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Cefoxitin, a Semisynthetic Cephamycin Antibiotic: Antibacterial Spectrum and Resistance to Hydrolysis by Gram-Negative Beta-Lactamases

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The in vitro activity of cefoxitin, 3-carbamolyloxymethyl-7-α-methoxy-7 [2-(2-thienyl)acetamido]-3-cephem-4-carboyxlic acid, was investigated. Activity against gram-positive organisms was less than that of cephalothin and cephloridine. It was highly active against gram-negative bacilli, with activity against *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae* equal to that of currently available cephalosporins. In addition, it was active against certain *Enterobacter* strains, *Serratia marcescens*, indole-positive *Proteae* and *Herellea*. The strains of these latter bacteria were strains susceptible to carbenicillin and ticarcillin. *Pseudomonas aeruginosa* and other *Pseudomonas* species were resistant. Changes in pH, inoculum size, and type of growth medium had no significant effect on the activity of the antibiotic. Cefoxitin was highly resistant to hydrolysis by various types of gram-negative beta-lactamases. The precise role of resistance to beta-lactamase hydrolysis varied from strain to strain. Bacterial resistance to cefoxitin was not necessarily related to hydrolysis of the antibiotic. However, the resistance of cefoxitin to hydrolysis did contribute to its activity. Cefoxitin could function as an inducer of beta-lactamase activity and effectively bound to purified beta-lactamases.

Cephalosporin antibiotics currently available for clinical use in the United States have an important role in the treatment of many serious infections caused by both gram-positive and gram-negative bacteria. There are certain members of the *Enterobacteriaceae*, such as *Enterobacter*, *Serratia*, and indole-positive *Proteae* species, that are consistently resistant to agents such as cephalothin, cephaloridine, and cefazolin. Cefoxitin, a semisynthetic cephamycin analogue, is a new cephalosporin-like antibiotic that has unique activity against some gram-negative strains (2, 9, 11). This paper reports studies of the overall antibacterial activity of cefoxitin in comparison with cephalosporin antibiotics and investigates the resistance of cefoxitin to hydrolysis by beta-lactamase-containing bacteria.

MATERIALS AND METHODS

Cefoxitin was obtained from Merck Sharp & Dohme Research Laboratories. Cephalothin, cephloridine, and cephalexin were gifts from Eli Lilly & Co. Ampicillin, carbenicillin, and ticarcillin were gifts from Beecham-Massengill Pharmaceuticals. Bacterial strains were isolates from patients hospitalized at the Columbia-Presbyterian Medical Center, New York City.

Susceptibility testing methods. The activity of cefoxitin was measured by a microtiter broth dilution technique. Serial twofold dilutions in brain-heart infusion broth (Difco) were used with an inoculum of 10^4 colony-forming organisms (CFU) from an overnight culture. Incubation was for 18 h at 35 C. The minimal inhibitory concentration (MIC) of antibiotic was defined as the lowest concentration that inhibited development of visible turbidity. The minimal bactericidal concentration was determined by plating clear wells from the microtiter plates. MIC values were also determined by the agar-dilution method with Mueller-Hinton agar. A 100-fold dilution of an overnight culture was applied with a replicating device.

Assay for antibiotic. Filter paper disks were dipped in test solutions and placed on brain-heart infusion agar seeded with *Staphylococcus aureus*. Zones of inhibition were measured with calipers, and concentration of antibiotic was determined from curves constructed from plots of standards run in an identical manner.

Beta-lactamase preparations. Purified beta-lactamases prepared by published methods (5, 7, 10) were used in some experiments. Crude beta-lactamase preparations were made by subjecting strains to sonic disruption and by using as enzyme the supernatant material from a high-speed centrifugation.

Assays of beta-lactamase. Hydrolysis of cefoxitin and other cephalosporin antibiotics was performed wih either a microiodometric modification of the

Novick method (8) or with purified enzymes by utilizing a spectrophotometric method (5).

