Restricted Intestinal Absorption of Some β-Lactam Antibiotics by an Energy-Dependent Efflux System in Rat Intestine

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Purpose. The purpose of this study was to examine factors limiting the intestinal absorption of orally inactive β-lactam antibiotics.
Methods. Permeation behaviors of various β-lactam antibiotics across rat intestinal segments were evaluated in vitro using diffusion cells.
Results. Poorly absorbed β-lactam antibiotics, like cephaloridine and cefoperazone, commonly exhibit greater serosal-to-mucosal permeation than mucosal-to-serosal permeation, while cephalexin permeation was greater in the mucosal-to-serosal direction. In the absence of D-glucose, secretory-oriented permeation of cephaloridine and cefoperazone disappeared. Addition of sodium azide into an experimental buffer including D-glucose significantly and selectively enhanced mucosal-to-serosal permeation of cephaloridine and cefoperazone. Although benzylpenicillin, ampicillin, and amoxicillin all showed secretory-oriented permeation, the tendency to permeation was greatest with benzylpenicillin and least with amoxicillin. Probenecid stimulated mucosal-to-serosal permeation of cephaloridine, but verapamil and p-aminohippuric acid had no significant effect on it.
Conclusions. It has been suggested that mechanisms which induce secretory-oriented permeation of orally inactive β-lactam antibiotics are factors limiting intestinal absorption of such antibiotics. This energy-demanding efflux system was distinct from P-glycoprotein-mediated transport. A free α-amino group in the molecule is an important factor for reducing an affinity with the efflux system.

KEY WORDS: β-lactam antibiotics; intestinal efflux; α-amino group; restricted absorption.

INTRODUCTION

There are remarkable differences in oral bioavailabilities among β-lactam antibiotics, ranging from poorly absorbed to completely absorbed (1). Generally, β-lactam antibiotics with a free α-amino group are well absorbed. Currently, these differences are explained by the hypothesis, derived from many studies using membrane vesicles, that well absorbed β-lactam antibiotics are transported as substrates of an intestinal oligopeptide transporter, whereas poorly absorbed analogs are not (2). However, it seems difficult to suppose that almost complete absorption of some β-lactam antibiotics depends on the peptide transport system alone, because membrane permeation of orally active β-lactam antibioticsis takes place via a non-saturable (passive) process when carrier-mediated transport is saturated (3). It has also been reported that passive diffusion significantly contributes to the total transport of cephalexin and cephradine (4).

Since most of the β-lactam antibiotics, including poorly absorbed analogs, possess similar degrees of hydrophilicity, the extent of passive diffusion of β-lactam antibiotics is not expected to vary greatly. Accordingly, the extremely low oral bioavailability of some β-lactam antibiotics like cefazolin and cephaloridine is curious. In addition, even relatively hydrophobic cefoperazone is not absorbed after oral administration.

Recently, we have shown in vitro that cefazolin, an orally inactive β-lactam antibiotic, was transported much greater amounts in the secretory direction than in the absorptive direction in the entire rat intestine, while orally active cephradine and cefaclor did not show such secretory-oriented permeation (5). Moreover, the permeation rate of cefazolin in the secretory direction was significantly greater than those of cephradine and cefaclor. Therefore, we have offered a possibility that a specialized efflux system plays an important role in the low oral bioavailability of cefazolin; that is, this system may restrict cefazolin absorption in vivo. This also implies that poor absorption of other β-lactam antibiotics might be due to transport by this intestinal efflux system. The present study has been undertaken to examine this possibility. Cephradine and cefaclor are amino β-lactam antibiotics. On the other hand, cefazolin lacks a free α-amino group. It was, therefore, necessary to evaluate the role of the α-amino group in the interaction between β-lactam antibiotics and the efflux system.

The results obtained are very consistent with those of cefazolin, demonstrating that intestinal absorption of poorly absorbed β-lactam antibiotics is due to the involvement of an energy-demanding efflux system, and that a free α-amino group on the side chain of a β-lactam antibiotic might be a determinant of an affinity for the efflux system.

