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Structure-Activity Relationship of Carbacephalosporins and Cephalosporins: Antibacterial Activity and Interaction with the Intestinal Proton-Dependent Dipeptide Transport Carrier of Caco-2 Cells

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An intestinal proton-dependent peptide transporter located on the lumenal surface of the enterocyte is responsible for the uptake of many orally absorbed β-lactam antibiotics. Both cephalexin and loracarbef are transported by this mechanism into the human intestinal Caco-2 cell line. Forty-seven analogs of the carbacephalosporin loracarbef and the cephalosporin cephalexin were prepared to evaluate the structural features necessary for uptake by this transport carrier. Compounds were evaluated for their antibacterial activities and for their ability to inhibit 1 mM cephalexin uptake and, subsequently, uptake into Caco-2 cells. Three clinically evaluated orally absorbed carbacephems were taken up by Caco-2 cells, consistent with their excellent bioavailability in humans. Although the carrier preferred the L stereoisomer, these compounds lacked antibacterial activity and were hydrolyzed intracellularly in Caco-2 cells. Compounds modified at the 3 position of cephalexin and loracarbef with a cyclopropyl or a trifluoromethyl group inhibited cephalexin uptake. Analogs with lipophilic groups on the primary amine of the side chain inhibited cephalexin uptake, retained activity against gram-positive bacteria but lost activity against gram-negative bacteria. Substitution of the phenylglycl side chain with phenylacetyl side chains gave similar results. Compounds which lacked an aromatic ring in the side chain inhibited cephalexin uptake but lost all antibacterial activity. Thus, the phenylglycl side chain is not absolutely required for uptake. Different structural features are required for antibacterial activity and for being a substrate of the transporter. Competition studies with cephalexin indicate that human intestinal Caco-2 cells may be a useful model system for initially guiding structure-activity relationships for the rational design of new oral agents.

Many β-lactams antibiotics are well absorbed orally. They are excellent substrates of an intestinal proton-dependent peptide transport carrier located on the lumenal (brush border) side of the enterocyte that lines the gastrointestinal tract (3, 36). This transporter also takes up dipeptide and tripeptide nutrients as well as angiotensin-converting enzyme inhibitors (3). The peptide transporter uses the proton gradient that exists across the brush border membrane to concentrate its substrates within the enterocyte prior to their exit through the basolateral surface into the bloodstream. This transporter has been extensively studied in intestinal membrane vesicles prepared from rabbits, rats, and humans (8, 17–19, 28, 32–34, 48). Also, the human intestinal cell line Caco-2 expresses the proton-dependent dipeptide transport carrier when grown to confluence. The peptide transporter of Caco-2 cells has all the salient features of the intestinal peptide transporter, and it takes up cephalexin, cefaclor, loracarbef, and cefixime (13–15). The structural requirements of the proton-dependent dipeptide transport carrier for binding of substrates have not been elucidated in detail (22).

The present report examines derivatives of two β-lactams that contain a phenylglycine side chain (Fig. 1). A series of analogs of the carbacephalosporin loracarbef and of the cephalosporin cephalexin were prepared and evaluated for their ability to compete with [^{14}C]cephalexin for uptake (38, 40). Subsequently, selected compounds were evaluated for their ability to be taken up by the proton-dependent dipeptide transport carrier of human intestinal Caco-2 cells (39). In addition, analogs were evaluated for their antibacterial activities against gram-positive and gram-negative bacterial strains.

MATERIALS AND METHODS

Chemistry. Analogs of loracarbef (compound 1) and cephalexin (compound 2) were prepared and are described in Tables 1 and 3 to 7. Compounds 1e, 1f, 2e, and 2f were prepared by adding triphenylbismuth diacetate (1.0 to 1.1 eq) and copper (0.1 eq) to the *para*-nitrobenzyl (PNB)-protected cephalexin or loracarbef and stirring at room temperature for 48 to 72 h, followed by chromatographic purification (5–7). Compounds 1g, 1h, and 2g to 2l were prepared by adding an alkylating agent, RX (0.8 to 2.5 eq), where R is benzyl for 1g, 1h, and 2g through 2j and R is methyl for 2k and 2l, and diisopropylethylamine (1.25 to 2.5 eq) to the PNB-protected cephalexin or loracarbef, where X is iodine for 2k and 2l and bromine for 1g, 1h, 2g, to 2j. The reaction mixture was heated to 50°C for 3 h; rinsed with water and brine, filtered, and evaporated to dryness; and then chromatographically purified. Compounds 1i to 1l, 2m to 2p, 1q, and 2t were prepared under standard Schotten-Baumann conditions by acylating the free amine of the PNB-protected nucleus of cephalexin or loracarbef with the desired acid chloride (41). Compound 2q was prepared by the method of Krishnamurthy (26). The diphenylbenzyl-protected nucleus of cephalexin was reacted with excess acetic formic anhydride to give the *N*-formyl derivative. Conversion of the formamide intermediate to the corresponding *N*-methylamine was achieved by reduction with borane-methyl sulfide. Acylation of the phenylglycine-protected amino acid was accomplished with 2,6-methoxy-4-chloro-1,2,5-triazine (24). The diphenylbenzyl ester was removed by stirring with trifluoroacetic acid (10 ml) and triethylsilane (3 ml) for 1 h at room temperature to give compound 2q. Compounds 1o and 2r were prepared by acylating the protected nucleus with carboxycyclohexane carbamic acid cyclic anhydride in hexamethylphosphoramide for 120 h. This side chain was prepared by reacting 1-amino-1-cyclohexane carboxylic acid and

