \frac{J_{max}(S)}{Kt + (S)} + Kd\,(S) \qquad (1)$$

where J is the initial uptake rate, (S) is the initial concentration of substrate in the medium, J_{max} is the maximum uptake rate, Kt is the Michaelis constant for saturable uptake, and Kd is the coefficient for non-saturable uptake. The solid line in Fig. 2 was

generated by using equation 1 with the NONLIN (Metzler et al 1974) fitted parameters; J_{max} = 318·0 ± 42·2 nmol min^{-1} g^{-1} of wet tissue, Kt = 1·43 ± 0·21 mM^{-1} and Kd = 11·4 ± 5·8 nmol min^{-1} g^{-1} of wet tissue mM^{-1}. These kinetic parameters were very close to those determined for cyclacillin (Nakashima et al 1984b).

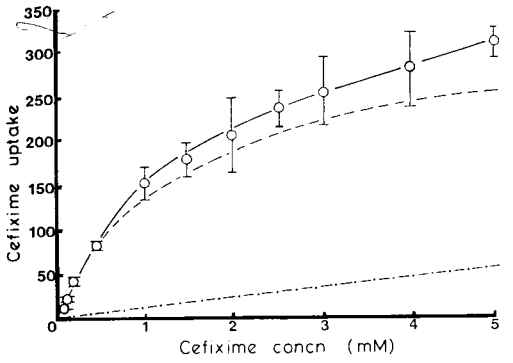

FIG. 2. Effect of cefixime concentration in the incubation medium on the cefixime uptake (nmol min^{-1} per g wet tissue) by isolated jejunum of rats. The curves were generated from equation 1, using the NONLIN fitted parameters described in the text. Solid line indicates the total uptake. The other lines indicate the carrier-mediated uptake (– – – –) and the non-mediated uptake (passive diffusion) (– · – · –). Each point shows the mean ± s.e.m. of three to seven determinations.

Inhibitions between permeants

As shown in Table 2, the uptake of 0·2 mM cefixime was significantly inhibited by 20 mM dipeptides (glycyl-L-proline and glycyl-glycine) and 20 mM amino-β-lactam antibiotics (cyclacillin and cephradine). Benzylpenicillin 20 mM and propicillin, which have been believed to be absorbed by intestinal lipoidal barrier diffusion (Tsuji et al 1978; Tsuji & Yamana 1981), also significantly inhibited the cefixime uptake. Replacement of Na^+ with choline in the buffer had only a slight but significant inhibitory effect ($P < 0·05$). Although there was no inhibitory effect of the amino acids, glycine and proline, on the cefixime uptake in the medium without Na^+, the dipeptide, glycyl-L-proline, showed significant inhibition under the same conditions.

The kinetic nature of the inhibition of cefixime uptake by the dipeptide and amino-β-lactam antibiotic was also studied with cyclacillin and glycyl-L-proline as the inhibitors and the results are given in Fig. 3 as Lineweaver-Burk plots. The effects of both cyclacillin and glycyl-L-proline on the cefixime uptake were to increase the apparent Kt for cefixime without altering J_{max}. Therefore, the inhibition of both compounds is clearly competitive. The inhibition constants (Ki) for cyclacillin and glycyl-L-proline were evaluated to be 3·83 ± 0·27 and 3·87 ± 0·58 mM, respectively, by NONLIN computer program incorporating the competitive inhibition kinetics. The Ki values obtained for cyclacillin and glycyl-L-proline are comparable with the Kt value of 1·15 mM at pH 7·0 reported for cyclacillin in rats (Nakashima et al 1984b) and that of 0·93 mM at pH 7·2 for glycyl-L-proline in rabbits (Rubino et al 1971).

Table 2. Effect of dipeptides (20 mM), amino acids (20 mM) and β-lactam antibiotics (20 mM) on the uptake rate of cefixime (0·2 mM) by isolated jejunum of rats in the presence and absence of Na^+.

Inhibitor	Uptake rate of cefixime J (nmol min^{-1} g^{-1} wet weight)[a]	Percentage of control	
		P^b	P^c
In the presence of Na^+[d]			
Non (control)	39·90 ± 6·67 (4)	100	
Glycyl-L-proline	8·06 ± 1·67 (4)	20	<0·05
Glycyl-glycine	14·69 ± 2·32 (4)	37	<0·05
Cyclacillin	9·23 ± 3·82 (4)	23	<0·05
Cephradine	16·04 ± 1·65 (4)	40	<0·05
Benzylpenicillin	22·13 ± 4·93 (4)	55	<0·05
Propicillin	11·99 ± 0·62 (4)	30	<0·05
In the absence of Na^+[e]			
Non (control)	24·15 ± 1·40 (5)	61	<0·05
Glycine	22·46 ± 1·93 (3)		93 N.S.[f]
Proline	26·25 ± 3·13 (4)		109 N.S.
Glycyl-L-proline	12·49 ± 0·99 (4)		52 <0·05

[a] Each value represents the mean ± s.e.m. The number of observations are given in parentheses.
[b] Statistical risk, compared with the control uptake rate in the presence of Na^+.
[c] Statistical risk, compared with the control uptake rate in the absence of Na^+.
[d] Determined in the medium of pH 5·0 containing 118 mM Na^+.
[e] Determined in the medium of pH 5·0 in which Na^+ was displaced with choline chloride.
[f] Not significant.