MATERIALS AND METHODS

Cephalexin, benzylpenicillin potassium, (±)-verapamil HCl, D-glucose, 3-o-methyl-D-glucose, p-aminohippuric acid, and probenecid were obtained from Wako Pure Chem. Ind. (Osaka, Japan). Cefoperazone was purchased from Sigma Chemical Co. (St. Louis, MO). Cephaloridine, ampicillin anhydrous, and amoxicillin were kindly donated. Structures of the β-lactam antibiotics tested are presented in Fig. 1. All other reagents were of the highest grade available.

Permeation experiments were performed as described previously (6) according to Grass and Sweetana (7) using diffusion cells from Costar. All permeation experiments were done with 1 mM drug solution.

Drug concentrations in the receiving chamber samples were determined by HPLC (Hitachi 655A-11) using UV absorbance detection (Hitachi 638-041), and the cumulative amount of drug permeating the intestine was calculated and plotted vs. time for each experiment. The permeation rate was determined by linear regression. Statistical comparisons were made using *t*-tests.

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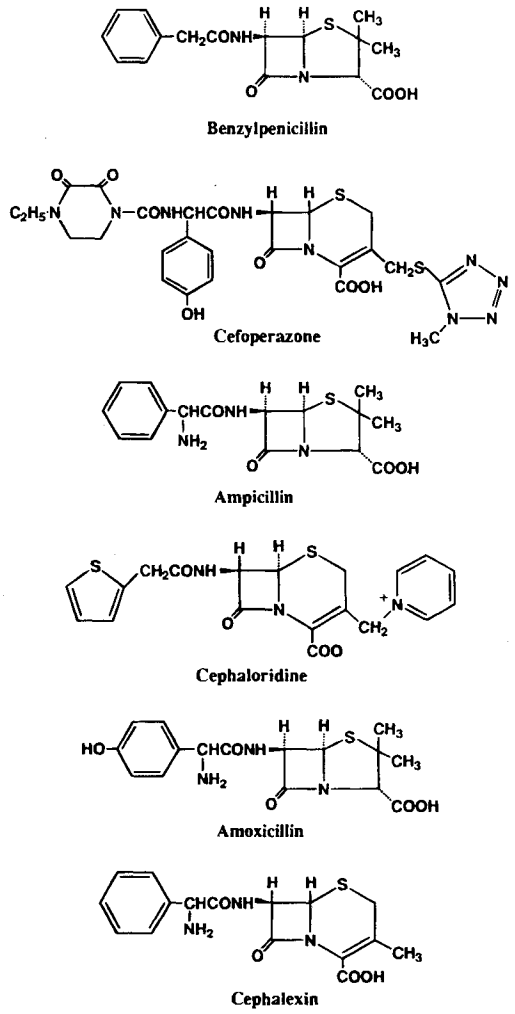

Fig. 1. Structures of β-lactam antibiotics tested.

HPLC conditions were as follows, a Nova-Pak C-18® (5 μm, 3.9 × 150 mm, Waters, Milford, MA) column was used at ambient temperature. Mobile phases were arranged according to compounds: acetonitrile/water/trifluoroacetic acid (15/85/0.1, pH 3) for cephaloridine and cefoperazone; methanol/0.01 M sodium acetate (20/80) for cephalexin and ampicillin; methanol/0.01 M sodium acetate (10/90) for amoxicillin; and methanol/0.01 M sodium acetate (35/65) for benzylpenicillin. Flow rate was 0.6 to 0.8 ml/min. The wavelength was 220 nm for ampicillin, amoxicillin, and benzylpenicillin; 230 nm for cefoperazone; 240 nm for cephaloridine; and 262 nm for cephalexin, respectively. The injection volume was 50 μl.