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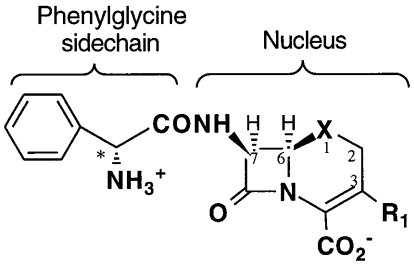


FIG. 1. General structure of the parent β-lactams containing a phenylglycine side chain. The cephalosporin, cephalexin, contains a sulfur at position X; the carbacephalosporin loracarbef contains a carbon at position X. R_1 is methyl for cephalexin and chlorine for loracarbef, respectively.

triphosgene in dioxane at 90°C for 4 h (2). Preparation of compounds 1a to 1d, 2a to 2d, 1m, 1n (1) 1p, 2s, 1r to 1z, and 1za was accomplished by acylation and deblocking of the protected nucleus with the desired protected amino acid (42). In general, PNB deprotection was accomplished by hydrogenation for the cephalexin derivatives and by Zn-HCl for the loracarbef derivatives (42). All final products were purified by preparative high-pressure liquid chromatography (HPLC) (42) to >95% purity.

clog P. The octanol-water partition coefficient, expressed numerically as the log P value, has been used to estimate and compare the lipophilicities of structural changes of compounds (21). Computer-assisted methods for calculation of log P values as the sum of substructural contributions to lipophilicity have been developed. The calculated log P (clog P) data are presented in Tables 4 and 5 to compare the lipophilicities of these compounds. The larger the number, the more lipophilic the compound. The program used to calculate these values was Weininger, D., CLOGP Daylight Chemical Information Systems Inc., Irvine, CA.

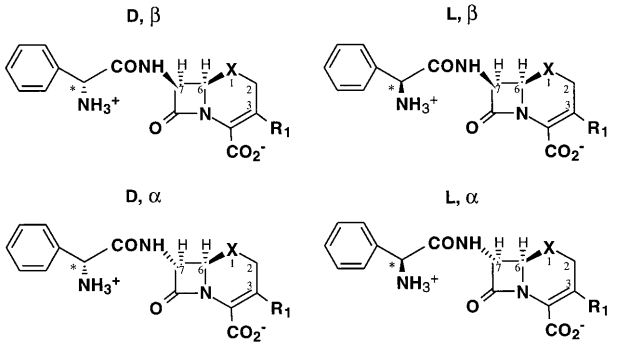
Antibacterial assays. MICs (in micrograms per milliliter) were determined by the agar dilution method (47) with Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.). The medium was supplemented with 1% supplement C (Difco Laboratories, Detroit, Mich.) to enhance the growth of nonenterococcal streptococci and *Haemophilus influenzae*. The antibiotics were incorporated directly into the melted agar prior to pouring the agar into the plates. Inocula of bacterial strains (approximately 10^4 CFU) were prepared by appropriate dilution of overnight broth cultures of organisms in fresh brain heart infusion broth (Difco) and were applied to plates with a 36-prong Cathra inoculating device (AutoMed Inc., Shoreview, Minn.). The plates were examined after 18 to 20 h of incubation at 37°C. The source of the bacterial strains was the collection of Eli Lilly and Company.

Transport measurements. Caco-2 cells were grown as described previously (13, 20). Drug uptake was measured in 2-week postconfluent Caco-2 cells by a cluster tray technique (13, 20). Initial rates of 1 mM [^{14}C]cephalexin uptake were determined over a 4-min time course. Uptake was measured in the absence or presence of 5 mM glycine-L-proline (Gly-L-Pro) or the indicated test compound. Incubations were for up to 2 h at 37 or 4°C. Cells were washed three times to remove excess drug and were lysed. Samples were taken for either protein determination (37) or scintillation counting. The percent inhibition for each test compound was normalized to that of 5 mM Gly-L-Pro, a concentration that gives complete inhibition of cephalexin uptake by the proton-dependent peptide transporter. Since a small percentage of cephalexin uptake is via diffusion which is not inhibitable, normalizing the data to Gly-L-Pro allows the interaction with the transporter to be assessed directly.

To measure the uptake of analogs, 1 mM test compound was added to the cells as described above. Uptake was measured in the absence or presence of 5 mM Gly-L-Pro or 25 mM cephalexin, to give maximum inhibition of the intestinal peptide transporter. Cells were washed and lysed. The amount of the compound taken up was quantitated by HPLC as described below. Uptake rates were corrected for "trapped" water by using [^{3}H]inulin (13). Steady-state drug levels were determined at 1 h at 37 and 4°C. A distribution ratio (DR) was calculated as follows to determine whether the compound was concentrated against a gradient (13): DR = intracellular drug concentration/extracellular drug concentration. A temperature- and energy-dependent mechanism exists when the DR is greater than 1 at 37°C and not at 4°C. When the DR is equal to or less than 1, diffusion is likely to be the only mechanism by which the compound enters the enterocyte.