Induction of beta-lactamase. Bacterial strains were grown in brain-heart infusion broth in side-arm flasks to a density of 10^8 CFU. Antibiotics were added in concentrations specified for each experiment. Induction was continued for 3 to 6 h, and organisms were removed by centrifugation. Bacteria were washed with room temperature, 0.05 M, pH 7.0, potassium phosphate buffer and then were resuspended in broth for studies with intact organisms or were sonically disrupted with a Branson sonifier. Growth of organisms was followed with a Klett spectrophotometer.

RESULTS

Cefoxitin is much less effective than are most cephalosporins against the common gram-positive coccal organisms; the MIC of cefoxitin for *S. aureus* was from 1.6 to 6.4 μg/ml, with 86% inhibited by 3.2 μg/ml (Table 1). This could be contrasted to an average cephalothin inhibitory concentration of 0.4 μg/ml, but is quite comparable to the 3.2 μg/ml cephalexin MIC for *S. aureus* (3). Cefoxitin showed greater activity against streptococci and pneumococci than it did against the staphylococci, but in general the activity was similar to that of cephalexin and less than that achieved with other cephalosporins.

Cefoxitin was active against a wide range of gram-negative organisms (Table 2). Indeed, only *Pseudomonas* species can be said to be uniformly resistant. *Escherichia coli* showed a fairly uniform susceptibility, with 35% susceptible to 3.1 μg/ml and 73% to 6.25 μg/ml. Cefoxitin at a concentration of 6.25 μg/ml inhibited 74% of *Proteus mirabilis* strains. Most *Salmonella* and *Shigella* species were susceptible to 1.6 μg/ml. Most *Proteus rettgeri* were susceptible to 25 μg/ml or less, and *Proteus morganii* and *Proteus vulgaris* were inhibited by 12.5 to 25 μg/ml. Activity against *Enterobacter* species was variable. The majority of strains tested were resistant. *Enterobacter aerogenes* was the most readily inhibited of the four species. *Serratia marcescens* strains also showed a variable resistance pattern. The strains had been bacteriocin typed to avoid a

TABLE 1. *Cumulative number of gram-positive isolates susceptible to cefoxitin*

Organism	No. of isolates	No. susceptible at MIC (μg/ml) of:							
		<0.8	0.8	1.6	3.2	6.4	12.5	25	>25
Staphylococcus aureus	23			8	12	3			
Staphylococcus epidermidis	10		1	4	5				
Streptococcus viridans	10	5	4	1					
Streptococcus pyogenes	10	3	6	1					
Diplococcus pneumoniae	10			1	8	1			
Streptococcus faecalis	10								10
Streptococcus bovis	10		1	2	4	1	1	1	

TABLE 2. *Cumulative number of gram-negative isolates susceptible*

Organism	No. of isolates	No. susceptible at MIC (μg/ml) of:								
		0.8	1.63	3.12	6.25	12.5	25	50	100	>100
Escherichia coli	89	2	8	22	33	19		3		2
Proteus mirabilis	55	1	2	18	20	7	3	2		2
Proteus vulgaris	16				3	5	2	1	1	4
Proteus morganii	32				1	7	12	8	2	2
Proteus rettgeri	8			1	3	2	2			
Providencia	15		3	1		4	1	1	1	4
Klebsiella pneumoniae	62		14	34	13	1				
Klebsiella ozeae	10		1	5	4					
Enterobacter	45		4	5	4	2	1	4	3	22
Serratia marcescens	37					7	6	5	9	10
Citrobacter freundii	24		2		5		3	2	6	6
Herellea	32			6	16	2				8
Shigella	10	1	4	3	2					
Salmonella	24	3	18	3						
Pseudomonas aeruginosa	30									30
Pseudomonas cepacia	10									10

single strain from the hospital producing an erroneous picture of either susceptibility or resistance. Some *Citrobacter* strains were inhibited by concentrations below 25 μg/ml, but in general *Citrobacter* strains had high MIC levels like those of *Enterobacter cloacae*. Sixty-eight per cent of *Herellea* strains were inhibited by 6.25 μg/ml. *Pseudomonas aeruginosa* and *Pseudomonas cepacia* were resistant to greater than 100 μg/ml.

