IN VITRO EVALUATION OF CEPHACETRILE, A NEW CEPHALOSPORIN ANTIBIOTIC

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Cephacetrile, 7-cyanacetamido-cephalosporanic acid, is a new cephalosporin active against many gram-positive and gram-negative organisms. *Staphylococcus aureus* and *Streptococcus pyogenes* were inhibited by less than 1 μg/ml, but 25 μg/ml were needed to inhibit enterococci. The majority of *Escherichia coli* and *Klebsiella* strains and *Proteus mirabilis* strains were inhibited by 12.5 μg/ml. *Serratia*, indole-positive *Proteus*, *Pseudomonas* and *Enterobacter* strains were resistant to more than 100 μg/ml. The *in vitro* activity of cephacetrile was influenced by inoculum size. Tube dilution and agar-plate dilution methods used to determine MIC differed by two to eight-fold. Cephacetrile was hydrolyzed by β-lactamases of intact *E. coli*, *S. typhymurium*, *E. cloacae*, *P. morganii*, *Ps. aeruginosa* strains. Hydrolysis of cephacetrile by purified β-lactamases showed that cephacetrile was not hydrolyzed as rapidly as cephalothin or cephaloridine.

A variety of cephalosporin derivatives have proved to be extremely useful in the treatment of serious infections. Cephalothin has produced remarkably few toxic reactions in humans, but is not well tolerated by the intramuscular route. Cephaloridine has been associated with renal toxicity which has tended to limit its usefulness[5]. Cephalothin has been used extensively at this institution for the past six years in treating cases of serious infection. For this reason we wished to determine the sensitivity of clinical isolates to cephacetrile (7-cyanacetamido-cephalosporanic acid), a compound which in preliminary investigations has been tolerated by intramuscular injection and has not been associated in animals with renal toxicity[1].

Cephalosporins have been found resistant to the β-lactamases of most gram-positive bacteria, but the β-lactamases of gram-negative organisms vary in their ability to hydrolyze cephalosporins. The ability of constitutive, inducible and episomally-mediated β-lactamases of several gram-negative organisms to hydrolyze cephacetrile was investigated.

Methods and Meterials

Cephacetrile (Fig. 1) was provided by Ciba-Geigy Corporation as a crystalline sodium salt which was prepared fresh daily in sodium phosphate buffer or medium. Cephalothin and cephaloridine were gifts from Eli Lilly.

Bacterial strains used were clinical isolates from blood, sputum and urine of patients hospitalized at the Columbia Presbyterian Medical Center during 1969~1971.

Susceptibility testing methods. The activity of cephacetrile was measured by tube

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dilution using trypticase soy broth (BBL). Serial two-fold dilutions were performed. An inoculum of 0.5 ml of a 10^4 dilution of an overnight culture was added to each tube giving a final volume of 1 ml. Incubation was for 16 hours at 35°C. The minimum inhibitory concentration (MIC) of the antibiotic was defined as the lowest concentration which inhibited visible turbidity. Bactericidal activity (MBC) was determined by streaking onto nutrient agar (Difco) a loopful of broth from the tubes which failed to become turbid.

Agar-plate dilutions were performed on trypticase soy agar (BBL) containing the antibiotic. Plates were used the same day they were prepared or refrigerated at 8°C and used the next morning. An inoculum of 0.01 ml of an overnight broth culture was spotted on the plates. The plates were incubated at 35°C for 16 hours and examined for growth. The effect of inoculum size on agar-plate sensitivities was determined by plating 0.01 ml of 10^2 and 10^4 dillutions of overnight cultures.

The susceptibilities of cephalosporins as substrates by β-lactamase were compared using equimolar concentrations of antibiotic prepared in sodium phosphate buffer, pH 7.0, 0.05 M using a modification of the microiodometric penicillinase assay[4],or by a spectrophotometric assay[3]. Partially purified β-lactamases previously prepared were used for hydrolysis.