HPLC analysis. For nonradiolabelled compounds, the amount of drug taken up was quantitated by HPLC with a Waters Nova-pak C_{18} RCM column (8 by 100 mm) with a Nova C_{18} guard insert (Waters Chromatography, Milford, Mass.). Data were collected on a P. E. Nelson Turbochrome chromatography software system (Perkin-Elmer Corporation, Norwalk, Conn.). The solvent system consisted of 90.5% H_2O–9% CH_3CN–0.5% $NH_4H_2PO_4$ with a run time of 4 min, a flow rate of 2.5 ml/min, and UV detection at 260 nm. The drug concentration was calculated from a standard curve by using the peak area.

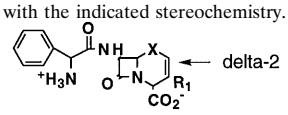
TABLE 1. Stereoisomers and structural isomers



Compound group and compound	Stereo-isomer[a]	C7 bond	R_1	% Inhibition of cephalexin uptake	MIC (μg/ml) for *S. aureus*
Loracarbef (compound 1)	D	β	Cl	52	0.5
Cephalexin (compound 2)	D	β	CH_3	47	4
Loracarbef analogs (X = CH_2)					
1a	L	β	Cl	100	16
1b[b]	D	β	Cl	35	128
1c[b]	L	β	Cl	92	128
1d	D	α	Cl	19	32
Cephalexin analogs (X = S)					
2a	L	β	CH_3	99	128
2b[b]	D	β	CH_3	26	32
2c[b]	L	β	CH_3	69	128
2d	D	α	CH_3	41	128

[a] The asterisks indicate stereocenters with the indicated stereochemistry.

[b] Delta-2 isomers of loracarbef and cephalexin:



Detection of the hydrolysis of loracarbef or cephalexin. To determine if the L isomers of loracarbef and cephalexin compounds (compounds 1a and 2a, respectively) were hydrolyzed, cells were incubated 2 h with each test compound, washed as indicated above, and lysed. Hydrolysis releases the nucleus (Table 1) of these β-lactams, which is difficult to detect and isolate by HPLC. Consequently, the *t*-butoxycarbonyl (t-Boc) derivative of the nucleus was prepared. Lysates were derivatized with an excess of di-*t*-butyldicarbonate and sodium bicarbonate in water and acetone at room temperature for 24 h and lyophilized prior to HPLC analysis. The treated lysates were compared to the t-Boc derivatives of the appropriate nuclei that served as standards. The solvent system for this study consisted of 81.5% H_2O–9% CH_3CN–9% CH_3OH–0.5% ammonium acetate (NH_4OAc) with a run time of 20 min and a flow rate of 2.5 ml/min. The standards for the loracarbef and cephalexin nuclei gave peaks with retention times of 12.8 and 14.9 min, respectively. The detection of the t-Boc derivatives of the L isomers in the lysates indicates that these compounds were hydrolyzed. To rule out the possibility that the compounds were hydrolyzed extracellularly and that the nucleus diffused into the cell, Caco-2 cells were also incubated for 2 h with the nucleus of loracarbef or the nucleus of cephalexin and subsequently washed, lysed, and derivatized. No peak was detected in these cell lysates.

RESULTS

Forty-seven analogs of loracarbef and cephalexin were prepared. They were evaluated for their antibacterial activities and also tested for their ability to compete for 1 mM cephalexin uptake via the intestinal proton-dependent dipeptide transporter. The parent compounds loracarbef (Table 1; X = CH_2, R_1 = Cl) and cephalexin (Table 1; X = S and R_1 = CH_3)

were modified at several key positions. Compounds are grouped in Tables 1 and 3 to 7 on the basis of the particular structural modification to the parent compound. Also presented in Tables 1 and 3 to 7 are the data for inhibition of cephalexin uptake and the antimicrobial activity by using the MIC for *Staphylococcus aureus*. This organism was chosen as a representative organism for comparison of the activities of compounds with structural changes. The analogs were tested for their ability to compete for cephalexin uptake via the human intestinal proton-dependent dipeptide transporter. The effect of a fivefold excess of the test compound on the uptake of 1 mM [^{14}C]cephalexin into human intestinal Caco-2 cells was examined. Previously, many orally absorbed β-lactam antibiotics have been shown to inhibit cephalexin uptake by 30% or greater (43).

These analogs were also evaluated for their activities against gram-positive and gram-negative bacteria, and these data are summarized in Table 2. Loracarbef and cephalexin are broad-spectrum antibiotics and are effective against both gram-positive and gram-negative pathogens.

Stereoisomers and structural isomers. The structures of loracarbef and cephalexin are presented in Table 1, along with those of a series of derivatives. Both loracarbef and cephalexin contain the D stereoisomer of the phenylglycine side chain, and this side chain is in the β position with respect to the nucleus. The nucleus of each contains a double bond at the delta-3 position. Modifications were made to these three sites within the parent compounds. They included (i) changing the stereochemistry of the side chain from D to L, (ii) altering the bond at C-7 from β to α, and (iii) changing the double-bond location within the nucleus from the delta-3 position to the delta-2 position.

The L stereoisomers of loracarbef and cephalexin, compounds 1a and 2a, respectively, inhibited cephalexin uptake approximately twofold more than their respective parent compounds, which are the D stereoisomers (12, 13). The 7-α stereoisomers of loracarbef (compound 1d) and cephalexin (compound 2d) inhibited cephalexin uptake by 19 and 41%, respectively. The 7-α isomer of loracarbef inhibited cephalexin uptake less than the parent compound did, while the inhibition of uptake by 7-α isomer of cephalexin was not significantly different from that of its parent.