Direct comparison of the activity of cefoxitin with the activity of cephalothin and cephloridine is given in Table 3. There was no significant difference in the MIC values against *E. coli*, *P. mirabilis*, and *Salmonella*. However, the activity of cefoxitin against indole-positive *Proteae* was significant in contrast to the general resistance of these species to cephalothin and cephloridine. The same is true of *Serratia*, with 45% inhibited by cefoxitin and none by cephalothin or cephloridine.

On the other hand, a better comparison of the activity of cefoxitin against these organisms may be seen in Table 4, which lists the MIC values of carbenicillin, ticarcillin, and ampicillin against *P. morganii*, *P. vulgaris*, and *Enterobacter*. In general, the organisms were more susceptible to the carbenicillin or ticarcillin, but totally resistant to the ampicillin. Isolates tested by the Kirby-Bauer single-disk technique (1) would be considered resistant to cefoxitin if the susceptibility determination had been on the basis of a cephalothin disk (Table 5). This is particularly true for the indole-positive *Proteae*, *Enterobacter*, and *Serratia* strains. In addition, strains of *Herellea*, *Citrobacter*, and a small number of *E. coli* would be considered resistant to cefoxitin if only cephalothin were the standard of susceptibility to cephalosporin antibiotics, as is the current practice.

The inoculum size was varied from 10^3 to 10^6 CFU. Strains of *E. coli*, *Klebsiella pneumoniae*, *S. marcescens*, *E. cloacae*, *E. aerogenes*, *P. mirabilis*, and *P. morganii* were tested. There was only a two- to fourfold increase in the cefoxitin MIC of susceptible strains when 10^6 CFU was used as compared with use of 10^3 CFU. However, strains with an MIC in excess of 100 μg/ml were as resistant with an inoculum of 10^3 CFU as with 10^6 CFU.

Use of brain-heart infusion, Trypticase soy, Mueller-Hinton, nutrient, and Columbia media yielded cefoxitin MIC values for *E. coli*, *K. pneumoniae*, *E. cloacae*, *P. morganii*, *P. mirabilis*, and *S. marcescens* which were all within a twofold dilution regardless of the medium used. This is within the error of the method.

Variation of the pH of the medium from 6 to 8 for both gram-positive and gram-negative orga-

TABLE 3. *Comparison of minimal inhibitory concentration of several cephalosporin antibiotics*[a]

Organism	Antibiotic	No. of isolates	Percent susceptible at MIC (μg/ml) of:							
			1.6	3.12	6.25	10.5	25	50	100	>100
Escherichia coli	Cefoxitin	24		12[b]	68	100				
	Cephalothin		4	16	50	96		100		
	Cephloridine		4	20	92			100		
Klebsiella pneumoniae	Cefoxitin	25	4	12	88	100				
	Cephalothin		32	96			100			
	Cephloridine		12	52	96		100			
Proteus mirabilis	Cefoxitin	25	8	20	68	72	96			100
	Cephalothin		20	28	72	84	88	96		100
Proteus, indole positive	Cefoxitin	30			10	26	73	86	100	
	Cephalothin									100
	Cephaloridine									100
Serratia marcescens	Cefoxitin	22				18	30	45	63	100
	Cephalothin									100
	Cephaloridine									100
Salmonella	Cefoxitin	24	84	100						
	Cephalothin		80	100						

[a] Values were determined by the agar plate method.
[b] Values represent percentage inhibited.

