EVIDENCE FOR A CARRIER-MEDIATED TRANSPORT SYSTEM IN THE SMALL INTESTINE AVAILABLE FOR FK089, A NEW CEPHALOSPORIN ANTIBIOTIC WITHOUT AN AMINO GROUP

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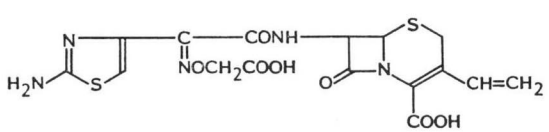
Transport of a new cephalosporin developed for oral use, FK089, has been studied with the rat everted small intestine *in vitro*. Uptake was found to be pH-dependent with the maximum rate at an acidic pH below 5 and with a 5-fold lower rate at pH 7.0. The shape of the pH-rate profile was very similar to that of cefixime and different from that of pH-lipophilicity profile of FK089. The saturation kinetics of the uptake of FK089 were demonstrated at pH 5.0. By correcting the nonsaturable rate process, the kinetics of the mutual inhibition of FK089 uptake by cefixime and cefixime uptake by FK089 were all consistent with competitive type inhibition. The results indicate that carrier-mediated transport is responsible for transport of cephem antibiotics without an α-amino group in the side chain at the 7-position of the cephem nucleus in the intestinal brush-border membrane.

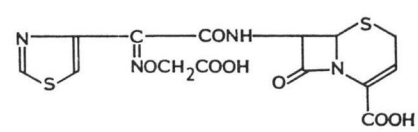
The oral cephalosporins available are all cephalexin type analogs having α-amino group in the side-chain at the 7-position of the cephem nucleus. Convincing evidence has accumulated that these amino-β-lactam antibiotics can be absorbed by the small intestine *via* a dipeptide carrier system(s).[1~6] Most recently, we have demonstrated,[7] using the rat intestinal everted sac method, that cefixime (Fig. 1), a new α-amino group deficient cephalosporin for oral use, can cross the intestinal brush-border membrane by utilizing a dipeptide carrier system(s), as in the case of amino-β-lactam antibiotics.

During the course of our research on the structural requirement of β-lactam antibiotics to be recognized by such dipeptide transporters in the intestinal brush-border membrane, we found another new oral cephalosporin, FK089, (6*R*,7*R*)-7-[(*Z*)-2-(4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo-5-thia-1-azabicyclo(4,2,0)oct-2-ene-2-carboxylic acid (Fig. 1). Single oral doses of 125 and 250 mg of FK089 in healthy volunteers give the mean peak serum concentrations of 1.52 and 2.16 μg/ml respectively within 2~4 hours and urinary recoveries during 24 hours of 43.7 and 33.6%, respectively, in a dose dependent manner.[8] In view of the interesting structure of FK089 in its lack of free amino group on the thiazole moiety at the 7-position and a vinyl group at the 3-position of cefixime, we studied the mechanism of intestinal transport of FK089 in comparison with that of cefixime.

Fig. 1. Structures of FK089 and cefixime.



Cefixime



FK089

Materials and Methods

Compounds

FK089 and cefixime were kindly supplied by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. All other chemicals were of the highest grade available commercially and used without further purification.

Uptake Experiments

Male Sprauge-Dowley rats (200~250 g) were fasted for about 20 hours prior to experiment and had free access to water. The preparation of everted intestine and the method of influx measurement were the essentially the same as those described in our previous paper.[5] The amounts taken up were corrected with the value of the volume of extracellular fluid adhering to the mucosal surface (inulin space) which has determined by using [^{14}C]inulin in each experiment under the same experimental conditions.

The incubation medium was a modified Krebs-Ringer Tris buffer solution of pH 5.0 containing 118 mM NaCl, 25 mM Tris, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 9.2 mM citric acid. In the uptake experiments at various pHs, varying proportions of Tris and citric acid were used to achieve and maintain the desired pH.