RESULTS

Intestinal Permeabilities of β-Lactam Antibiotics

In vitro intestinal permeation in mucosal-to-serosal (absorptive) and serosal-to-mucosal (secretory) directions were compared for cephaloridine, cefoperazone, and cephalexin. Cephaloridine and cefoperazone were models of orally inactive cephalosporins. Cephalexin is well absorbed in the small intestine. As shown in Table I, the permeation rates of cephaloridine and cefoperazone were 2 to 3 fold greater in serosal-to-mucosal direction than in mucosal-to-serosal direction, suggesting that the net movement of these drugs across rat intestine was secretory-oriented. In cephalexin, on the other hand, mucosal-to-serosal permeation was greater than serosal-to-mucosal permeation, showing that cephalexin permeation was absorptive-oriented. It was considered that this absorptive-oriented permeation leads to the good oral bioavailability of cephalexin. The permeation behaviors of cephaloridine and cefoperazone were similar to that of cefazolin, as recently reported (5). Therefore, secretory-oriented intestinal permeation is a common feature of orally inactive cephalosporins. It should be noted that serosal-to-mucosal permeation of cephaloridine and cefoperazone was greater than that of cephalexin in spite of their lower mucosal-to-serosal permeation as compared with cephalexin.

Table I. Comparison of Mucosal-to-Serosal (M-to-S) and Serosal-to-Mucosal (S-to-M) Permeation Rates for Three Cephalosporins Using 1 mM Drug, pH 7.4 Mucosal and Serosal Buffers, and Rat Jejunum

	Permeation rate (nmol/min)[a]		
	M-to-S	S-to-M	S-to-M/M-to-S
Cephaloridine	0.472 ± 0.052	1.064 ± 0.070[b]	2.25
Cefoperazone	0.237 ± 0.011	0.812 ± 0.073[b]	3.43
Cephalexin	0.914 ± 0.046	0.671 ± 0.059[c]	0.73

[a] Mean ± S.E. of 3 or more experiments.
[b] $p < 0.001$, significantly different from M-to-S.
[c] $p < 0.05$, significantly different from M-to-S.

Figure 2 illustrates the effect of the replacement of D-glucose in Tyrode's buffer with 3-o-methyl-D-glucose on the jejunal permeation of three cephalosporins. In cephaloridine and

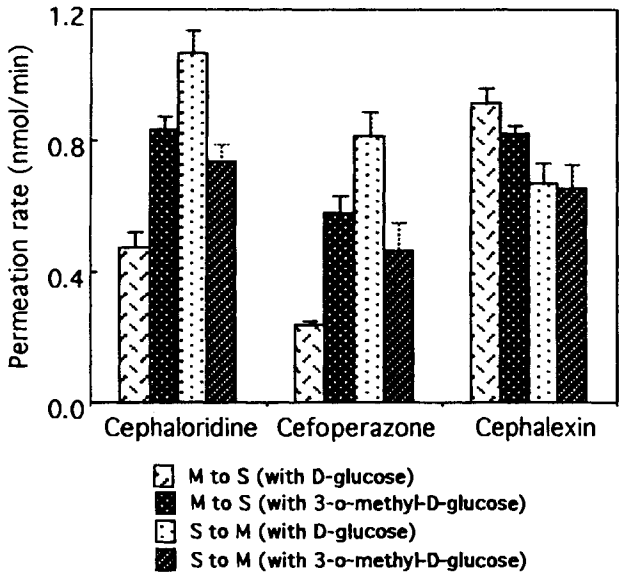

Fig. 2. Effects of 3-o-methyl-D-glucose instead of D-glucose on mucosal-to-serosal (M to S) and serosal-to-mucosal (S to M) permeation of three β-lactam antibiotics across rat jejunum. Drug permeation was determined from 1 mM solution at pH 7.4. Each column represents the mean ± SE of 4 experiments.

cefoperazone, mucosal-to-serosal permeation was significantly increased and serosal-to-mucosal permeation decreased. As a result, secretory-oriented permeation of both antibiotics was abolished. On the other hand, both rates of mucosal-to-serosal and serosal-to-mucosal permeation of cephalexin were not affected significantly. Therefore, D-glucose was necessary for secretory-oriented intestinal permeation of cephaloridine and cefoperazone.