TABLE 4. *Cumulative percentage of isolates susceptible to cefoxitin, carbenicillin, ticarcillin, and ampicillin*

Organism	Antibiotic	No. of isolates	Percent susceptible at MIC (μg/ml) of:							
			1.6	3.12	6.2	12.5	25	50	100	>100
Proteus morganii	Cefoxitin	16				12	75	87	100	
	Carbenicillin		37	62			75	87	100	
	Ticarcillin		25		50	75	100			
	Ampicillin									100
Proteus vulgaris	Cefoxitin	16			12	62	87	100		
	Carbenicillin		50				100			
	Ticarcillin				50	75		100		
	Ampicillin									100
Enterobacter	Cefoxitin	22		9	18					100
	Carbenicillin		9	36		45	63	81		100
	Ticarcillin			9	45	54	63		81	
	Ampicillin									100

TABLE 5. *Susceptibility of gram-negative bacteria to cefoxitin, cephalothin, and carbenicillin*[a]

Organism	No. of isolates	No. of isolates susceptible		
		Cefoxitin	Cephalothin	Carbenicillin
Escherichia coli	36	36	33	29
Klebsiella pneumoniae	24	20	20	0
Enterobacter	72	12	4	46
Serratia	33	8	1	12
Citrobacter	20	6	5	15
Herellea	14	11	0	3
Proteus vulgaris	12	7	0	12
Proteus morganii	6	5	0	6
Proteus rettgeri	4	4	0	4
Pseudomonas	25	0	0	21

[a] The Kirby-Bauer method of disk susceptibility was used. Disks contained 30 μg of cefoxitin, 30 μg of cephalothin, and 50 μg of carbenicillin. A zone diameter of 18 mm or greater was used.

nisms revealed no significant effect, and organisms were not rendered susceptible or resistant by alteration of pH of the medium from acid to alkaline.

Resistance to hydrolysis by beta-lactamases. Organisms which were resistant to cephalosporins were selected for study to determine the degree of hydrolysis of cefoxitin by intact bacteria. Table 6 shows the susceptibility of the organisms to ampicillin, cephaloridine, cephalothin, and cefoxitin compared with the amount of antibiotic hydrolyzed. In spite of the resistance of many of these oragnisms to 100 μg of cefoxitin per ml, only 5 of the 17 strains hydrolyzed cefoxitin, and the amount hydrolyzed was trivial. *E. coli*, which possess different types of beta-lactamases according to the Richmond et al. (10) classification, although they hydrolyzed penicillins or cephalosporins efficiently did not appreciably destroy cefoxitin, although two of the strains were resistant to over 200 μg/ml. Both *E. cloacae* and *E. aerogenes* were resistant or susceptible to cefoxitin without regard to the activity of the beta-lactamase of the intact cell against the antibiotic. The lack of correlation of beta-lactamase activity and resistance to cefoxitin was also seen with *Serratia* strains, all of which were resistant to cephalothin and one of which was susceptible to ampicillin and cefoxitin. Thus, in these species, *E. coli*, *Enterobacter*, *Serratia*, and *Proteus*, the organisms can be resistant in spite of the stability of cefoxitin to the gram-negative beta-lactamase.

Although intact cells did not hydrolyze cefoxitin, it was possible that strategically placed beta-lactamases could hydrolyze the compound as it entered the cell. For this reason partially purified beta-lactamase preparations were used to determine the resistance of cefoxitin to destruction. Table 7 demonstrates that cefoxitin is also resistant to hydrolysis by different types of beta-lactamase. An *E. coli* enzyme of the Richmond type III or TEM does not hydrolyze the compound. The activity of this enzyme against penicillins would be at least 100-fold greater. A beta-lactamase from *E. cloacae*, which is primarily a cephalosporinase even in a purified, isolated state, does not effectively hydrolyze cefoxitin. The induced beta-lactamase from *P. aeruginosa*, which is primarily a cephalosporinase, also does not destroy cefoxitin. This resist-

TABLE 6. *Susceptibility of organisms to antibiotics in comparison with the amount of antibiotic hydrolyzed*[a]