Next, the effect of moving the double bond from the delta-3 position to the delta-2 position was examined by using both the L and D stereoisomers of the 7-β-phenylglycine side chain. The delta-2-(L) isomers of loracarbef (compound 1c) and cephalexin (compound 2c) inhibited uptake well, by 92 and 69%, respectively. The delta-2-(D) isomers of loracarbef (compound 1b) and cephalexin (compound 2b), however, inhibited cephalexin uptake less than the parent compounds: 35% for the loracarbef delta-2-(D) isomer (compound 1b) and 26% for the cephalexin delta-2-(D) isomer (compound 2b). When the double bond was placed in the delta-3 position, the delta-3-(L) isomers of loracarbef and cephalexin, compounds 1a and 2a, respectively, inhibited uptake completely. Thus, the L stereochemistry of the phenylglycine side chain enhanced the inhibition of cephalexin uptake. By contrast, the delta-2 and 7-α stereochemistries had no effect on or decreased the ability of the compounds to inhibit cephalexin uptake. For all of these stereoisomers, the antibacterial activities were dramatically decreased to little or no activity (Table 2). Thus, the three-dimensional placement of the substituents of these compounds is absolutely critical for antibacterial activity.

Hydrogen replacement. The role of the protons in the phenylglycyl side chain was examined on different enantiomers of loracarbef and cephalexin, as illustrated in Table 3. The methine proton at position R_5 was replaced with a methyl group on both the D and L stereoisomers of loracarbef to give compounds 1m and 1n, respectively. For compound 2q, the proton at R_6 of cephalexin was replaced with a methyl group. Methyl group replacement at R_5 for both the D and L isomers of loracarbef resulted in complete inhibition of cephalexin uptake (97 to 98%), whereas compound 2q, with a methyl substitution on the amide nitrogen at R_6 of cephalexin, inhibited 1 mM [^{14}C]cephalexin uptake by 58%, close to that of the parent compound, cephalexin (47%). Interestingly, methyl replacement at either of these two positions resulted in nearly complete loss of antibacterial activity for these compounds. However, the hydrogens at these two positions evidently do not play a role in binding to the transporter since the methyl group either had no effect or enhanced the interaction with the intestinal peptide transporter.

Lipophilicity. In the acidic milieu of the lumen of the small intestine, the parent compounds exist as zwitterions, and as such, they are highly polar. The structure in Table 1 is shown with the carboxyl group and the amino group in the zwitterionic form. In order to decrease the polarity without altering their ionic characteristics, lipophilic substitutions were made to both loracarbef and cephalexin at the phenylglycine amine, as indicated in Table 4. Modifications included the addition of methyl, phenyl, or benzyl groups to the amine, compounds 1e to 1h and 2e to 2l. All of these compounds competed for cephalexin uptake. The diphenyl, dibenzyl, and dimethyl compounds were more inhibitory than their respective mono derivatives, 82 to 100% and 41 to 76%, respectively, indicating that the increased lipophilicity and decreased polarity enhanced their binding to the cephalexin transporter.

When these compounds were examined for their antimicrobial activities, all but one of the compounds retained activity against *S. aureus*. The loracarbef diphenyl compound (compound 1f) had significantly less *S. aureus* activity. Examination of the data for gram-negative organisms in Table 2 showed that the mono and dibenzyl derivatives of loracarbef, compounds 1g and 1h, respectively, have good activity against *H. influenzae*, but in general, activities against gram-negative organisms decreased for these compounds. Next, a carboxyl group was added to the primary amine of loracarbef to assess the effect of an amide at this position. Compound 1i did not compete for the uptake of 1 mM cephalexin (9%); moreover, the MIC for *S. aureus* increased from 0.5 μg/ml for loracarbef to 16 μg/ml for compound 1i. Overall, for this set of compounds, lipophilicity had little effect on their activities against gram-positive pathogens, but diminished their effectiveness against gram-negative pathogens.

Alteration of ionic character to neutral phenylacetyl derivatives. Previously, peptides and peptidyl mimetics which lack a free α-amino group (certain β-lactams and angiotensin converting enzyme inhibitors) have been shown to be transported by the intestinal peptide transporter (4, 30). Consequently, modifications were made to evaluate the necessity for β-lactams to be zwitterions. The phenylglycyl side chain was replaced with a phenylacetyl side chain which lacks a free amino group and therefore cannot exist as a zwitterion. These compounds, 1j to 1l and 2m to 2p, interestingly, inhibited cephalexin uptake by 33 to 93% and retained antibacterial activity, as indicated in Table 5, confirming that a free amino group is not absolutely required for antibacterial activity or for binding to the intestinal peptide transporter.

Analogs with side chains of marketed compounds. Three side chains present in marketed β-lactams were used to replace the phenylglycine of loracarbef and cephalexin. These results are presented in Table 6. The side chains chosen were those of