Sodium azide is known as a metabolic inhibitor. When 10 mM sodium azide was added to Tyrode's buffer, including 6 mM D-glucose, the permeation rate of cephaloridine in the mucosal-to-serosal direction increased from 0.47 ± 0.05 to 0.73 ± 0.09 (mean ± S.E.) nmol/min, while that of cephalexin remained the same (0.91 ± 0.05 vs. 0.91 ± 0.13). D-Glucose is used as an energy source while sodium azide inhibits energy-production. Accordingly, the enhancing effect of sodium azide on mucosal-to-serosal permeation of cephaloridine indicates that the secretory-oriented permeation was an energy-demanding process.

Effects of Verapamil on Mucosal-to-Serosal Permeation of Cephaloridine and Cefoperazone

We recently reported that intestinal absorption of a water-soluble cyclic peptide was restricted by an intestinal P-glycoprotein-mediated secretory transport (6). If secretory-oriented permeation of a poorly absorbed β-lactam antibiotic was mediated by P-glycoprotein, the addition of verapamil would enhance mucosal-to-serosal permeation. According to our previous study (6), verapamil exhibited its greatest effect in inhibiting intestinal P-glycoprotein at 0.2 mM. However, 0.2 mM verapamil did not alter the permeation behaviors of cephaloridine, cefoperazone, or cephalexin significantly (data not shown). Verapamil similarly had no effect on the secretory-oriented permeation of cefazolin (5). Therefore, the efflux system responsible for secretory transport of orally inactive cephalosporins was not P-glycoprotein-mediated.

The Role of Free α-Amino Group in Intestinal Permeabilities of Penicillins

Table II demonstrates permeation behaviors of three penicillins, benzylpenicillin, ampicillin, and amoxicillin. Benzylpenicillin lacks a free α-amino group. Each of these three antibiotics had greater serosal-to-mucosal permeation than mucosal-to-serosal permeation. However, the tendency toward secretory-oriented permeation was most remarkable in benzylpenicillin. Since degradation of benzylpenicillin in the mucosal solution was negligible, preferential serosal-to-mucosal permeation was not due to a decrease in the mucosal concentration. The serosal-to-mucosal/mucosal-to-serosal permeation rate ratio was much lower for ampicillin than for benzylpenicillin, with the only structural difference being an α-amino group. This resulted from a slight increase in absorptive permeation and significant decrease in secretory permeation relative to benzylpenicillin. In the case of amoxicillin, which has a hydroxy group attached to the ampicillin structure, the difference in permeation rates in mucosal-to-serosal and serosal-to-mucosal directions became even smaller. These results suggest that the presence of a free α-amino group may influence the interaction of these antibiotics and the intestinal efflux system.

Mutual Interaction Between Cephaloridine and Benzylpenicillin

Table III presents the results of the mutual interaction studies for jejunal permeation of cephaloridine and benzylpenicillin. Mucosal-to-serosal permeation of 1 mM cephaloridine was significantly enhanced in the presence of 10 mM benzylpenicillin. However, the same concentration of benzylpenicillin did not significantly alter cephalexin permeation. These results possibly suggest that the increase in absorptive permeation of cephaloridine was due to a competitive inhibition of secretory transport by benzylpenicillin. Since it is still unclear why 10 mM cephaloridine did not affect significantly the permeation of 1 mM benzylpenicillin, another study should be undertaken using higher concentrations of cephaloridine.

Effects of Probenecid and p-Aminohippuric Acid on the Mucosal-to-Serosal Permeation of β-Lactam Antibiotics

It has been reported that various β-lactam antibiotics are transported by the renal organic anion transport system (8), which is known to be inhibited by probenecid. As shown in Fig. 3, mucosal-to-serosal permeation of benzylpenicillin, cephaloridine, and ampicillin were each significantly enhanced in the presence of 5 mM probenecid. This enhancement was 200% or more of control. Although cephalexin permeation was slightly increased in the presence of probenecid, the enhancement was only 20%, indicating that the enhancing effect of probenecid was much more remarkable for secreted β-lactam antibiotics. Probenecid itself had much greater mucosal-to-

Table II. Comparison of Mucosal-to-Serosal (M-to-S) and Serosal-to-Mucosal (S-to-M) Permeation Rates for Three Penicillins Using 1 mM Drug, pH 7.4 Mucosal and Serosal Buffers, and Rat Jejunum