Determination of the Partition-coefficient

For the determination of the apparent partition-coefficient (Papp) of FK089 in 2-methylpropanol - water system, aqueous phase was buffered with citrate to maintain the desired pH of 2.0~4.5. In each aqueous phase, the Na^+ concentration was held constant at 0.1 M by the addition of sodium chloride. FK089 was 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. FK089 in aqueous phase at partition equilibrium was assayed by HPLC method. The detailed procedures for the determination and the calculation of the partion-coefficient were described previously.[9]

Analytical Procedures

The amount of FK089 or cefixime taken up by the intestinal tissue was assayed by HPLC method. The intestinal tissue was homogenized in a homogenizer (Ultra Turrax, Ika-Werk, Janke & Kunkel) with 1/15 M phosphate buffer (pH 7.4) to give a 20% (w/v wet weight) homogenate after centrifugation of 15,600×g for 15 minutes, the amount of FK089 or cefixime in the supernatant fraction was determined after deproteinization with acetonitrile (at a volume ratio of 1 to 1) by HPLC.

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 and with a reversed phase column (3.9 mm × 30 cm, μBondapak C_{18}, Waters Associate, Milford, Mass.) packed in this laboratory. A guard column, C_{18}/Corasil (Waters Associate) was used between the analytical column and the injector. The mobile phase was acetonitrile - water system, 30 : 70 for FK089 and 25 : 75 for cefixime containing 10 mM tetra-*N*-butylammonium bromide, 10 mM ammonium acetate and 167 mM acetic acid. The injection volume was 20 μl. The peak areas recorded with an integrator (model Chromatopak C-R3A, Shimadzu Co., Kyoto, Japan) were used for quantification. Standard curves were generated after similar deproteinization with acetonitrile by the use of blank tissue sample containing known amount of FK089 and cefixime and were linear between the range of 10 to 1,000 nmol/g of wet tissue weight with a coefficient of variation of 4.5% for cefixime and 6.1% for FK089 including a day-to-day variation of the slopes.

In a study for determination of partition-coefficient of FK089, the mobile phase was 5% acetonitrile - 95% water containing 10 mM ammonium acetate.

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-(5-phenyloxazole)]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

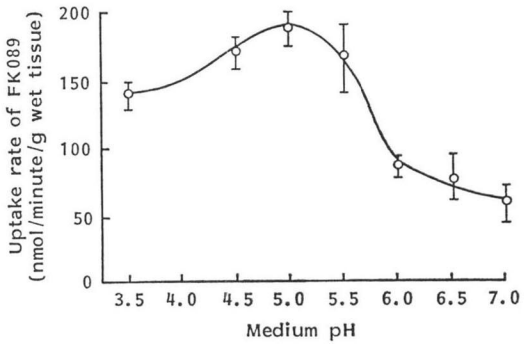
Table 1. Apparent partition-coefficient (Papp) of FK089 between 2-methylpropanol and water as a function of pH of the aqueous phase at 37°C.

pH[a]	Papp[b]
2.0	1.535±0.119
3.0	0.454±0.016
3.5	0.064±0.002
4.0	0.017±0.002
4.5	0.004±0.001

[a] Determined at equiliblium.
[b] Initial concentration of FK089 was 0.05 mM. Each value represents the mean±S.E.M. of 3~6 experiments.

Fig. 2. Effect of pH of the incubation medium on the rate of 1 mM FK089 uptake by isolated jejunum of rats.

Each point shows the mean±S.E.M. of four determinations.



scintillation counter (model LSC-700, liquid scintillation counter, Aloka Co.).

Data Analysis

The kinetic parameters for the uptake of FK089 were estimated by solving the following equations by a nonlinear least-square method, using NONLIN computer program.[10]

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

where J_{max} and Kt are the maximum uptake rate and Michaelis constant, respectively, for mediated uptake and Kd is the coefficient for nonmediated uptake. Statistical comparisons were made with Student's t-test. P value of <0.05 was accepted as significant.