TABLE 2. MICs

Compound	MIC (μg/ml)					
	Staphylococcus aureus X1.1[a]	*Streptococcus pyogenes* C203	*Streptococcus pneumoniae* PK	*Enterococcus faecalis* 2041	*Haemophilus influenzae*[b]	
					−	+
1	0.5	0.25	1	32	1	1
2	4	1	1	128	16	8
1a	16	1	4	128	8	8
1b	128	1	64	128	128	128
1c	128	128	128	128	128	128
1d	32	1	16	128	8	16
1e	0.25	0.015	0.015	16	0.5	0.25
1f	64	64	64	64	64	64
1g	0.25	0.015	0.03	16	1	2
1h	0.25	0.015	0.015	16	8	4
1i	16	0.25	2	128	4	4
1j	2	0.125	0.5	64	2	2
1k	0.5	0.03	0.125	64	2	8
1l	2	0.125	1	128	4	8
1m	128	64	64	128	128	128
1n	128	8	32	128	128	64
1o	128	128	128	128	128	128
1p	32	0.25	0.25	4	0.5	0.5
1q	2	0.125	1	128	2	2
1r	2	0.06	1	64	1	2
1s	0.5	0.125	2	128	8	8
1t	2	0.06	0.125	16	2	2
1u	16	1	0.5	128	8	8
1v	2	0.03	0.03	8	1	1
1w	1	0.03	0.06	4	0.5	0.5
1x	2	0.06	0.25	16	1	2
1y	2	0.25	1	32	2	1
1z	2	8	8	128	1	1
1za	2	0.25	0.25	128	1	2
2a	128	8	64	128	128	64
2b	32	32	128	128	128	64
2c	128	128	128	128	128	128
2d	128	128	128	128	128	128
2e	0.5	0.03	0.03	32	4	0.5
2f	1	0.25	0.125	128	128	128
2g	1	0.25	0.25	128	16	2
2h	1	0.5	0.5	128	128	64
2i	8	1	2	128	128	32
2j	8	1	0.5	128	128	128
2k	4	0.5	8	128	32	4
2l	8	8	128	128	128	32
2m	0.5	0.015	0.06	32	0.25	0.25
2n	0.125	0.03	0.06	16	2	4
2o	0.5	0.5	1	64	2	2
2p	0.25	0.06	0.125	32	1	0.5
2q	128	128	128	128	128	128
2r	128	128	128	128	128	128
2s	8	0.008	0.03	16	0.06	0.015
2t	0.125	0.125	0.25	32	8	2

[a] Penicillin-sensitive strain.
[b] −, β-lactamase nonproducing; +, β-lactamase producing.
[c] Ampicillin-sensitive strain.
[d] TEM, β-lactamase-producing strain.

(i) cyclacillin, the 1-aminocylohexyl side chain present in this oral penicillin; (ii) the cefotaxime side chain aminothiazolemethoxyoxime (ATMO); and (iii) the cefamandole side chain *O*-formyl mandelic acid. When the phenylglycyl side chains of loracarbef and cephalexin were replaced with the cyclacillin side chain, compounds 1o and 2r (2) competed for 1 mM cephalexin uptake quite well, giving complete inhibition (98% for 1o and 100% for 2r), while antibacterial activity was completely lost.

Compounds with an ATMO side chain are not normally orally absorbed (27). Replacement of the phenylglycyl side chains of loracarbef and cephalexin with the ATMO side chain along with modification of R_1 with a cyclopropyl group gave compounds 1p and 2s. Compounds 1p and 2s inhibited cephalexin uptake by 40 and 35%, respectively, which are similar to the values for their respective parent compounds. These results indicate that compounds with ATMO side chains may warrant further studies because they are poten-

TABLE 2—*Continued*

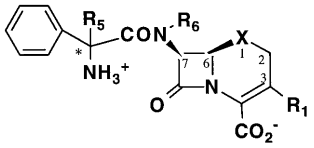
MIC (μg/ml)						
Escherichia coli		*Klebsiella pneumoniae* X26	*Enterobacter cloacae* EB5	*Salmonella* sp. X514	*Serratia marcescens* SE3	*Morganella morganii* PR
EC14[c]	TEM[d]					
0.25	1	0.5	2	0.25	32	128
4	8	4	64	2	128	128
1	4	1	128	4	128	128
64	128	64	128	128	128	128
128	128	128	128	128	128	128
8	16	4	64	4	128	128
64	32	1	128	32	128	128
128	128	64	128	128	128	128
32	32	1	128	64	128	128
128	32	4	128	128	128	128
8	128	4	128	2	128	128
8	16	1	128	2	128	128
64	128	4	128	128	128	128
32	32	2	128	64	128	128
128	128	128	128	128	128	128
64	32	32	128	64	128	128
128	128	128	128	128	128	128
0.5	0.5	0.125	4	1	16	0.5
1	2	0.5	16	0.5	128	128
0.5	1	1	16	0.5	128	128
4	4	4	128	8	128	128
0.5	1	0.5	16	2	32	8
32	32	8	32	16	64	16
1	2	0.25	16	1	32	8
0.5	1	0.125	8	0.125	16	4
0.5	1	0.5	8	0.5	16	32
0.5	2	1	32	0.5	128	128
0.25	0.25	0.25	32	0.125	128	128
0.5	0.5	0.25	4	1	32	128
128	128	128	128	128	128	128
128	128	64	128	128	128	128
128	128	128	128	128	128	128
128	128	128	128	128	128	128
128	64	1	128	128	128	128
128	128	128	128	128	128	128
128	64	8	128	128	128	128
128	128	128	128	128	128	128
128	128	64	128	128	128	128
128	128	128	128	128	128	128
32	16	8	128	32	128	128
128	128	128	128	128	128	128
16	16	1	128	4	128	128
128	128	4	128	128	128	128
128	64	4	128	128	128	128
64	64	2	128	128	128	128
128	128	128	128	128	128	128
128	128	128	128	128	128	128
0.25	0.125	0.015	1	0.5	8	8
8	4	2	64	8	128	64

tial oral antibiotics. The activities against *S. aureus* were diminished, with an MIC of 0.5 μg/ml for loracarbef compared to an MIC of 32 μg/ml for compound 1p and an MIC of 4 μg/ml for cephalexin compared to an MIC of 8 μg/ml for compound 2s. Overall, though, their spectra of activity were the same or improved over those of the parent compounds, as indicated in Table 2. In this case, the *S. aureus* pathogen was not useful as a comparison of overall activity. ATMO-type compounds are generally not as active against *S. aureus* as β-lactams with a phenylglycine side chain (i.e., cephalexin and loracarbef) (10).