	Permeation rate (nmol/min)[a]		
	M-to-S	S-to-M	S-to-M/M-to-S
Benzylpenicillin	0.419 ± 0.053	1.026 ± 0.170[b]	2.45
Ampicillin	0.477 ± 0.066	0.778 ± 0.061[c]	1.63
Amoxicillin	0.548 ± 0.031	0.787 ± 0.024[d]	1.44

[a] Mean ± S.E. of 4 experiments.
[b] $p < 0.001$, significantly different from M-to-S.
[c] $p < 0.01$, significantly different from M-to-S.
[d] $p < 0.05$, significantly different from M-to-S.

Table III. Mutual Interaction among β-Lactam Antibiotics in Mucosal-to-Serosal Permeation across Rat Jejunum

	Permeation rate (nmol/min)[a]		
Additives	Cephaloridine	Benzylpenicillin	Cephalexin
Control	0.473	0.419	0.903
10 mM Benzylpenicillin	0.830 (176)[b]	—	0.950 (105)
10 mM Cephaloridine	—	0.426 (102)	—

Note: Experiments were done with 1 mM drug at pH 7.4.
[a] Mean of 4 experiments.
[b] % of control in parentheses.

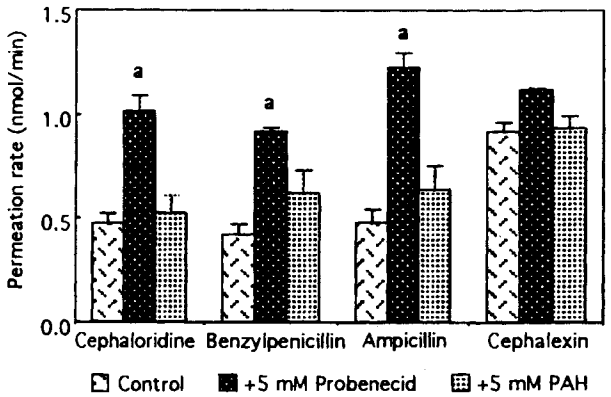

Fig. 3. Effects of probenecid and p-aminohippuric acid on mucosal-to-serosal permeation of four β-lactam antibiotics. Drug permeation was determined from 1 mM solution at pH 7.4. The concentration of probenecid and p-aminohippuric acid was 5 mM. Each column represents the mean ± SE of 4 experiments. a; $p < 0.01$ significantly different from control.

serosal permeation than serosal-to-mucosal permeation through rat intestine, so the secretory-oriented transport of probenecid is not significant (5). In order to further examine the relevance of renal organic anion transport, the effect of 5 mM p-aminohippuric acid (PAH) on the mucosal-to-serosal permeation of cephaloridine, benzylpenicillin, ampicillin, and cephalexin was evaluated. PAH is a typical substrate of organic anion transport in the kidney. However, PAH had no significant effect on the intestinal permeation of these four antibiotics, suggesting that a specialized efflux system for β-lactam antibiotics in the intestine is distinct from renal anion transport, at least in substrate specificity.

DISCUSSION

In this study, orally inactive β-lactam antibiotics, like cephaloridine and cefoperazone, exhibited secretory-oriented permeation in rat intestine. Interestingly, serosal-to-mucosal permeation of these two antibiotics was greater than that of orally active cephalexin, in spite of their lower permeation in the absorptive direction. Moreover, replacing D-glucose with 3-o-methyl-D-glucose abolished the directional dependence of cephaloridine permeation, indicating that D-glucose plays an important role in the secretory transport of cephaloridine and cefoperazone. Moreover, the directional dependence of permeation rates was also reduced in the presence of sodium azide, a metabolic inhibitor. On the other hand, well absorbed cephalexin exhibited absorptive-oriented permeation, and glucose removal and sodium azide had no significant effects on its permeation. These findings provide evidence of the involvement of a specialized efflux system, which is an energy-demanding process, in impeding the absorption of cephaloridine and cefoperazone; that is, the efflux system is in part responsible for low bioavailability of orally inactive β-lactam antibiotics. This efflux system appears distinct from the P-glycoprotein-mediated transport system, because verapamil failed to improve absorptive permeation of these antibiotics.