DISCUSSION

The apparent pH-dependence of cefixime uptake by the rat everted intestine (Fig. 1) may be ascribed to an increase in the fraction of the lipophilic form of cefixime which is more permeable through lipid membrane according to the pH-partition hypothesis and/or an increase of permeability of cefixime at low pH by some specialized transport mechanism. Since the apparent partition-coefficient (P_{app}) of cefixime is comparable with that of the poorly absorbable ceftizoxime (Table 1), and pH-dependence of the P_{app} value of cefixime showed the distinct shape from the pH uptake rate curve of cefixime, it is safe to say that an increase in lipid solubility of cefixime contributes little to its intestinal uptake dependent on pH. Therefore, the second possibility is more

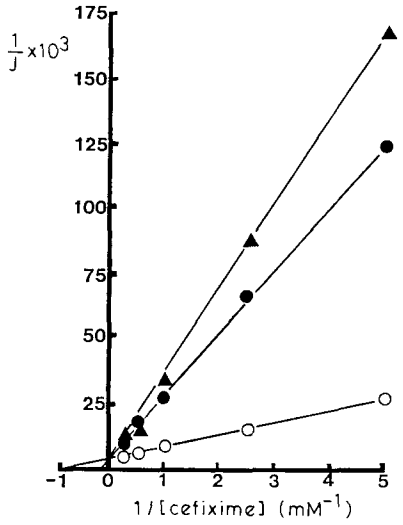

FIG. 3. Lineweaver-Burk plots of uptake of cefixime by isolated jejunum of rats in the absence (○, control) and in the presence of 20 mM cyclacillin (●) and 20 mM glycyl-L-proline (▲). The values are corrected for non-mediated uptake. The line for the control experiment was calculated from the mediated uptake parameters (J_{max}, Kt) described in the text. Each point shows the mean of three to five determinations. Units of J are nmol min^{-1} per g wet tissue.

likely to participate in the intestinal uptake of cefixime.

As shown in Figs 2 and 3 and Table 2, all the kinetic evidence, the saturable uptake and the inhibitory effects, suggest that cefixime is transported by the intestinal brush-border membrane via a peptide carrier system(s) which has been claimed by several investigators (Nakashima et al 1984a, b; Nakashima & Tsuji 1985) to play a role in the transport systems for amino-β-lactam antibiotics. Recent investigations (Ganapathy & Leibach 1983; Ganapathy et al 1984; Takuwa et al 1985) using intestinal brush-border membrane vesicles (BBMVs), showed that small peptides (di or tripeptide) are co-transported with H^+ and that the peptide transport is Na^+-independent and dependent on pH gradient. However, Addison et al (1972) and Matthews et al (1974) using everted intestinal tissue showed that transport of dipeptide is Na^+-dependent. The present observation in intact tissue preparations shows a significant reduction in uptake of cefixime when Na^+ is replaced with choline. Although Na^+-dependent transport of cefadroxil, one of the amino-cephalosporins, was observed in uptake by everted intestinal tissue (Kimura et al 1983), the same authors found Na^+-independent transport of cefadroxil in BBMVs (Kimura et al 1985). These discrepancies in Na^+ effects on intestinal transport of peptides and amino-β-lactam antibiotics between the experiments using intact tissues or cells and BBMVs may be due to the participation of Na^+, K^+-ATPase and Na^+-H^+ exchanger and, in part, to solvent drag driven by Na^+-dependent bulk flow of fluid (Hoshi 1985), both of which take place only in intact tissue cells.

In view of the significant inhibition of uptake of cefixime by a dipeptide (glycyl-L-proline), but the lack of an effect by the constituting amino acids in Na^+-free medium (Table 2) and the clear pH-dependent uptake (Fig. 2), it is anticipated that the pH gradient between the mucosal side and intracellular fluid stimulated cefixime absorption, or the acidic environment around the mucosal surface enhances its absorption. Although the luminal bulk pH of the intestine is considered to be unfavourable for cefixime absorption after oral administration, the existence of a microclimate pH of 5–6 (Lucas et al 1975) in the close vicinity of the intestinal brush-border membrane seems to facilitate the cefixime transport in-vivo.

This is the first report showing evidence of saturable uptake of cephem antibiotics without an α-amino group in the side chain, most likely via a dipeptide carrier system(s). The present results suggest that some β-lactam antibiotics which have no α-amino group in their side chain can be absorbed via dipeptide carrier(s), if the antibiotics regardless of the existence of an α-amino group have a sufficient affinity to the carrier system(s). The above speculation may be supported by the observation of the significant inhibition of cefixime uptake by benzylpenicillin and propicillin (Table 2). This also suggests that carrier-mediated transport through the intestine may, in part, contribute to the absorption of benzylpenicillin and propicillin, in addition to the simple diffusion through the intestinal lipoidal barrier as shown previously (Tsuji et al 1978; Tsuji & Yamana 1981).

However, the apparent saturation kinetics interpreted here as evidence for carrier-mediated transport could be produced by saturable binding at the mucosal surface or other kinetically equivalent mechanisms.

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