Next, cefamandole side chain derivatives of loracarbef and cephalexin, compounds 1q and 2t, respectively, were prepared. The loracarbef derivative with a Cl at R_1 (compound 1q) gave poor inhibition (25%) of cephalexin uptake into Caco-2 cells, consistent with the poor bioavailability of cefamandole observed in humans (29). By contrast, the cephalexin derivative which had also been modified with a cyclopropyl group at R_1 (compound 2t) inhibited cephalexin uptake by 55%. The cyclopropyl group appears to enhance the interaction with the cephalexin transporter, as has also been demonstrated with the cefotaxime analogs, compounds 1p and 2s. These analogs retained activity against the same spectrum of organisms as their parent compounds.

Derivatives with the cyclacillin, cefotaxime, and cefamandole side chains competed for cephalexin uptake and, with the

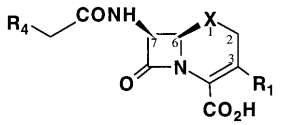
TABLE 3. Hydrogen replacement



Compound group and compound	Stereoisomer[a]	R_1	R_5	R_6	% Inhibition of cephalexin uptake	MIC (μg/ml) for *S. aureus*
Loracarbef analogs (X = CH_2)						
1	D	Cl	H	H	52	0.5
1m	D	Cl	CH_3	H	98	128
1n	L	Cl	CH_3	H	97	128
Cephalexin analogs (X = S)						
2	D	CH_3	H	H	47	4
2q	D	CH_3	H	CH_3	58	128

[a] The asterisk indicates the stereocenter with the indicated stereochemistry.

TABLE 5. Alteration of ionic character to neutral phenylacetyl derivatives



Compound group and compound	R_1	R_4	% Inhibition of cephalexin uptake	MIC (μg/ml) for *S. aureus*	clog P
Loracarbef analogs (X = CH_2)					
1	Cl		52	0.5	−0.47
1j	Cl	Ph[a]	52	2	2.48
1k	Cl	*m*-(CF_3)Ph	34	0.5	3.36
1l	Cl	*o*-(Cl)Ph	87	2	3.19
Cephalexin analogs (X = S)					
2	CH_3		47	4	−1.90
2m	Cl	Ph	33	0.5	1.25
2n	Cl	*m*-(CF_3)Ph	60	0.13	2.14
2o	Cl	*o*-(Cl)Ph	88	0.5	1.97
2p	Cl	*p*-(Cl)Ph	93	0.25	1.97

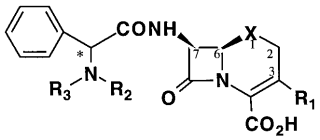
[a] Ph, phenyl.

exception of the cyclacillin derivatives, had antibacterial activities similar to those of loracarbef and cephalexin.

Substituted phenyl derivatives of the side chain and R_1 modification of loracarbef. Next, 10 loracarbef analogs were evaluated. As indicated in Table 7, modifications were made by replacement of the phenyl group with a dihydrophenyl group (compound 1r) or substitutions on the phenyl group of the side chain (position R_4; compounds 1s to 1x), and the chloride at R_1 was also replaced with a trifluoromethyl or a cyclopropyl group. Compounds 1r and 1y to 1za were modified only at the phenyl group of the side chain. All of the compounds retained antibacterial activity and also inhibited cephalexin uptake.

Uptake by the intestinal peptide transporter. Eleven compounds were selected for a further study to directly examine their ability to be taken up by the proton-dependent peptide transport carrier of human intestinal Caco-2 cells. These re-

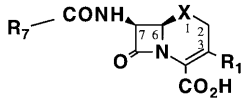
TABLE 4. Lipophilicity



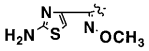
Compound group and compound	Stereoisomer[a]	R_1	R_2	R_3	% Inhibition of cephalexin uptake	MIC (μg/ml) for *S. aureus*	clog P
Loracarbef analogs (X = CH_2)							
1	D	Cl	H	H	52	0.5	−0.47
1e	D	Cl	H	Ph[b]	68	0.25	3.73
1f	D	Cl	Ph	Ph	100	64	5.86
1g	D	Cl	H	CH_2Ph	74	0.25	3.71
1h	D	Cl	CH_2Ph	CH_2Ph	82	0.25	5.54
1i	D	Cl	H	$COCH_3$	9	16	1.77
Cephalexin analogs (X = S)							
2	D	CH_3	H	H	47	4	−1.90
2e	D	CH_3	H	Ph	76	0.5	2.30
2f	D	CH_3	Ph	Ph	100	1	4.43
2g	D	CH_3	H	CH_2Ph	55	1	2.28
2h	D	CH_3	CH_2Ph	CH_2Ph	97	1	4.11
2i	L	CH_3	H	CH_2Ph	43	8	2.28
2j	L	CH_3	CH_2Ph	CH_2Ph	100	8	4.11
2k	D	CH_3	H	CH_3	41	4	−1.38
2l	D	CH_3	CH_3	CH_3	71	8	−0.99

[a] The asterisk indicates the stereocenter with the indicated stereochemistry.
[b] Ph, phenyl.