Whole cell hydrolysis studies were conducted by incubating washed cells with the compounds, removing an aliquot and assaying the antibiotic remaining in a cell-free filtrate. Assay of unhydrolyzed antibiotic was performed by the agar-well diffusion technique using *Bacillus subtilis* as test organism.

Results

The activity of cephacetrile against gram-positive organisms is shown in Table 1 and against gram-negative organisms in Table 2. All staphylococci were sensitive to 1.28 μg/ml or less of cephacetrile. Seven of the strains tested were methicillin resistant. *Strept. pyogenes* were sensitive but had a broad MIC range. Enterococci had a high cephacetrile MIC. *Staph. epidermidis* strains[10] tested by an agar-plate method were all susceptible to 1.28 μg/ml of cephacetrile.

Among the Enterobacteriaceae, utilizing a 10^4 dilution in the broth method, 36 percent of *E. coli* were susceptible to 6.25 μg/ml and 64 percent to 12.5 μg/ml. *Klebsiella pneumoniae* showed a similar pattern with 60 percent of strains susceptible to 12.5 μg/ml of cephacetrile. *Proteus mirabilis* strains showed a uniform susceptibility to 12.5 μg/ml of the antibiotic. This contrasts with the rather broad range of susceptibility of *E. coli*, *Klebsiella*, *Salmonella* and *Shigella*. Indole-positive *Proteus* strains (*P. morganii*, *P. rettgeri*, *P. vulgaris*) were uniformly resistant to cephacetrile as were *Pseudomonas aeruginosa*, *Enterobacter* species (*E. cloacae*, *E. aerogenes*, *E. hafnia*, *E. liquifaciens*) and most *Serratia marcescens* (both pigmented and nonpigmented strains). *Providence* and *Herellea* strains were also resistant to cephacetrile.

Direct comparison of the susceptibility of various bacteria to cephacetrile and

Table 1. Cephacetrile dilution studies with gram-positive organisms

Organism	No. of strains	Minimum inhibitory concentration (μg/ml)*									
		0.04	0.08	0.16	0.32	0.64	1.28	2.56	6.25	12.5	25
Strept. pyogenes	22	3	4	9	5	1	—	—	—	—	—
Staph. aureus	19	—	—	—	—	12	7	—	—	—	—
Enterococci	23	—	—	—	—	—	—	—	2	4	17

* Tube dilution method with trypticase soy broth using an inoculum of a 10^4 dilution of an overnight culture.

Table 2. Cephacetrile dilution studies with gram-negative organisms

Organism	No. of strains	Minimum inhibitory concentration (μg/ml)*							
		<3.12	3.12	6.25	12.5	25	50	100	>100
Escherichia coli	58	2	—	19	16	7	1	6	7
Klebsiella	25	1	1	6	7	2	1	3	4
Enterobacter	28	—	—	—	—	—	—	3	25
Proteus mirabilis	22	—	—	—	21	1	—	—	—
Salmonella	20	—	3	12	2	2	—	—	1
Shigella	8	—	—	3	3	2	—	—	—
Pseudomonas	29	—	—	—	—	—	—	—	29
Serratia	20	—	—	—	2	—	—	1	17
Citrobacter	10	3	—	—	—	—	—	—	7
Proteus morganii	10	—	—	—	—	—	—	—	10
Proteus vulgaris	5	—	—	—	—	—	—	—	5
Proteus rettgeri	5	—	—	—	—	—	—	—	5
Providence	5	—	—	—	—	—	—	—	5
Herellea	3	—	—	—	—	—	—	—	3

* Tube dilution method utilizing an inoculum of a 10^4 dilution of an overnight culture performed in trypticase soy broth.

Table 3. Antibacterial comparison of cephacetrile and cephalothin

Organism	Minimal inhibitory concentration (μg/ml)*	
	Cephacetrile	Cephalothin
Staph. aureus	0.64	0.16
Staph. epidermidis	0.64	0.64
Strept. pyogenes	0.16	0.08
Strept. faecalis	25.0	12.50
E. coli	6.25	12.50
Klebsiella	3.12	3.12
Enterobacter	200.	400.
S. typhimurium	6.25	12.5
P. mirabilis	12.5	3.12
P. vulgaris	100.	100.
Pseudomonas	400.	400.

