THE COMPARATIVE β-LACTAMASE RESISTANCE AND INHIBITORY ACTIVITY OF 1-OXA CEPHALOSPORIN, CEFOXITIN AND CEFOTAXIME

KWUNG P. FU and HAROLD C. NEU*

Division of Infectious Diseases, Departments of Medicine and Pharmacology,
College of Physicians and Surgeons, Columbia University,
New York, N. Y. 10032, U.S.A.

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The β-lactamase stability and inhibitory activity of 1-oxa cephalosporin, (6R,7R)-7-[[carboxy(4-hydroxyphenyl)acetyl]amino]-7-methoxy-3-[[(1-methyl-1H-tetrazol-5-yl)thio]methyl]-8-oxo-5-oxa-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, was investigated and compared to that of cefoxitin and cefotaxime. There was no detectable β-lactamase hydrolysis of 1-oxa cephalosporin, cefotaxime and cefoxitin when incubated with β-lactamases of plasmid or chromosomal origin which were primarily cephalosporinases or enzymes which hydrolyzed both penicillins and cephalosporins. The β-lactamase inhibitory activity of 1-oxa cephalosporin was comparable to that of cefoxitin and cefotaxime. At equal molar concentration of substrate and inhibitor, cefoxitin, cefotaxime and 1-oxa cephalosporin effectively inhibited cephalosporinase hydrolysis of cephaloridine. Cefoxitin and cefotaxime were more effective inhibitors than the 1-oxa cephalosporin against a *Providencia* enzyme, whereas cefotaxime and 1-oxa cephalosporin were more effective inhibitors of a *Citrobacter* cephalosporinase.

The ability to produce β-lactamases has been associated with the resistance of Gram-negative organisms to β-lactam antibiotics[4,7,9]. Although β-lactamases are not the only factors contributing to the resistance of Gram-negative bacteria to β-lactam antibiotics, there is a growing interest in cephalosporins that are resistant to β-lactamase hydrolysis. Recently we reported that a cephalosporin, cefotaxime, not only was resistant to β-lactamase hydrolysis, but was also an effective cephalosporinase inhibitor[3]. The development of the 1-oxa cephalosporin, (6R,7R)-7-[[carboxy(4-hydroxyphenyl)acetyl]amino]-7-methoxy-3-[[(1-methyl-1H-tetrazol-5-yl)thio]methyl]-8-oxo-5-oxa-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, with the replacement of sulfur by oxygen at position one in the six membered dihydrothiazine component of the cephem nucleus prompted us to evaluate its β-lactamase stability and its ability to inhibit β-lactamases compared to cefoxitin and cefotaxime.

Materials and Methods

Organisms:

All the organisms tested were clinical isolates from Columbia-Presbyterian Medical Center, New York City. The organisms were selected on the basis of their resistance to either cephaloridine or cephalothin and their production of β-lactamase as determined by use of the chromogenic cephalosporin, nitrocefin[6].

Antibiotics:

Cephaloridine, cephalothin and 1-oxa cephalosporin were gifts from Lilly Research Laboratories.

* Address reprint requests to: Department of Medicine, 630 West 168 Street, New York, N.Y. 10032, U.S.A.

Cefoxitin was supplied by Merck Sharp & Dohme. Cefotaxime was obtained from Hoechst-Roussel. Antibacterial activity was determined by the agar dilution method in MUELLER-HINTON agar using an inoculum of 10^5 colony forming units (CFU)[1]. Synergy tests were determined by a checkerboard method described earlier[1].

β-Lactamase Preparation and Assay:

The β-lactamases from *Shigella sonnei* 10-101, *Pseudomonas aeruginosa* 1882, *Proteus morganii* 771, *Klebsiella pneumoniae* 3971, *Citrobacter freundii* 2732, and *P. aeruginosa* 3901 have been described previously[3]. The *Acinetobacter* 4303 β-lactamase was a constitutive enzyme. *Providencia* 3905 β-lactamase was a constitutive enzyme and *Providencia* 2943 enzyme was induced by 25 μg/ml cephaloridine by the method described earlier[3]. All enzymes were partially purified from sonic extracts which had been subjected to centrifugation and Sephadex G-50 column elution. Substrate specificity determination and inhibitor profile showed that the *Providencia* 2943 enzyme functioned primarily as a cephalosporinase; whereas the *Providencia* 3905 enzyme was a broad spectrum β-lactamase hydrolyzing both penicillins and cephalosporins.

