Beta-Lactamase Stability of HR 756, a Novel Cephalosporin, Compared to That of Cefuroxime and Cefoxitin

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Received for publication 15 March 1978

The stability to β -lactamase hydrolysis of HR 756, a new cephalosporin antibiotic, was compared to the β -lactamase stability of cefoxitin and cefuroxime. HR 756, cefoxitin, and cefuroxime were not hydrolyzed by Richmond type I, III, IV, and V β -lactamases. Antibacterial activity of HR 756 correlated well with resistance to β -lactamase hydrolysis except against *Pseudomonas aeruginosa*. HR 756, cefoxitin, and cefuroxime inhibited type I β -lactamases, but not type III, IV, or V enzymes. HR 756 was the most active inhibitor.

Cephalosporin antibiotics are important chemotherapeutic agents because of their broad antibacterial spectrum, which includes many species of gram-negative organisms that have become the major species causing hospital infections in the past 2 decades. Changes in the chemical structure of the cephalosporins have resulted in an increased antibacterial spectrum, more useful pharmacokinetic properties, and, in some cases, a high level of resistance to β -lactamase hydrolysis. The presence of β -lactamases in gram-negative organisms has been associated with resistance to cephalosporins, although these enzymes are not the only factor contributing to the resistance of gram-negative bacilli to β -lactam compounds (3, 9, 10, 15). There is great interest in antibiotics that are resistant to β -lactamase hydrolysis and in compounds that are inhibitors of β -lactamases (2, 5, 11). Certain β -lactamases of gram-negative bacilli are inhibited by β -lactam antibiotics such as cloxacillin or methicillin (11). Recently, naturally occurring inhibitors of β -lactamases produced by streptomyces have been discovered (1, 14). Cefoxitin and cefuroxime, two new β -lactam compounds, have been reported to be resistant to β -lactamases of gram-negative bacilli (3, 12).

HR 756 (Fig. 1) has been shown to be active against many bacteria resistant to the older cephalosporins (6; H. C. Neu, N. Aswapokee, and K. P. Fu, manuscript in preparation). We wished to assess the β -lactamase resistance of this compound and to compare its resistance to β -lactamase hydrolysis with the resistance of cefoxitin and cefuroxime.

MATERIALS AND METHODS

Organisms. *Shigella sonnei* 10-101, *Pseudomonas aeruginosa* 3901, *Proteus morganii* 771, *Proteus mi-*

rabilis 3378, *Citrobacter freundii* 2732, *Klebsiella pneumoniae* 3973, and *P. aeruginosa* 1822 were clinical isolates that had been selected for study on the basis of their resistance to one or more β -lactam antibiotics and the presence of β -lactamases (4).

Antibiotics. Cephaloridine, cephalothin, and cefazolin were gifts from Lilly Research Laboratories. Cefoxitin was supplied by Merck Sharp & Dome. Cefuroxime was a gift of Glaxo Research Ltd., England, and HR 756 was obtained from Hoechst-Roussel, New Jersey. Antibacterial activity was determined in Mueller-Hinton broth using an inoculum of 10^5 colony-forming units. Synergy tests were determined by a checker-board method as described previously (4).

β -Lactamase preparation. The *Shigella* 10-101 and *Pseudomonas* 1822 β -lactamases were plasmid-mediated, constitutive enzymes. *P. morganii* 771 β -lactamase was a constitutive enzyme. *Klebsiella* 3973 β -lactamase was induced by 25 μ g of cephalothin per ml, *P. mirabilis* 3378 β -lactamase was induced by 50 μ g of cephaloridine per ml, *Citrobacter* 2732 β -lactamase was induced by 25 μ g of cephaloridine per ml, and *Pseudomonas* 3901 β -lactamase was induced by 500 μ g of penicillin G per ml. Overnight cultures were diluted 10-fold into 300 ml of brain heart infusion broth and grown for 2 h in a gyratory shaker at 37°C. Inducers were added, and the organism was grown for another 3 h. The cultures were harvested by centrifugation, washed twice with phosphate buffer (0.05 M; pH 7.0), and suspended in phosphate buffer at a 10-fold concentration. The bacteria were sonically disrupted by a Branson sonifier for three 30-s intervals in an ice bath. The extracts were centrifuged for 2 h at 4°C at 40,000 $\times g$, and the supernatant was dialyzed against phosphate buffer. The dialyzed material was used as the β -lactamase preparation. *Pseudomonas* 1822 and *P. morganii* 771 preparations were purified to homogeneity through diethylaminoethyl-cellulose and hydroxyapatite column chromatography as described previously (8).