Microorganism	Susceptibility				Antibiotic hydrolyzed (%)			
	Ampicillin	Cephloridine	Cephalothin	Cefoxitin	Ampicillin	Cephloridine	Cephalothin	Cefoxitin
Escherichia coli 109	R	S	S	S	100	100	10	0
E. coli 1927	R	R	R	R	100	100	75	0
E. coli 1929	S	R	R	R	20	85	100	10
Enterobacter cloacae 670	R	R	R	R	75	75	50	0
E. cloacae 673	R	R	R	S	0	0	0	0
E. cloacae 1374	R	R	R	R	70	100	65	5
Enterobacter aerogenes 1373	S	R	R	S	0	50	25	0
E. aerogenes 1675	R	R	R	R	10	20	10	0
Serratia marcescens 1109	R	R	R	R	20	45	50	10
S. marcescens 1613	S	R	R	S	0	0	5	0
S. marcescens 1101	R	R	R	R	0	10	10	0
Proteus mirabilis 1367	R	R	R	R	0	0	0	0
P. mirabilis 1077	R	R	R	R	100	50	10	0
P. vulgaris 684	R	R	R	S	100	10	10	0
P. morganii 1619	R	R	R	R	100	100	100	20
P. rettgeri 671	R	R	R	R	20	20	10	5
Citrobacter freundii 2017	S	R	R	S	0	72	50	0

[a] S, Susceptible; R, resistant.

TABLE 7. *Hydrolysis of cephalosporin antibiotics by partially purified beta-lactamases*

Beta-lactamase	Substrate hydrolyzed (μmol/min)			
	Cefoxitin	Cephalothin	Cephloridine	Cephalexin
Escherichia coli	<0.2	4.1	7.0	0.3
Enterobacter cloacae	0.7	2.6	9.0	7.6
Proteus morganii	0.2	33.1	7.8	5.6
Salmonella typhimurium	<0.1	2.3	12.0	0.2
Pseudomonas aeruginosa	0.2	4.1	18.4	0.7

TABLE 8. *Effect of previous exposure to cefoxitin and cephalothin*

Organism	Inducer	Inhibitor	Growth (h)[a]		
			2	3.5	6.5
Serratia marcescens 1631	None	None	28	122	153
	Cephalothin	Cefoxitin	16	23	29
	Cefoxitin	Cefoxitin	8	78	120
Proteus morganii 1618	None	None	16	94	153
	Cephalothin	Cefoxitin	0	0	14
	Cefoxitin	Cefoxitin	33	98	128
Enterobacter cloacae 670	None	None	22	98	134
	Cephalothin	Cefoxitin	23	76	128
	Cefoxitin	Cefoxitin	21	87	128

[a] Values represent Klett readings. Organisms were grown in the presence of cephalothin (200 μg/ml) and cefoxitin (25 μg/ml) to serve as inducing agent. After 3.5 h, a sample from each was removed, washed, and resuspended in medium to which 25 μg of cefoxitin per ml was added. Organisms were placed in side arm flasks on a water bath shaker at 35 C, and growth was followed with a Klett spectrophotometer.

ance to hydrolysis is not due to lack of affinity of cefoxitin for beta-lactamases. By using an *E. coli* TEM beta-lactamase cefoxitin had, with penicillin as substrate, a K_i/K_m of 1×10^{-3}, and with cephaloridine as substrate a K_i/K_m of 1.5×10^{-4}. With the same substrates oxacillin had a K_i/K_m of 2×10^{-4}. This demonstrates that the compound does act as an inhibitor of the hydrolysis of other penicillins and cephalosporins, albeit less efficient than oxacillin.

A possible explanation of the low level of resistance of species such as *Enterobacter* and *Serratia* to cefoxitin, compared with their resistance to other cephalosporin type antibiotics, was investigated by determining the effect of cefoxitin on induction of beta-lactamase. An *E. cloacae* strain of intermediate susceptibility, cefoxitin MIC of 50 μg/ml, was exposed to four cephalosporins, namely, cephalothin, cefazolin, cephalexin, and cefoxitin, at a concentration of 25 μg/ml for 2 h. The organisms were disrupted by sonic treatment, and the beta-lactamase activity was determined. The amount of cephalothin, cephloridine, cephalexin, and cefoxitin hydrolyzed was the same regardless of the inducing cephalosporin. Thus cefoxitin was as effective an inducer of beta-lactamase activity as were cephalothin, cephalexin, and cefazolin.