Results

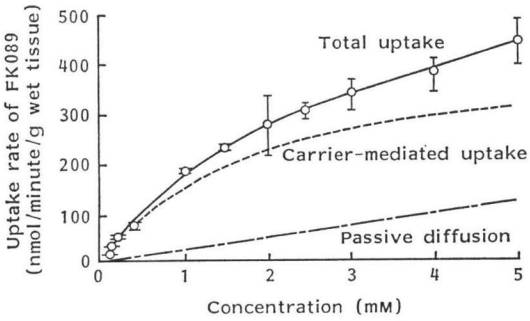
The effect of pH on the rate of uptake of FK089 (1 mM) by the rat everted intestine is shown in Fig. 2. As seen in this figure, maximum uptake was observed at an acidic pH around 5. At neutral pH 7, the rate was significantly reduced compared with the rates at the pH of 3.5, 4.5, 5 and 5.5 ($P<0.05$). The shape of the pH-rate profile and the magnitude of the rate for FK089 uptake is very similar to those obtained previously for cefixime under the same conditions.[7] There was no direct relationship between the pH-dependency for the intestinal uptake rate of FK089 and that for the apparent partition-coefficient of this antibiotic determined in the 2-methylpropanol - water system (Table 1). The pH-dependent uptake of FK089 suggests the participation of some specialized transport mechanism rather than lipid membrane transport mechanism according to the pH-partition theory.

The initial uptake rates for cefixime at pH 5.0, which were calculated from the amount determined at 2 minutes, are plotted in Fig. 3 against the concentration of FK089 in the incubation medium. As observed previously for amino-β-lactam antibiotics,[5] the uptake rate was curvilinear over the concentration range of 0.05~5 mM. The solid line in Fig. 3 was generated by using equation 1 with the NONLIN[10] fitted parameters; $J_{max}=413.4\pm97.8$ nmol/minute/g wet tissue, $Kt=1.73\pm0.42$ mM and $Kd=24.1\pm12.3$ nmol/minute/g wet tissue/mM.

The results of a mutual inhibition study of FK089 and cefixime are given in Figs. 4 and 5 as Lineweaver-Burk plots for saturable uptake. In the presence of 10 mM cefixime, the uptake of FK089 was inhibited competitively. Furthermore, in the presence of FK089, cefixime uptake was also inhibited competitively. The inhibition constant (Ki) of FK089 for cefixime uptake and that of cefixime for FK089 uptake were evaluated to be 2.01 ± 0.25 and 1.29 ± 0.06 mM, respectively. The values of

Fig. 3. Effect of FK089 concentration in the incubation medium on the FK089 uptake by isolated jejunum of rats at the pH of 5.0.

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 mediated uptake (---) and the nonmediated uptake (- - -). Each point shows the mean±S.E.M. of three to seven determinations.



Ki are in good agreement with the *Kt*, determined for each antibiotic (*Kt*=1.73 mM for FK089 and *Kt*=1.43 mM for cefixime[7]) under the same condition.

All the kinetic evidence obtained in this study indicates that FK089 and cefixime are transported *via* a common carrier-system(s) in the intestinal brush-border membrane.

Discussion

Orally active cephalosporins such as cephalexin and its analogs are less active against Gram-negative bacteria and less stable to β-lactamases than injectable second-generation and newly developed cephalosporins. Although much attention has been focused on searching for orally active cephalosporins with a broad spectrum, high potency and significant resistance to β-lactamases, there was no report on such orally active cephalosporins before recent findings for FK089[8] and cefixime.[11]

Fig. 4. Lineweaver-Burk plots of uptake of FK089 at the pH of 5.0 by isolated jejunum of rats in the absence (○) and in the presence (●) of 5 mM cefixime.

The values are corrected for nonmediated 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 six determinations.