Assay of β -lactamase. β -Lactamase activity was determined by the spectrophotometric method in a temperature-controlled spectrophotometer at 30°C.

The reaction mixture contained 0.5 ml of 0.2 mM cephalosporin in 0.5 ml of phosphate buffer (0.05 M, pH 7.0). The decrease in optical density at 255 nm was recorded after addition of 15 to 50 μ l of crude or purified β -lactamase. Inhibition of the hydrolysis of cephaloridine was determined by addition of cefoxitin, cefuroxime, or HR 756 to the reaction mixture. Enzyme was added last. Kinetic parameters were estimated from a least-squares fit to Lineweaver-Burk plots with substrate concentrations of 0.05 to 0.2 mM.

RESULTS

The in vitro susceptibility of the organisms used is given in Table 1. HR 756 was the most active cephalosporin. Only the *Pseudomonas* isolates, with inhibitory levels of 50 μ g/ml, might be considered resistant to HR 756. All of the organisms except the *Shigella* 10-101 were resistant to cephaloridine, cephalothin, and cefazolin.

FIG. 1. Chemical structure of HR 756.

Table 2 illustrates the hydrolysis of the representative cephalosporins by the β -lactamases. The rate of hydrolysis of the antibiotics was expressed as micromoles hydrolyzed per minute relative to the rate of hydrolysis of cephaloridine, which was given a value of 100. Cefoxitin, cefuroxime, and HR 756 were resistant to hydrolysis by the β -lactamases. Two enzymes, *Pseudomonas* 3901 and *P. morganii* 771, hydrolyzed cephalothin more rapidly than cephaloridine, but with other enzymes cephaloridine was the best substrate. β -Lactamase hydrolysis of cephaloridine, cephalothin, and cefazolin correlated with the minimal inhibitory concentrations given in Table 1. The resistance to hydrolysis of cefoxitin and cefuroxime did not correlate with the minimal inhibitory concentrations for the *Proteus* and *Pseudomonas* isolates. The resistance to β -lactamase hydrolysis of HR 756 correlated with its activity against the *Enterobacteriaceae* but not against *Pseudomonas*.

Since cefoxitin, cefuroxime, and HR 756 were resistant to β -lactamase hydrolysis, we investigated the β -lactamase inhibitory activity of the three compounds. Table 3 shows that cefoxitin, cefuroxime, and HR 756 inhibited the β -lactamase activity of a constitutive *P. morganii* and

TABLE 1. Comparative activity of cephalosporin antibiotics

Organism, enzyme type ^a	Minimum inhibitory concn (μ g/ml) of drug:					
	Cephaloridine	Cephalothin	Cefazolin	Cefoxitin	Cefuroxime	HR 756
<i>C. freundii</i> 2732, type I	100	50	50	100	0.8	0.2
<i>P. aeruginosa</i> 3901, type I	>800	>800	>800	>800	>800	50
<i>P. morganii</i> 771, type I	>400	>400	>400	>200	12.5	0.1
<i>P. aeruginosa</i> 1822, type III	>800	>800	>800	>800	>800	50
<i>K. pneumoniae</i> 3973, type V	200	100	50	12.5	12.5	0.2
<i>P. mirabilis</i> 3378	200	100	200	25	50	3.1
<i>S. sonnei</i> 10-101, type V	50	200	3.1	6.3	3.1	0.05

^a Richmond classification.