Assay of β-Lactamase:

β-Lactamase activity was determined by the spectrophotometric method in a temperature controlled spectrophotometer at 30°C[2,3]. The decrease in optical density at 255 nm was recorded for the first 5 minutes. Inhibition of the hydrolysis of cephaloridine was determined by the addition of cefoxitin, cefotaxime or 1-oxa cephalosporin. Kinetic parameters were estimated from a least squares fit to LINEWEAVER-BURK plots utilizing a computer program.

Results

The mean activity of the compound against β-lactamase producing bacteria is given in Table 1. The β-lactamase stability of 1-oxa cephalosporin is presented in Table 2. The 1-oxa cephalosporin was not hydrolyzed by any of the β-lactamases, and had the same stability that cefoxitin and cefotaxime had. The stability of cefoxitin, cefotaxime and 1-oxa cephalosporin toward β-lactamase hydrolysis prompted us to investigate their ability to inhibit the β-lactamase hydrolysis of cephaloridine (Table 3). At equimolar concentration of cephaloridine(0.2 mM), cefoxitin, cefotaxime and 1-oxa cephalosporin produced significant inhibition of the hydrolysis of cephaloridine by *Citrobacter, Providencia* 2943 and *P. morganii* β-lactamases. On the other hand, no significant inhibitory activity was noted against the *Shigella* and *Klebsiella* enzymes. More than 70% of the *Acinetobacter* 4303 β-lactamase activity and 40% of *Pseudomonas* and *Providencia* 3905 enzyme activity were inhibited by the three antibiotics.

The comparative inhibitory activity of the compounds against a β-lactamase with primary cephalo-

Table 1. Activity of 1-oxa cephalosporin against β-lactamase producing bacteria.

Organism (No.)	MIC Mean	Organism (No.)	MIC Mean
Staphylococcus aureus (5)	6.2	*Proteus morganii* (10)	0.1
Staphylococcus epidermidis (5)	25	*Proteus inconstans* (10)	0.1
Haemophilus influenzae (3)	0.01	*Citrobacter freundii* (10)	0.1
Neisseria gonorrhoeae (3)	0.01	*Serratia marcescens* (10)	12.5
Escherichia coli (10)	0.1	*Salmonella typhimurium* (10)	0.1
Klebsiella pneumoniae (10)	0.1	*Shigella sonnei* (10)	0.1
Enterobacter cloacae (10)	0.1	*Pseudomonas aeruginosa* (10)	12.5
Proteus vulgaris (10)	0.1	*Bacteroides fragilis* (10)	6.2
Proteus rettgeri (10)	0.1		

Table 2. Comparative β-lactamase hydrolysis of 1-oxa cephalosporin with cephaloridine, cephalothin, cefoxitin and cefotaxime.

β-Lactamase source	Type of β-lactamase	Relative hydrolysis rate (%)*		
		Cephalothin	Cefoxitin+ cefotaxime	1-Oxa-cephalosporin
Citrobacter freundii 2732	Cephalosporinase	89	0	0
Proteus morganii 771	Cephalosporinase	160	0	0
Providencia stuarti 2943	Cephalosporinase	30	0	0
Acinetobacter 4303	Penicillinase	212	0	0
Providencia stuarti 2395	Penicillinase plasmid	113	0	4
Klebsiella pneumoniae 3973	Cephalosporinase & penicillinase	45	0	0
Pseudomonas aeruginosa 1882	Cephalosporinase & penicillinase plasmid	30	0	0
Shigella sonnei 10–101	Cephalosporinase & penicillinase plasmid	45	0	0
Staphylococcus aureus	Penicillinase plasmid	0	0	0

* The hydrolysis of cephaloridine is given a value of 100. The concentration of all substrates are 0.1 mM.

sporinase activity was further investigated. Fig. 1 shows the inhibitory activity against β-lactamase hydrolysis of cephaloridine at different concentrations of cefoxitin, cefotaxime and 1-oxa cephalosporin. Against the *Providencia* 2943 enzyme the inhibitory activity of cefoxitin was greater than that of cefotaxime and the 1-oxa cephalosporin. On the other hand, 1-oxa cephalosporin and cefotaxime were equivalent in inhibitory activity against the *Citrobacter* enzyme and ten-times more effective than was cefoxitin. The 1-oxa cephalosporin competitively inhibited both the *Providencia* (not shown) and *Citrobacter* enzyme hydrolysis of cephaloridine (Fig. 2). For the *Providencia* enzyme, the 1-oxa cephalosporin had a *Ki* of 7.5 μM and a *Km* of 0.66 μM. Against the *Citrobacter* enzyme the 1-oxa cephalosporin had *Ki* value of 2.27×10^{-2} μM and a *Km* value of 0.192 μM. In contrast, cefoxitin tested with the *Citrobacter* enzyme had a *Ki* of 1.36 μM and a *Km* of 0.244 μM, indicating cefoxitin was a less effective inhibitor. The *Ki* of dicloxacillin against this *Citrobacter* cephalosporinase was 1.6×10^{-3} μM.