TABLE 6. Side chain analogs of marketed compounds

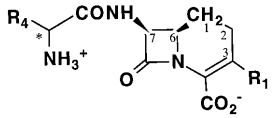


Compound group and compound	R_1	X	R_7	% Inhibition of cephalexin uptake	MIC (μg/ml) for *S. aureus*
Cyclacillin analogs					
1o	Cl	CH_2		98	128
2r	CH_3	S		100	128
Cefotazime side chain analogs					
1p	Δ	CH_2	ATMO[a]	40	32
2s	Δ	S	ATMO	35	8
Cefamandole side chain analogs					
1q	Cl	CH_2	PhCH(OCHO)[b]	25	1
2t	Δ	S	PhCH(OCHO)	55	0.125

[a] ATMO, 

[b] Ph, phenyl.

TABLE 7. Substituted side chain derivatives and R_1 modifications of loracarbef



Compound	Stereoisomer[a]	R_1	R_4	% Inhibition of cephalexin uptake	MIC (μg/ml) for *S. aureus*
1	D	Cl	Ph[b]	52	0.5
1r	D	Cl		43	2
1s	D	Δ	Ph	58	0.5
1t	D	CF_3	*m*-(CH_3SO_2NH)Ph	80	2
1u	L	CF_3	*m*-(CH_3)Ph	96	16
1v	D	CF_3	*m*-(CH_3)Ph	66	2
1w	D	CF_3	*p*-(OH)-*m*-(Cl)Ph	88	1
1x	D	CF_3		37	2
1y	D	Cl	*p*-(OH)Ph	84	2
1z	D	Cl	*m*-(CH_3SO_2NH)Ph	63	2
1za	D	Cl	*m*-($EtSO_2NH$)Ph[c]	76	2

[a] The asterisk indicates the stereocenter with the indicated stereochemistry.
[b] Ph, phenyl.
[c] Et, ethyl.

sults are presented in Table 8. For comparison, cephalexin, loracarbef, and three carbacephems (compounds 1y to 1za) were chosen; all of these compounds are well absorbed orally in humans (9, 16, 25, 31) (unpublished data). Four compounds from Table 1 were selected on the basis of their ability to inhibit cephalexin uptake and their antibacterial activities. The 7-α stereoisomers (compounds 1d and 2d) did not inhibit cephalexin uptake and had little antibacterial activity, and the L stereoisomers of loracarbef and cephalexin (compounds 1a and 2a) showed complete inhibition of cephalexin uptake but lacked antibacterial activity. Two compounds, the D and L stereoisomers of loracarbef with the methine proton on the side chain (R_5; Table 5) replaced with a methyl group (compounds 1m and 1n, respectively), were also selected to determine if the methyl groups might affect uptake or hydrolysis.

The results in Table 8 indicate that the uptake of loracarbef and cephalexin was inhibited by the presence of either Gly-L-Pro or cephalexin and that these drugs were concentrated intracellularly at 37°C, with DRs of 4.1 and 3.4, respectively. The three analogs of loracarbef that are absorbed orally in humans (compounds 1y to 1za) had DRs ranging from 3.9 to 6.2, indicating that the dipeptide transporter also concentrated these drugs intracellularly. The 7-α isomers (compounds 1d and 2d) were not concentrated within the cell, consistent with the lack of inhibition of cephalexin uptake (Table 1).

By contrast, the L stereoisomers (compounds 1a and 2a) were not detected in cell extracts, even though the L stereoisomers of loracarbef and cephalexin inhibited cephalexin uptake by 99 to 100% (Table 1). One reason might be due to intracellular hydrolysis by peptidases to liberate the nucleus and the side chain. The intracellular hydrolysis of these compounds was confirmed by preparing the t-Boc derivatives of the cell lysate after uptake and comparing them with the t-Boc derivatives of the loracarbef and cephalexin nuclei (data not shown). The loracarbef and cephalexin nuclei have reduced antibacterial activity, consistent with the MICs presented in Tables 1 and 2. If hydrolysis occurred as indicated, then the placement of a methyl group on R_5 would be expected to reduce hydrolysis (Table 5). This modification of the D isomer of loracarbef (compound 1m) inhibited cephalexin uptake by 98% and was concentrated well intracellularly (DR = 5.3; Table 8). The same modification of the L isomer of loracarbef (compound 1n) inhibited cephalexin uptake by 97% and was concentrated 2.2-fold intracellularly. In both cases, the level of accumulation of the methylated L stereoisomer was reduced compared to that of the D stereoisomer. Taken together, these data indicate that the L stereoisomer is transported and rapidly hydrolyzed and that placement of a methyl group apparently reduces the level of hydrolysis, allowing the methylated compound to be concentrated within the cell. Thus, the transporter is capable of taking up the more metabolically stable L stereoisomer.