Although the present observations do not fully characterize the intestinal efflux system involved, several interesting aspects were demonstrated. Previously, an α-amino group was regarded as a key structural component for interaction with the absorptive peptide transporter on the brush-border membrane. However, this idea has been disproven with the development of orally active β-lactam antibiotics without a free α-amino group, such as ceftibuten and cefixime. These α-amino lacking analogs are also reported to be transported by the peptide transport system in the intestine (2). Moreover, using dipeptide analogs Bai *et al.* (11) demonstrated direct evidence that a free α-amino group was not absolutely essential for transport by the intestinal peptide transporter. A similar conclusion was presented by Oh *et al.* (12). As shown in Table II, serosal-to-mucosal permeation was much lower for ampicillin than for benzylpenicillin. Therefore, our present results suggest another possibility that the presence of a free α-amino group might reduce the affinity for the energy-dependent efflux system. It should be noted that as long as a β-lactam antibiotic is the substrate with a high affinity for this intestinal efflux system, the efflux system can pump it out efficiently from cytosol, even if an absorptive peptide transport system on the brush-border membrane mediates their translocation from the lumen into the cytosol. Alternatively, it may be possible that amino β-lactam antibiotics have a way to escape such an intestinal efflux system; for example, binding to a cytosolic component as reported previously by Iseki *et al.* (13) could prevent amino β-lactam antibiotics from interacting with the energy-dependent efflux system, stimulating absorption of these antibiotics. The role of the α-amino group should be evaluated in more detail.

Interestingly, the mucosal-to-serosal permeation of amoxicillin was greater than that of ampicillin, in spite of there being no difference in serosal-to-mucosal permeation between them, suggesting that the introduction of a hydroxy group is effective in enhancing the absorptive movement of these β-lactam antibiotics. This observation seemed consistent with the fact that amoxicillin is absorbed in much greater amounts than ampicillin in vivo.

Recently, Hidalgo *et al.* (14) reported benzylpenicillin fluxes across excised rabbit small intestine in Ussing chambers. They showed benzylpenicillin exhibited identical fluxes in both absorptive and secretory directions, indicating the lack of secretory-oriented movements of benzylpenicillin in rabbit intestine. On the other hand, in a recent study with rabbit small intestinal brush-border membrane vesicles, it has been shown that benzylpenicillin uptake is mediated via a common transport system shared by peptides, penicillins, and cephalosporins (15). It is often reported that the proton-coupled transport of β-lactam antibiotics is more pronounced in brush-border membrane vesicles from rabbit intestine than those from rat intestine (16). Therefore, it is probable that equal contributions by absorptive peptide transport and an energy-dependent efflux across excised rabbit intestine could make benzylpenicillin permeation identical in both directions.

β-Lactam antibiotics can be transported by carrier-mediated excretory processes in liver (17), brain (18), and kidney (8). These transport systems have been characterized as organic anion transport systems. Benzylpenicillin has been used as a typical substrate for these transporters. The energy-dependent efflux system in rat intestine described in this study exhibits some similarities to these other organs in that: (1) this efflux system can recognize both penicillins and cephalosporins as substrates; (2) probenecid is a potent inhibitor of the efflux system; and (3) the efflux system is not shared by oligopeptides

as previously reported (5). However, the lack of PAH effect on secretory-oriented permeation of benzylpenicilin, cephaloridine, and ampicillin in this study indicates the unique features of the intestinal efflux system for orally inactive β-lactam antibiotics. Further studies are now underway in order to characterize the mechanism and substrate specifity of the intestinal efflux system in more detail.

In conclusion, it was demonstrated that oral ineffectiveness of some β-lactam antibiotics is in part due to restricted intestinal absorption by an energy-demanding efflux system, which is distinct from P-glycoprotein-mediated transport system. The side chain structure of β-lactam antibiotics is capable of altering affinities to the efflux system. The present findings, together with our previous observations, strongly suggest that there might be multiple intestinal efflux systems which efficiently restrict the absorption of their substrates.

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