However, previous exposure to specific cephalosporins does affect the resistance of some

strains. *P. morganii* and *S. marcescens* strains which had been grown in the presence of cephalothin were inhibited by cefoxitin (Table 8). But the same strains which had grown in the presence of cefoxitin now grew as well as control organisms. It was not determined whether the strains had increased hydrolytic activity, but other experiments which failed to demonstrate increased beta-lactamase induction would indicate that this was not the explanation. In contrast, an *E. cloacae* strain which is completely resistant to all cephalosporins was unaffected by previous cefoxitin exposure.

DISCUSSION

Cefoxitin is an analogue of cephamycin C, a family of antibiotics similar to the cephalosporins, but which have been said to exhibit increased resistance to hydrolysis by beta-lactamases of gram-negative organisms (2, 9). This study shows that cefoxitin is less effective than cephalosporin antibiotics such as cephalothin or cephaloridine against gram-positive coccal organisms, but the activity is comparable with that of cephalexin. Cefoxitin activity against *E. coli*, *P. mirabilis*, and *K. pneumoniae* is comparable with the activity of cephalothin, but cefoxitin has activity against an appreciable number of strains of *Enterobacter*, *Citrobacter*, indole-positive *Proteae*, and *Herellea*. It should be pointed out, however, that these strains are ones which are susceptible to carbenicillin and ticarcillin. Indeed, as shown in Table 4, most strains of *P. morganii* and *P. vulgaris* have a carbenicillin MIC significantly lower than that of cefoxitin. However, use of cephalothin as a standard of cephalosporins would cause a number of cefoxitin-susceptible organisms to be mislabeled as resistant.

Resistance of cefoxitin to hydrolysis by cephalothin-resistant bacteria is easily demonstrated. However, some bacterial strains which failed to hydrolyze cefoxitin were nonetheless resistant. This was true particularly of certain *Enterobacter cloacae* and *Serratia marcescens* strains. It is not possible to explain the lack of hydrolysis of cefoxitin by intact bacteria by failure of the cefoxitin to get inside the cell since isolated enzymes did not hydrolyze the antibiotic. Cefoxitin can act as a competitive inhibitor of the hydrolysis of penicillins by beta-lactamases, showing that it does bind to the enzymes. Indeed it might be possible that strategically placed beta-lactamase binds the entering cefoxitin preventing it from reaching its target site in resistant strains. Such a concept could be tested with penicillins which are good beta-lactamase inhibitors, but inactive against the strains.

The data obtained do not permit one to state conclusively that resistance of cefoxitin to hydrolysis is the important factor in its activity. Preliminary studies in our laboratory with several related compounds suggest that derivatives of this type have a high affinity for receptor sites because these other compounds are highly active, although readily hydrolyzed.

It is not possible from the data presented or from previous investigations (2, 9, 11) to predict whether a particular strain of *Enterobacter*, *Serratia*, or *P. morganii* will be susceptible to cefoxitin. However, strains of these species which are susceptible to carbenicillin or ticarcillin tend to be susceptible to cefoxitin and to another agent, cefamandole (H. C. Neu, manuscript in preparation).

Cefoxitin acts as an inducer of beta-lactamase comparable with other cephalosporin molecules. In one series of experiments, however, the data suggested that prior exposure to cefoxitin caused the organism to become increasingly resistant. Because destruction of antibiotic is not involved, selection of cells with altered cell wall or receptor sites is a possible explanation. Selection of such resistant mutants has occurred in clinical settings with carbenicillin therapy (6).

Extensive animal experiments will have to be conducted to determine the usefulness of cefoxitin in actual infections. The studies of Miller et al. (4) have shown that cefoxitin protects mice against *E. cloacae*. However, further clinical investigation of cefoxitin is clearly indicated.

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