Table 3. Comparative-β-lactamase inhibitory activity of 1-oxa cephalosporin, cefoxitin and cefotaxime

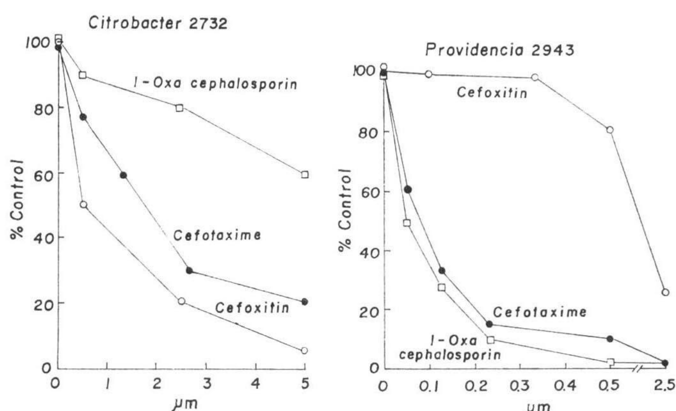
β-Lactamase source[a]	Relative hydrolysis rate[b]		
	1-Oxa cephalosporin	Cefoxitin	Cefotaxime
Citrobacter freundii 2732	0	0	0
Proteus morganii 771	0	0	0
Providencia stuarti 2943	1	1	0
Acinetobacter 4303	19	19	28
Providencia stuarti 3905	58	73	50
Klebsiella pneumoniae 3973	70	89	65
Pseudomonas aeruginosa 1882	56	56	43
Shigella sonnei 10–101	64	89	80

[a] The type of β-lactamase is the same as in Table 1.

[b] Reaction mixture contains 0.5 ml of 0.2 mM cephaloridine plus 0.5 ml of 0.05 M phosphate buffer (pH 7) as control or plus 0.5 ml of 0.2 mM of inhibitors. The hydrolysis of cephaloridine is given a value of 100.

The inhibition of cephaloridine hydrolysis by the 1-oxa cephalosporin suggested that it might act synergistically with other β-lactam agents as do the β-lactamase resistant penicillins. In order to determine if the inhibition found with the isolated enzymes was significant in intact growing cells, the compound was combined with cephaloridine and cephalothin and tested against selected β-lactamase

Fig. 1. Comparative β-lactamase inhibitory activity of 1-oxa cephalosporin, cefoxitin and cefotaxime. Substrate cephaloridine concentration is 0.01 mM.
β-Lactamase activity was measured spectrophotometrically for the first 5 minutes.



producing organisms in which the β-lactamase had primarily cephalosporinase activity (Table 4). Indifference and partial synergy was found when the compound was combined with cephaloridine and cephalothin against *Pseudomonas*, *Acinetobacter*, *Klebsiella*, *Citrobacter*, and *Providencia*. No antagonism was observed. However, the combination of 1-oxa cephalosporin with either cephalothin or cephaloridine showed a synergistic bactericidal inhibition of the *Citrobacter freundii* 2732 which produced a β-lactamase that was inhibited by the 1-oxa cephalosporin (Fig. 3). However, the low inhibitory level that the 1-oxa cephalosporin had against this organism would make this form of synergy unnecessary.

Fig. 2. Competitive inhibition of *Citrobacter* β-lactamase hydrolysis of cephaloridine by 0.5 μM of 1-oxa cephalosporin, 0.5 μM of cefoxitin and 0.05 μM of cefotaxime.

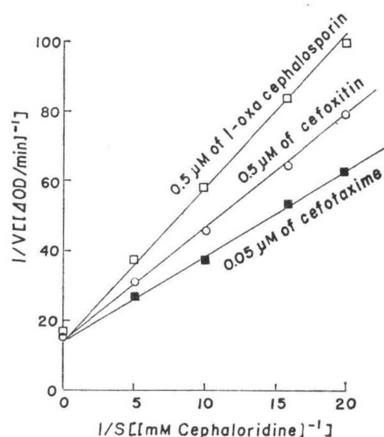


Table 4. Antibacterial activity of 1-oxa cephalosporin combined with cephaloridine and cephalothin.