DISCUSSION

In the present study, a wide array of analogs of loracarbef and cephalexin were examined for their antimicrobial activities

TABLE 8. Uptake by the intestinal peptide transporter

Compound	Inhibition by Gly-L-Pro or cephalexin	DR	
		37°C	4°C
1	52	4.1	0.5[a]
1a	100	Not detected	Not detected
1d	19	0.5	0.2
1m	97	5.3	0.1[a]
1n	98	2.2[b]	0.3[a]
1y	84	6.2	0.2[a]
1z	63	4.6	0.1[a]
1za	76	3.9	0.2[a]
2	47	3.4	0.5[a]
2a	99	Not detected	Not detected
2d	41	0.3	0.5

[a] Significantly different ($P < 0.05$) than DR measured at 37°C.
[b] Significantly different ($P < 0.05$) than loracarbef.

and their ability to inhibit cephalexin uptake by the proton-dependent peptide transporter of human intestinal Caco-2 cells. Alterations to the parent β-lactams included changes at several stereocenters, changes in lipophilicity and ionic character, as well as other modifications. The structure-activity relationships for the analogs indicate that the properties necessary for antibacterial activity and binding to the intestinal proton-dependent peptide transporter are not necessarily the same.

Stereochemistry plays a major role both in antibacterial activity and in the transport of compounds (23). The intestinal transporter has an increased affinity for compounds with the L stereochemistry over those with the D stereochemistry (13, 45, 46). The L epimers of both loracarbef and cephalexin are taken up by the transporter of Caco-2 cells, although they are readily hydrolyzed intracellularly to release their biologically inactive nuclei. Similar findings have been reported with an in vitro and in vivo rat intestinal system (44). Stereoselectivity has also been observed for *cis*- and *trans*-ceftibuten; the *cis* isomer is transported, while the *trans* isomer is not (11, 49). Replacement of the hydrogens on the side chain at R_5 or R_6 had no effect on binding compared to the binding of the parent compounds; moreover, both the D and L epimers were actively transported by the transport carrier and were concentrated intracellularly. Furthermore, the present study indicates that the hydrolysis of L-cephalexin can be reduced by replacement of the hydrogen at R_5 with a methyl group (Tables 3 and 8). Although this enhances their metabolic stability in human intestinal cells, these compounds were devoid of antibacterial activity, as reported previously (39). Thus, these hydrogens appear to be absolutely required for antimicrobial activity. Changes at the 7 position of the nucleus also had dramatic effects on the transport or antimicrobial properties of the analogs. They were not substrates of the intestinal peptide transporter, even though some were well recognized by the transporter. Evidently, the stereochemistry at this center is absolutely critical for transport. Moreover, these compounds had substantially reduced antimicrobial activities. Movement of the double bond from delta-3 to delta-2 was well tolerated by the peptide transporter; however, there was a substantial reduction in antibacterial activity compared to the activities of the parent compounds. Thus, stereochemistry is extremely important for both antimicrobial activity and uptake by the human intestinal peptide transporter.

The peptide transport carrier bound compounds with a broad range of polarities. When the polarity was decreased by adding lipophilic groups to the amine on the side chain or by removing the zwitterionic amine to give a neutral phenylacetyl compound, all but one of these compounds were bound to the transporter. In general, the less polar compounds competed best for drug uptake. Less polar compounds or those that lack an ionic character showed a narrower spectrum of antibacterial activity. Generally, the activities against gram-positive microorganisms were retained with the modification; however, the activities against gram-negative microorganisms decreased. Apparently, a free α-amine is not absolutely necessary for antibacterial activity or for recognition by the peptide transporter. This is consistent with the properties of two marketed β-lactam antibiotics, cefixime and ceftibuten. The free amino group may not be necessary because the intestinal peptide carrier may recognize and transport amino β-lactam antibiotics, such as cephalexin, as a hydrated molecule, as proposed by Sakane et al. (35). By computer simulation, cefixime and ceftibuten were well superimposed on the hydrated structure of cephalexin (35). Amidon and coworkers (4) also provide evidence that an N-terminal α-amino group on the substrate is not essential for transport by the intestinal mucosal peptide transporter. By contrast, molecular modeling studies of the substrate specificity of the renal oligopeptide transporter indicate a direct interaction of the α-amino group with the transport carrier (22).

A number of loracarbef analogs that contained a substituted phenyl group or a dihydrophenyl group were evaluated. All of these analogs inhibited cephalexin uptake well. When three loracarbef analogs were evaluated for their ability to be taken up by the peptide carrier, they were actively transported and concentrated intracellularly. This is consistent with earlier studies that Caco-2 cells take up oral β-lactams well. Loracarbef, cephalexin, cefaclor, and cefixime are concentrated against a gradient (13–15). Moreover, the present studies with these three carbacephems are in agreement, because they are well absorbed orally in both rodents and humans (9, 16, 25, 31, 42) (unpublished data).

The results also indicate that inhibition of cephalexin uptake may be used initially to screen for the likelihood of absorption by this mechanism. Compared to evaluation in animal models, the assay requires small amounts of test compound and is a rapid first indication of the potential for absorption. The more interesting compounds can then be studied in more detail for uptake and transcellular transport through Caco-2 monolayers (45, 46). In vivo oral absorption studies would be limited to the most promising compounds.

The in vitro Caco-2 model is useful for the quick development of a structure-activity relationship. Traditionally, the development of a structure-activity relationship for a new oral antibiotic has proceeded sequentially by focusing on the antimicrobial activity first, followed by evaluation for oral absorption. The present study emphasizes the need to evaluate both properties simultaneously during a structure-activity relationship study to uncover the best compounds more rapidly. This study clearly demonstrates the different structural requirements for antibacterial activity and for interaction with the peptide transporter. Developing an understanding of the requirements for each activity should lead to a rapid and rational design of new oral antibiotics.

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