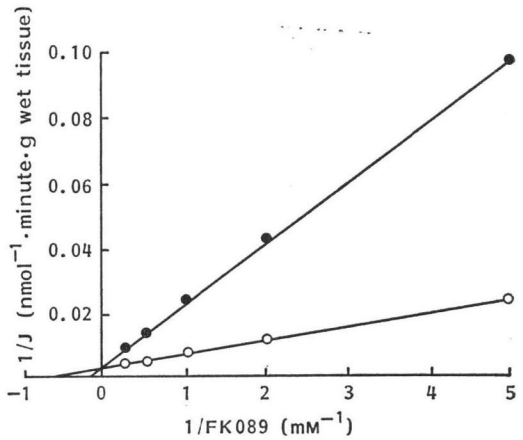
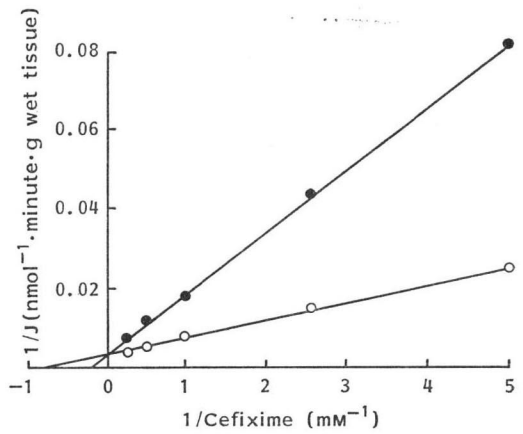


Fig. 5. Lineweaver-Burk plots of uptake of cefixime at the pH of 5.0 by isolated jejunum of rats in the absence (○) and in the presence (●) of 5 mM FK089. For detailes see Fig. 4.



On the basis of the previous transport studies performed in our laboratory by using intact animals and *in vitro* everted intestinal techniques,[3~5,12~16] the mechanism for the absorption of β-lactam antibiotics from the alimentary tract can be summarized as follows. The antibiotics containing highly lipophilic penicillins such as phenoxy and isoxazole derivatives can cross the brush-border membrane by the rate-limiting processes of lipid membrane barrier and the unstirred water layer existing in front of the mucosal surface.[12,13,16] This passive transport process is commonly available for highly lipid-soluble drugs. Several prodrugs of low lipophilic penicillins and parenteral cephalosporins are con-

sidered to be transported by this simple diffusion route.[13,16,17] By contrast, amino-β-lactam antibiotics such as amoxicillin, ciclacillin, cephalexin, cephradine and cefadroxil, which have very low lipophilicity and a zwitterionic structure, can cross *via* a dipeptide carrier-system(s) in the intestinal brush-border membrane.[1~6] Such carrier-mediated transport of β-lactam antibiotics across the intestine has been generally believed to be responsible only for β-lactam antibiotics having an α-amino group in the side-chain at the 6-position of penicillins or the 7-position of cephem antibiotics. However, our previous study[7] using the rat intestinal everted sac technique showed evidence that cefixime, of which the structure is quite different from that of amino-β-lactam antibiotics, can be absorbed also *via* the dipeptide carrier-system(s).

FK089 has two ionizable carboxylic acids (both *pKa*'s are less than 4). As seen from the value of the apparent partition-coefficient of FK089 as a function of pH (Table 1), this antibiotic becomes lipid soluble below pH 3, whereas above this pH the lipophilicity decreases remarkably with increase of the fractions of monoanionic and dianionic species. The apparent elevation of the maximum rate for the intestinal uptake of FK089 in the vicinity of pH 5, where this antibiotic is almost completely ionized and has very low lipid solubility, could not be explained by a contribution of the rate-limiting transport across the lipid barrier of the intestinal brush-border membrane.

In the present study, we obtained evidence that FK089 possessing a structure very similar to that of cefixime and lacking any amino group can be transported also *via* a carrier-mediated process. The results on the competitive inhibition kinetics obtained in this study for FK089 uptake by cefixime and cefixime uptake by FK089 and those reported previously[7] for cefixime uptake by ciclacillin and glycyl-L-proline indicate strongly that these three antibiotics and the dipeptide can share the same carrier-system(s).

The maximum uptake of FK089 by the *in vitro* everted intestine occurred at an acidic pH around 5. Although the luminal bulk pH condition in the intestine is unfavorable for the absorption of both antibiotics after oral administration, the existence of a microclimate pH of 5.9~6.0[18] in the close vicinity of the brush-border membrane of enterocytes is thought to facilitate the transport of FK089 and cefixime *in vivo*.

In conclusion, FK089 can be transported in a pH-dependent manner across the intestinal brush-border membrane *via* a carrier-mediated system which is a transporter of dipeptide, cefixime and amino-β-lactam antibiotics.

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