Organism*	MIC (μg/ml)				
	1-Oxa cephalosporin	Cephaloridine	Cephalothin	1-Oxa cephalosporin + cephaloridine	1-Oxa cephalosporin + cephalothin
Acinetobacter 4303	50	≥400	≥400	12.5	25
Citrobacter 3532	0.2	200	12.5	0.1	0.1
Providencia 2943	0.05	≥400	50	0.1	0.05
Providencia 3905	0.05	>400	12.5	0.05	0.05
Pseudomonas 4404	12.5	≥400	≥400	12.5	6.3
E. coli 3994	0.8	3.2	6.3	0.4	0.8

* All the organisms tested here are β-lactamase producing organisms.

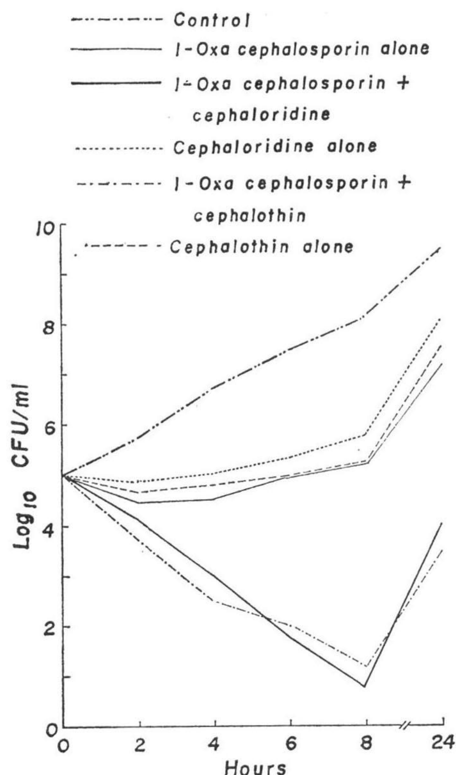
Discussion

We have shown that cefoxitin, cefuroxime and cefotaxime not only are resistant to β-lactamase hydrolysis, but also are inhibitors of β-lactamases which are of chromosomal origin and act primarily as cephalosporinases[3]. This study demonstrates that the 1-oxa cephalosporin also is resistant to β-lactamase hydrolysis and is a β-lactamase inhibitor. The methoxy group in the 1-oxa cephalosporin may confer the resistance to β-lactamase hydrolysis as it does with cefoxitin.

The competitive nature of 1-oxa cephalosporin inhibition of certain β-lactamases indicates that it competes with the substrate for the catalytic site in some β-lactamases. However, the fact that like cefoxitin and cefotaxime, 1-oxa cephalosporin is not a substrate for other types of β-lactamases and is not an inhibitor, suggests that it may not enter the catalytic site.

The inhibition of β-lactamase which primarily have cephalosporinase activity would be of clinical importance. Organisms producing these type of β-lactamases are major causes of serious Gram-negative infection in the hospital and are resistant to most penicillins and cephalosporins[8]. The recently developed β-lactamase inhibitors, clavulanic acid and the penicillanic acid sulfone, do not inhibit these β-lactamases[2,5]. Preliminary studies by our group indicate that this new compound does not induce β-lactamases which hydrolyze other cephalosporins. Unfortunately, the combination of the 1-oxa cephalosporin with an agent such as cephaloridine, which readily enters many Gram-negative bacilli but is β-lactamase unstable, did not result in synergy. It is possible that this is due to the greater affinity of the 1-oxa cephalosporin for β-lactam receptor proteins in the intact organism than for the β-lactamase. Thus although this agent is almost as effective an inhibitor for certain β-lactamases as are the β-lactamase-resistant antistaphylococcal penicillins, the use of this compound with other β-lactam compounds will not result in increased antibacterial activity.

Fig. 3. Synergistic activity of 1-oxa cephalosporin combined with cephaloridine and cephalothin at the concentration of one-fourth MIC of each antibiotic against *Citrobacter* 2732.
The MIC values against *Citrobacter* is 1-oxa cephalosporin 0.2 μg/ml; cephaloridine 50 μg/ml and cephalothin 25 μg/ml.



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