* Determined by agar-plate technique using a 10^2 dilution of an overnight culture. Each organism refers to a single isolate.

Table 4. Comparison of minimum inhibitory activity in broth-dilution and agar-plate dilution*

Organism	No. of strains tested	No. of fold higher MIC obtained by agar-plate			
		0	2	4	8
Escherichia coli	40	10	9	15	6
Klebsiella	25	7	6	6	6
Proteus mirabilis	8		6	2	
Enterococci	23	4	17	2	
Serratia	3	3			
Enterobacter	3	3			
Pseudomonas	3	3			

* Broth dilution performed with a 5×10^4 CFU per ml from an overnight culture in trypticase soy broth. Agar-plate dilution performed with 0.01 ml of an overnight culture (approximately 5×10^6 CFU).

cephalothin is shown in Table 3. The MIC of cephacetrile and cephalothin varied by a single tube in most cases.

Broth dilution and agar-plate dilution methods did not yield comparable results. Cephacetrile MIC values by the agar-plate method with 0.01 ml of an overnight culture being utilized as inoculum were eight-fold greater than those obtained by tube dilution where 5×10^4 colony forming units were used (Table 4).

The effect of inoculum size upon the agar-plate method was also particularly pronounced. For example, a 100-fold dilution of *Proteus* cultures produced a two to eight-fold reduction in the cephacetrile MIC. This was true of strains that lacked β-lactamase and of those that possessed either a constitutive β-lactamase or a β-lactamase which was inducible. A 100-fold dilution produced a similar effect with *E. coli* and *Klebsiella*.

The cephacetrile minimum inhibitory concentration and minimum bactericidal concentration for gram-negative bacteria was either identical or varied by only two to

Table 5. Relative activity of several β-lactamases against cephalosporins

β-Lactamase from	Relative activity				
	Penicillin	Cephacetrile	Cephalothin	Cephaloridine	Cephalexin
P. morganii	100	12.3	39	14.7	13.4
Ps. aeruginosa	100	4.1	7.85	23.8	1.34
E. coli	100	0	0.23	14.9	0

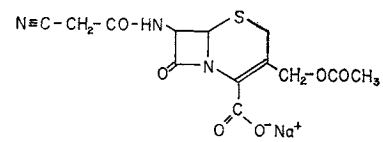
Equimolar concentrations of antibiotics were prepared in 0.05M. pH 7.0 potassium phosphate buffer. Purified β-lactamase from each organism was incubated at 30℃ wiith the substrate. Relative activity is compared to hydrolysis of benzylpenicillin.

Table 6. MICHAELIS constant for various β-lactamases

β-Lactamase from	Km (M)		
	Cephacetrile	Cephaloridine	Ampicillin
S. typhimurium	1.05×10^{-4}	4.3×10^{-4}	2.6×10^{-5}
P. morganii	2.36×10^{-5}	1.0×10^{-4}	2.7×10^{-5}
Ps. aeruginosa	1.5×10^{-4}	5×10^{-4}	1.6×10^{-5}

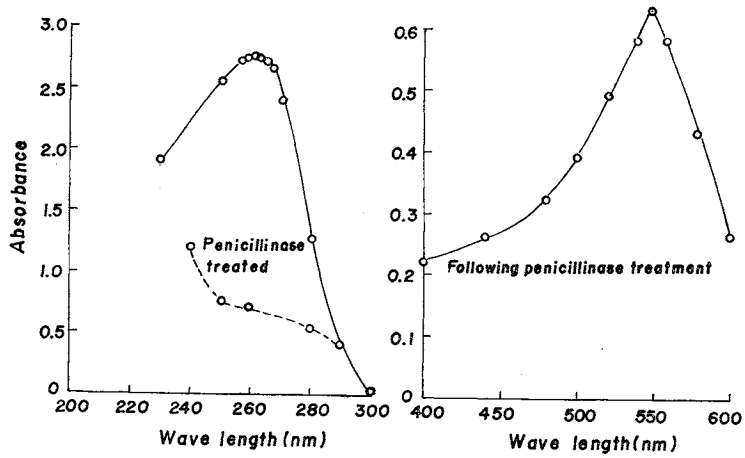
β-Lactamase assays were performed using the spectrometric assay[3] for cephacetrile and cephaloridine. The microidometric assay[4] was used for ampicillin. Km was calculated by standard LINEWEAVER-BURK plots.