TABLE 2. β -lactamase hydrolysis of HR 756 compared with other known cephalosporins^a

Organism	Type of β -lactamase	Antibiotic						
		Cephaloridine (μ mol/min) ^b	Cephalothin		Cefazolin		HR 756, cefuroxime, cefoxitin	
			μ mol/min ^b	RHR ^c	μ mol/min ^b	RHR ^c	μ mol/min ^b	RHR ^c
<i>Klebsiella</i> 3973	Induced; type IV	13.7	8.6	63	3.8	28	0	0
<i>Citrobacter</i> 2732	Induced; type I	4.76	4.70	98	2.98	63	0	0
<i>Pseudomonas</i> 1822	Type III; plasmid	3.56	0.60	17	0.39	11	0	0
<i>P. mirabilis</i> 3378	Induced	12.06	3.03	25	0.21	2	0	0
<i>P. morganii</i> 771	Type I	4.9	11.1	227	2.58	53	0	0
<i>Shigella</i> 10-101	Type V; plasmid	3.9	0.91	23	0.4	10	0	0
<i>Pseudomonas</i> 3901	Induced	5.1	8.3	163	6.5	127	0	0

^a β -Lactamase activity was determined by the spectrophotometric method, measuring optical density decreased at 255 nm at 30°C. Reaction mixture contains 0.5 ml of 0.4 mM cephalosporin substrate in 0.5 ml of phosphate buffer (0.05 M, pH 7).

^b These values do not indicate the relative activity of different organisms

^c RHR, Relative hydrolysis rate compared with cephaloridine, which is given a value of 100.

an induced *Citrobacter* β -lactamase. Cefoxitin, cefuroxime, and HR 756 at equimolar concentrations inhibited 50% of the hydrolysis of cephaloridine by the *Pseudomonas* 3901 β -lactamase. We did not find inhibition of the β -lactamase activity of *Shigella* 10-101 (type V), *Pseudomonas* 1822 (type III), and *P. mirabilis* 3378 by these three compounds. Cefoxitin did not inhibit hydrolysis of cephaloridine by the *Klebsiella* β -lactamase tested at equimolar concentrations, but cefuroxime and HR 756 inhibited 67 and 84% of the β -lactamase activity.

P. morganii 771 and *Citrobacter* 2732 β -lactamases were used to determine the kinetics of the inhibitory activity of cefoxitin, cefuroxime, and HR 756 (Fig. 2). HR 756 and cefuroxime were of equal effectiveness as inhibitors of the two β -lactamases: both inhibited 80% of activity at a concentration of 0.1 μ M, whereas cefoxitin was approximately 50-fold less effective than the other two agents, inhibiting 80% of activity at the concentration of 5 μ M. Cefoxitin and cefuroxime were competitive inhibitors of both the type I β -lactamases (Fig. 3 and 4). HR 756 was a noncompetitive inhibitor of the β -lactamase of

Citrobacter (Fig. 3) and was a competitive inhibitor of the β -lactamases of *P. morganii* (Fig. 4). HR 756 had K_i values of 1.17×10^{-2} μ M for *Proteus* β -lactamase and 7.7×10^{-3} μ M for *Citrobacter* β -lactamase, indicating HR 756 is a potent inhibitor although not of the order of the semisynthetic penicillins. Cefoxitin had K_i of 1.42 μ M for the *Proteus* enzyme and 1.17 μ M for *Citrobacter* enzyme. Cefuroxime had K_i of 4×10^{-2} μ M for *Proteus* and 1.26×10^{-2} μ M for the *Citrobacter* enzyme.

To determine whether the β -lactamase inhibition produced by HR 756 was significant in intact bacteria, HR 756 was combined with ampicillin, cephalothin, and cephaloridine and tested against *P. mirabilis*, *Citrobacter*, and *Klebsiella*. Table 4 shows the minimal bactericidal concentrations. Although HR 756 inhibited the β -lactamase of *Citrobacter*, it did not act synergistically with any of the three compounds. The minimal bactericidal concentration of HR 756 against *C. freundii* 2732 was greater in the presence of cephalothin. HR 756 acted synergistically against *P. mirabilis* with ampicillin but not with cephaloridine or cephalothin. HR 756

TABLE 3. Inhibition of β -lactamase hydrolysis of cephaloridine by HR 756 compared with cefoxitin and cefuroxime^a