Fig. 1.



Cephacetrile

Fig. 2.



four-fold. This was not correlated with β-lactamase production or the level of the MIC; that is, strains with a high MIC did not have a greater discrepancy between MIC and MBC.

The lowest cephacetrile MIC was consistently found with nutrient medium. No differences were seen with the other media used. For example, *Klebsiella* 888 had an MIC of 12.5 μg/ml on nutrient agar and 100 μg/ml on trypticase soy (BBL), brain-heart infusion (BBL) and MUELLER-HINTON (BBL) agars. The results with *E. coli* and *P. mirabilis* were similar. Values obtained in tube dilutions performed in 50% human serum showed no appreciable difference from those found in control broth tubes.

β-Lactamase effect. The hydrolysis and inactivation of cephacetrile is easily followed since the active molecule has an absorption maximum at 260 nm which disappears with the hydrolysis of the β-lactam ring (Fig. 2). A pink color appears when the β-lactam ring is opened that has an extinction maximum at 550 nm. The

hydrolysis of cephacetrile by both intact cells and purified β-lactamases was investigated. Cephacetrile is hydrolyzed readily by intact *E. cloacae*, *P. morganii*, *E. coli* and *P. aeruginosa*. Cephacetrile is more resistant to hydrolysis by β-lactamases purified from these bacteria than are cephalothin or cephaloridine (Table 5). Cephacetrile has a high affinity for β-lactamases, and can function as a competitive inhibitor of the hydrolysis of benzylpenicillin or ampicillin by resistance-factor mediated β-lactamases of *E. coli* or *Salmonella*. Comparison of the Km of cephacetrile, cephaloridine and ampicillin shows that cephacetrile has an affinity for the enzymes which is between that of ampicillin and cephaloridine (Table 6).

Discussion

Cephacetrile has an excellent *in vitro* activity against gram-positive species, but gram-negative organisms vary markedly in susceptibility. The cephacetrile MIC for gram-negative organisms is influenced by inculum size both in the agar plate and tube dilution methods. The type of medium used in the assay also affects the level of the MIC. These effects do not appear to be mediated by penicillinase only but also by the intrinsic resistance of the organisms.

The higher cephacetrile MIC values we obtained compared to those reported in a previously published study[1] may be due to differcences in assay medium and the size of inoculum used. Some differences may also be due to the level of resistance to cephalosporins seen in our institution where cephalosporins have been extensively employed as chemotherapeutic agents for the past six years. However, direct comparison of the MIC of cephacetrile and cephalothin for various bacteria showed no significant differences.

Comparison of the hydrolysis of cephacetrile by various gram-negative β-lactamases shows that it is readily hydrolyzed by intact bacteria. Whether the β-lactamase is an induced enzyme (*Enterobacter*), constitutive (*P. morganii*) or episomally mediated (*E. coli*) has no influence. The location of the β-lactamase, whether an internal enzyme, that is, confined within the cytoplasmic membrane, located in the periplasmic space, or surface bound did not prevent hydrolysis of cephacetrile. The fact that cephacetrile is not significantly protein bound[1] may contribute to its ready entry into the bacterial cell.

Cephacetrile is more resistant to hydrolysis by episomal β-lactamases (*E. coli*) of primary penicillin affinity than is cephaloridine.

The value of cephacetrile in the clinical setting will have to be dependent upon its lack of toxicity and ease of administration as compared with other cephalosporins.

Acknowledgement

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