Organism	Type of β -lactamase	Cephaloridine (μ mol/min)	Cephaloridine + cefoxitin		Cephaloridine + cefuroxime		Cephaloridine + HR 756	
			μ mol/min	RHR ^b	μ mol/min	RHR	μ mol/min	RHR
<i>Citrobacter</i> 2732	Type I induced	4.76	0	0	0	0	0	0
<i>Pseudomonas</i> 3901	Type I induced	4.1	1.88	46	1.82	44	2.02	0
<i>P. morganii</i> 771	Type I constitutive	5.11	0	0	0	0	0	0
<i>Pseudomonas</i> 1822	Type III constitutive	5.74	6.1	106	5.5	96	5.67	98
<i>P. mirabilis</i> 3378	Induced	6.2	6.2	100	6.2	100	5.8	94
<i>Klebsiella</i> 3973	Type IV induced	5.8	5.8	100	3.86	67	4.88	84
<i>Shigella</i> 10-101	Type V constitutive	3.7	3.16	85	3.23	87	3.03	82

^a 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 cefoxitin, cefuroxime, and HR 756.

^b RHR, Relative hydrolysis rate. See Table 2, footnote c.

FIG. 2. Inhibition of β -lactamase hydrolysis of cephaloridine by cefoxitin, cefuroxime, and HR 756. Symbols: (●) *Citrobacter* 2732; (▲) *P. morganii* β -lactamases.

FIG. 3. (A) Competitive inhibition of *Citrobacter* 2732 β -lactamase hydrolysis of cephaloridine by cefoxitin and cefuroxime. Symbols: (○) cefoxitin (2.5 μM); (△) cefuroxime (0.01 μM); (●) control. (B) Noncompetitive inhibition of *Citrobacter* 2732 β -lactamase hydrolysis of cephaloridine by HR 756. Symbols: (○) 0.1 μM ; (△) 0.05 μM ; (●) control.

FIG. 4. Competitive inhibition of *P. morganii* β -lactamase hydrolysis of cephaloridine by 0.01 μM HR 756 (△), 0.01 μM cefuroxime (×), and 2.5 μM cefoxitin (○); (●) control.

showed no synergy against *Klebsiella* when combined with other antibiotics.

DISCUSSION

HR 756, a novel cephalosporin, has been shown to be as resistant to hydrolysis by the common gram-negative β -lactamases as are cefoxitin and cefuroxime. These findings suggest that the 7 α methoxy group is not crucial in determining β -lactamase resistance, as has been postulated for the β -lactamase stability of cefoxitin (3, 7).

Darland and Birnbaum (3) and Olsson et al. (13) reported that cefoxitin was a competitive inhibitor of *Bacteroides* β -lactamases. Our results also indicate that cefoxitin is a competitive inhibitor of Richmond type I β -lactamases, but that HR 756 and cefuroxime are more potent inhibitors.

Cole et al. (2) reported that some semisynthetic penicillins highly resistant to the β -lactamase of *Escherichia coli* and *Klebsiella aerogenes* were not inhibitors. Although cefoxitin, cefuroxime, and HR 756 were resistant to β -lactamase hydrolysis of type III, IV, and V enzymes, they did not inhibit the enzymes, suggesting that there may not be a correlation between β -lactamase stability and effectiveness as inhibitors. Since cefoxitin, cefuroxime, and HR 756 inhibited *Proteus* and *Citrobacter* type I β -

TABLE 4. Bactericidal activity of HR 756 combined with other β -lactam antibiotics

Organism	Minimum bactericidal conc (μ g/ml) of antibiotic:					
	HR 756	Ampicillin	Cephalothin	Cephalexidine	HR 756 + ampicillin	HR 756 + cephalothin HR 756 + cephaloridine
<i>P. mirabilis</i> 3378	12.5	≥ 800	200	≥ 800	1.6/1.6 ^a	12.5/12.5
<i>K. pneumoniae</i> 3973	12.5	≥ 800	400	200	12.5/12.5	12.5/0.2
<i>C. freundii</i> 2732	0.8	400	400	400	0.8/0.8	3.1/200

^a First number is minimum bactericidal concentration of HR 756; second is that of the other agent.

lactamases but were not substrates, it is suggested that they did bind to the active site in the enzymes, whereas with type III, IV, and V enzymes they did not bind to these types of β -lactamases. Since HR 756 did not act as a *Klebsiella* β -lactamase inhibitor, it was not surprising that it did not act synergistically against *Klebsiella* when combined with other β -lactam antibiotics. Its lack of synergy with β -lactam antibiotics against *Citrobacter* may be related to poor transport of the other agents to their receptor sites.

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